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REMOTE PRECONDITIONING: EVALUATING THE EFFICACY OF CARDIOPROTECTION IN TYPE-2 DIABETES AND EXPLORING THE MECHANISTIC ROLE OF EXOSOMES

by

JOSEPH WIDER

DISSERTATION

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of Wayne State University,

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for the degree of

DOCTOR OF PHILOSOPHY

2017

MAJOR: PHYSIOLOGY

Approved By:

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Advisor Date

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DEDICATION

I dedicate this to my family.

Unrelenting support.

Unconditional acceptance.

Much Love.

To Maranda and Mac.

ACKNOWLEDGMENTS

First and foremost, I would like to express my gratitude to my dissertation advisor and mentor. Dr. Karin Przyklenk hired me as a new graduate and encouraged me to pursue a doctoral degree. As an advisor, Karin has provided me with guidance and opportunities I could not have anticipated. She has instructive and supportive while being been patient, lenient and provided me with freedom in the laboratory. I fully appreciate the culture of excellence in her lab, which demands hard work and productivity. Most importantly, Karin is an advocate for students, which is apparent in her dedication as an advisor.

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My gratitude goes to members of the lab whose support has been essential to my success. Dr. Christian Reynolds stands as a primary role model, always willing to discuss science and life; Dr. Yi Dong gave me crucial troubleshooting advice, without which I would be lost; Mr. Vishnu Undyala helped train me in several important laboratory techniques; the brilliant Dr. Peter Whittaker set a standard for his invaluable perspective and range of knowledge. His surgical training was also part of the

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CHAPTER 1: ISCHEMIC CONDITIONING: THE CHALLENGE OF PROTECTING THE DIABETIC HEART

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1. Clinical Significance: Acute Myocardial Infarction and Type-2 Diabetes

Cardiovascular disease (CVD) remains the leading cause of death in the United States and globally, with acute myocardial infarction (AMI) accounting for a substantial portion of CVD-associated mortality.[1] Timely reperfusion is the standard clinical therapy for AMI and, although necessary to rescue ischemic tissue, restoration of blood flow can paradoxically exacerbate cell death (rather than initiate salvage) in populations of ischemic myocytes, a phenomenon termed lethal ischemia-reperfusion (I/R) injury.[4-8] The volume of myocardium rendered necrotic following ischemia-reperfusion (i.e., myocardial infarct size) is a primary determinant of mortality and morbidity associated with AMI.[8-10] However, despite decades of preclinical and clinical investigation aimed at elucidating the mechanisms of I/R injury and developing mechanisms-based therapies to augment the benefits of early reperfusion and reduce myocardial infarct size, no advances have been translated into clinical practice.[10-12]

In 1986, Murry et al. made the landmark observation that the heart could be 'preconditioned' or rendered resistant to lethal I/R injury, by exposure to a brief and non-lethal, antecedent ischemic insult.[14] Subsequent studies expanded the paradigm of myocardial 'conditioning' beyond the phenomenon of ischemic preconditioning to encompass postconditioning and remote conditioning.[7,15,16] Overwhelming experimental evidence, obtained in multiple models and species, has demonstrated that all three forms of myocardial conditioning induce potent cardioprotection.[7,8,11] However, the vast majority (>90%) of these studies have been conducted using healthy, juvenile or adult populations that do not manifest the risk factors and comorbid conditions

typically seen in patients with CVD and suffering AMI.[17] A major, well-established independent risk factor for CVD and AMI, the prevalence of which has ~doubled over the past 20 years, is type-2 diabetes, [1,18] and there is emerging concern that the efficacy of conditioning-induced cardioprotection may be compromised in the diabetic heart.[6,17,20-22] Our aim in the current review is to focus on this important issue: we provide a synopsis of our present understanding of the effect of type-2 diabetes on the infarct-sparing effect of ischemic conditioning.

2. What is Ischemic Conditioning?

2.1 Definitions and Basic Concepts

Ischemic conditioning renders the myocardium resistant to I/R injury via the up-regulation of as-yet incompletely understood, endogenous cardioprotective signaling cascades.[7,8] There are three permutations of conditioning that differ in terms of the site and timing of the brief ischemic stimulus [7]. Classic ischemic preconditioning, as first described by Murry and colleagues,[14] is initiated by subjecting the heart to 2-4 repeated episodes of brief (2-5 minute) ischemia, interrupted by intervening ~5 minute periods of reperfusion, before the onset of a sustained ischemic insult. By contrast, postconditioning is not a preischemic intervention but, rather, as described in the seminal publication by Zhao *et al*.,[15] is a form of modified reperfusion. i.e., Blood flow to the ischemic myocardium is restored in a stuttered or staccato manner, with a typical algorithm of 3-6 cycles of [10-30 seconds of reperfusion interspersed with 10-30 second periods of reocclusion], before establishing full and complete reperfusion. For preconditioning and postconditioning, both the protective stimulus (brief antecedent ischemia or stuttered reflow) are applied in heart, at the same site and in the same vascular territory as the sustained period of ischemia. Remote conditioning differs

importantly from both of the aforementioned strategies in that cardioprotection is evoked by brief ischemia applied at a distant site, such as a different and distinct myocardial vascular bed (as first reported by Przyklenk et al. [16]) or a remote and less vulnerable tissue or organ such as skeletal muscle.[25] With regard to timing, protection with remote conditioning can be achieved when the stimulus is applied before sustained coronary occlusion, during the sustained ischemic insult, or at the time of reperfusion (remote preconditioning, perconditioning or postconditioning, respectively)[7] (Figure 1-1).

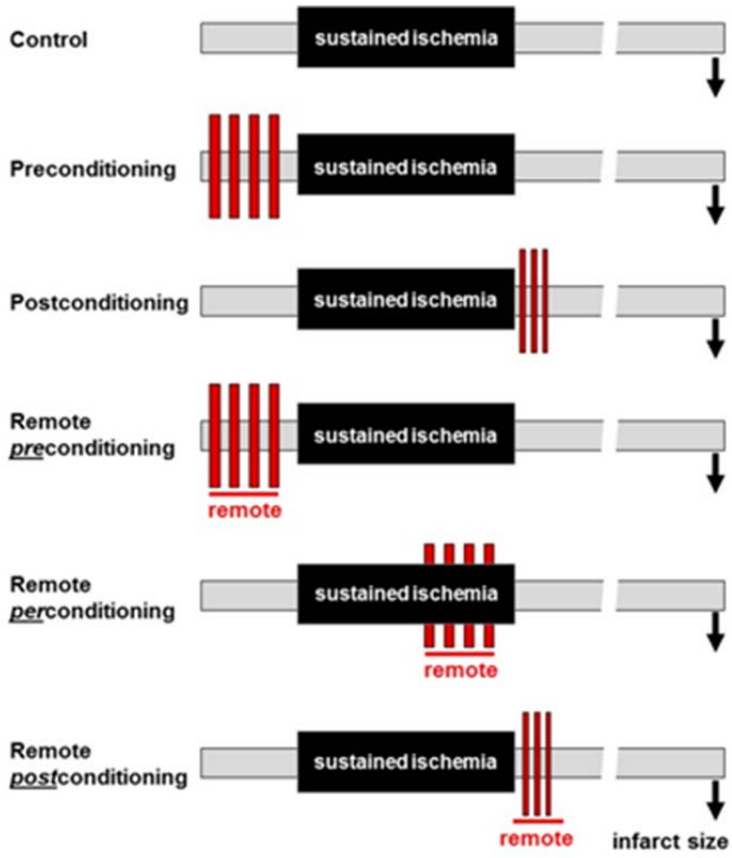


Figure 1–1: Schematic diagram illustrating the key temporal aspects of preconditioning, postconditioning, and remote pre-, per- and postconditioning. Red boxes denote the conditioning stimulus.

2.2 Reduction of Infarct size: the Hallmark of Ischemic Conditioning

Despite these temporal and spatial differences, all three facets of ischemic conditioning share a common and overarching theme: all are profoundly effective in reducing myocardial infarct size beyond that achieved by reperfusion alone[7,8]. Conditioning-induced cardioprotection has, with rare exceptions, been documented and

confirmed in countless laboratories and in all models and species that have been tested. There are reports that the benefits of ischemic conditioning (in particular, preconditioning and postconditioning) may extend to other pathophysiologic aspects of I/R injury, including postischemic myocardial 'stunning', arrhythmias, microvascular damage and endothelial dysfunction. [21,26,27] However, there is no question that the most robust endpoint for the assessment of cardioprotection is infarct size, and the established hallmark of ischemic conditioning is its infarct-sparing effect.[7,8] (Figure 1-2)

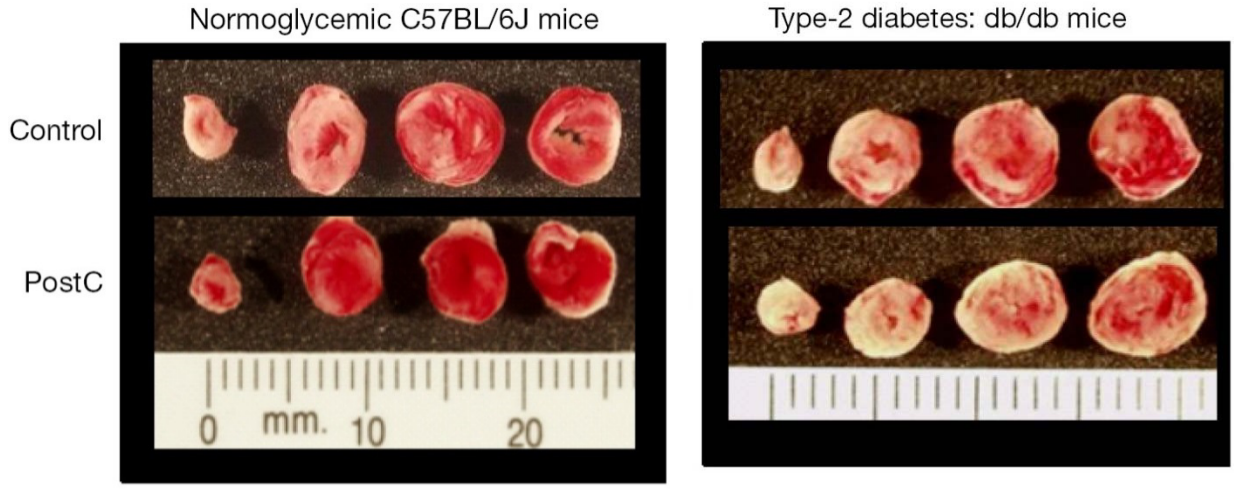


Figure 1–2: Original images of control and postconditioned (PostC) mouse hearts. Hearts were cut into transverse slices and stained with triphenyltetrazolium chloride; using this method, viable myocardium is stained red, whereas necrotic myocardium remains unstained so appears pale. Infarct size was reduced with PostC in normoglycemic C57BL/6J mice. In contrast, in hearts from db/db mice (model of type-2 diabetes), infarct sizes were comparable in control and PostC groups. Reprinted with permission from reference [2].

2.3 Cellular Mechanisms: The Major Players

In addition to sharing a common primary endpoint – reduction of infarct size – the three facets of ischemic conditioning also appear to share common elements in terms of cellular mechanisms. Not surprisingly, the greatest insight has been gained into the mechanisms of ischemic preconditioning (comprehensive reviews provided in [6,7,27-30]). There is a consensus that preconditioning is initiated through ligand-receptor interactions including, most notably, stimulation of G_i-protein coupled receptors. The

archetypal trigger for preconditioning, first described in 1991, is release of adenosine from myocardium rendered ischemic during the brief antecedent ischemic stimulus and binding to adenosine A_1 or A_3 receptors on the cardiomyocyte membranes.[31,32] In the ensuing years, redundancies in the ligands capable of triggering preconditioning via binding to their respective receptors were identified, including (but not limited to) bradykinin, opioids, acetylcholine and TNF-α.[33-36] Ligand-receptor binding subsequently activates multiple signaling cascades in a complex, biphasic and possibly redundant manner, following the general paradigm of: i) initial up-regulation of phosphatidylinositol 3-kinase (PI3 kinase)/Akt, nitric oxide-mediated activation of protein kinase G (PKG) and subsequent activation of the ε isoform of protein kinase C (PKC) during the early minutes of sustained ischemia, and ii) receptor re-population and up-regulation of the so-called 'RISK' (reperfusion injury salvage kinase) and/or 'SAFE' (survival activating factor enhancement) pathways during the early seconds-minutes following restoration of blood flow.[30,37-39] There appears to be minimal overlap or intersection between these latter two reperfusion-associated signaling cascades: key components of the RISK pathway include PI3 kinase-Akt, extracellular signal regulated kinase (ERK), p70S6 kinase and glycogen synthase kinase 3β (GSK3β), while the pivotal constituents of the SAFE pathway are janus activated kinase (JAK) and signal transducer and activator of transcription (STAT).[6,7,27-30,38,39] Nonetheless, both pathways converge on the mitochondria, the proposed end-effector of ischemic preconditioning, with specific molecular targets including the mitochondrial adenosine triphosphate-sensitive potassium (K_{ATP}) channel, mitochondrial connexin 43, and, most notably, the mitochondrial permeability transition pore (mPTP).[28,40-43] Cardioprotection is purportedly conferred by a resultant stabilization of mitochondrial membranes (including

suppression of mPTP opening) and better maintenance of mitochondrial integrity.[6,7,27-30]

The discovery of infarct size reduction with postconditioning, and observations of a comparable magnitude of cardioprotection with both preconditioning and postconditioning, provided compelling and provocative evidence that pretreatment – and up-regulation of kinase signaling at the onset of ischemia – is not required to render the heart resistant to I/R injury.[15] Moreover, the lack of an additive effect of combined administration of preconditioning + postconditioning suggests that common (or redundant) reperfusion-associated mechanisms may underlie the infarct-sparing effect of the two interventions.[15] Indeed, receptor-mediated up-regulation of the RISK and/or SAFE pathways, culminating in stabilization of mitochondria (with an emphasis on inhibition of mPTP opening) are hypothesized to play critical roles in the reduction of infarct size achieved with postconditioning.[6,7,21,28,30,44-46]

Perhaps not surprisingly, the three common themes of G-protein coupled receptor stimulation on myocyte membranes, activation of multiple kinases including members of the RISK and/or SAFE pathways, and mitochondria as end-effectors have also been implicated as key mechanistic components of remote conditioning (reviewed in references 7,47-50). There is, however, an inherently unique aspect of remote conditioning not shared by pre- and postconditioning: the cardioprotective signaling cascades are initiated by communication or transfer of a protective signal from the site of the conditioning stimulus to the heart. Details concerning the identity of the signal(s) and mode of communication remain elusive, but two leading theories are under investigation: remote conditioning may be triggered by blood- or perfusate-borne transport of one or more unknown humoral factor (possibly including a small, <15 kDa hydrophobic molecule)[51-53], and communication via neuronal stimulation. [7,47-55] Of note,

these two hypotheses are not mutually exclusive, are in all likelihood model-dependent, and, in at least some models, both humoral and neuronal communication may be involved. [7,47,48,56,57]

2.4 Ancillary and Alternative Mediators of Conditioning-induced Cardioprotection

As summarized in the preceding paragraphs, intensive interest and attention has focused on the involvement of RISK and SAFE signaling to the infarct-sparing effect of ischemic pre-, post- and remote conditioning. However, additional and less well-characterized mediators have also been postulated to contribute to conditioning-induced cardioprotection, either in concert with or as possible alternatives to the RISK and SAFE cascades. For example, isoforms of PKC (in particular, PKCε) may play a broader role in conditioning-induced cardioprotection, beyond the well-described early activation following brief preconditioning ischemia: PKC has been implicated as a component of kinase signaling initiated in response to both postconditioning and remote preconditioning. [29,58-62] Generation of low, sub-lethal levels of reactive oxygen species (ROS) and signaling via nitric oxide have, similarly, been proposed to integrate with both PKC and the RISK and STAT pathways as mediators of pre- and postconditioning.[30,59,60,62-64]

A long-standing concept that may be relevant to the issue of cardioprotection in diabetic cohorts, particularly for ischemic preconditioning, is that alterations in myocardial metabolism play a causal role. Metabolic hallmarks of cardiac ischemia include the rapid (within seconds-minutes) shift from aerobic to anaerobic metabolism and the resultant, progressive temporal decline in myocardial ATP concentration as ATP synthesis via anaerobic glycolysis is insufficient to meet the diminished, residual energy consumption of the ischemic tissue. [65] The first report of infarct size reduction

with preconditioning was accompanied by evidence of an increase in metabolic efficiency: i.e., preconditioning slowed myocardial energy demand during the subsequent period of sustained ischemia, thereby attenuating the rate of ATP utilization and reducing the rate of anaerobic glycolysis.[14,66,67] Ensuing studies argued against the concept of a cause-and-effect relationship between reduced energy demand during prolonged ischemia and infarct size reduction with preconditioning.[68] Nonetheless, there is evidence for a mechanistic link between metabolism and preconditioning-induced cardioprotection. Ischemic preconditioning is associated with an increase in glucose uptake during sustained ischemia, an effect that has been attributed to: i) co-activation of Akt and AMP-activated protein kinase (AMPK: the 'metabolic master switch' that responds to ATP depletion and an increase in the ratio of ADP/ATP); ii) translocation of the glucose transporter protein GLUT4 to the cardiomyocyte surface; and iii) subcellular redistribution of hexokinase to mitochondria, where it phosphorylates and facilitates sequestration of glucose.[69-76] This metabolic signaling is purportedly required for the infarct-sparing effect of ischemic preconditioning,[69-73] and has been implicated to play a secondary role in postconditioning.[77]

3. Cardiac Consequence of Diabetes

3.1 The Big Picture: Diabetes, Cardiovascular Disease and Acute Myocardial Infarction

The successful clinical translation of novel interventions to attenuate myocardial I/R injury has been identified as a major unmet need.[10-12] This issue is of particular relevance and importance to patients with type-2 diabetes, as underscored by: i) the $\geq$2-fold greater incidence of CVD, acute coronary syndromes and AMI in this patient cohort; ii) evidence of larger infarct sizes and exacerbated necrotic and apoptotic cell death; and thus, perhaps not surprisingly, iii) a 2- to 4-fold greater incidence of CVD-related

mortality in diabetic *versus* non-diabetic subjects.[1,78-89] Indeed, CVD is the leading cause of death and disability among diabetics, a poor prognosis that has not been appreciably influenced by the current trend of an overall reduction in mortality associated with AMI.[1,78,79,88,89] Recent statistics reveal that, at present, Americans have an estimated ~40% lifetime risk for the development of diagnosed diabetes, and a sustained increase in the incidence of type-2 diabetes in the USA and worldwide is predicted for the next 20-30 years. This anticipated escalation in the numbers of patients with diabetes may have the potential to diminish the gains that have been made attenuating the overall prevalence of CVD-related death.[1,90-92]

3.2 The Cellular/Molecular Perspective

The hallmarks of type-2 diabetes are insulin resistance and accompanying metabolic dysregulation. Reduced insulin sensitivity has both direct effects on glucose uptake and insulin-mediated signaling in cardiac cells, and indirect cellular effects that are secondary to the accompanying hyperglycemia, hyperinsulinemia and hyperlipidemia.[93-96] Most importantly, in terms of cardioprotection, type-2 diabetes has been associated with impaired PI3 kinase/Akt signaling (components of both insulin signaling and the RISK pathway) and GLUT4 protein expression and/or translocation, as well as defects in AMPK and, indeed, essentially all kinases proposed to contribute to the infarct-sparing effect of ischemic conditioning. For example, impaired phosphorylation of PKC, PI3 kinase/Akt, ERK, STAT3, and GSK-3β has been described in diabetic hearts, possibly due to reported increases in activities of multiple phosphatases including phosphatase and tensin homolog (PTEN), MAPK phosphatases (MKPs) and protein phosphatase-2C (PP2C). There is evidence to suggest that downstream targets and proposed end-effectors of conditioning-induced

cardioprotection are also modified by type-2 diabetes, including alterations in expression and activity of mitochondrial K_{ATP} channels and increased propensity of mPTP opening in response to increased intracellular Ca^{2+} concentrations in the diabetic myocardium.[96-102] Although these insights have, not surprisingly, been largely derived from genetic rodent models of type-2 diabetes, including db/db and ob/ob mice and strains of fatty and lean rats (Zucker Fatty, Otsuka Long-Evans-Tokushima fatty and Goto-Kakizaki),[96-100] analysis of myocardial tissue samples collected from diabetic patients at the time of cardiac surgery have yielded corroborative results.[101,102]

4. Diabetes, Infarct Size and Ischemic Conditioning

4.1 Diabetes and Ischemic-Reperfusion Injury

The effect of type-2 diabetes on the infarct-sparing effect of ischemic conditioning has, almost exclusively, been assessed in the aforementioned rodent models. Accordingly, meaningful discussion of conditioning-induced cardioprotection in these models first requires an understanding of the effect of type-2 diabetes on infarct size in untreated control animals.

In contrast to the clinical consensus that diabetes is associated with larger infarct sizes and poor outcomes when compared with non-diabetic patients,[1,78-89] data obtained in preclinical models is mixed: diabetes has been reported to increase, decrease or have no effect on cardiomyocyte death.[22,103-105] These disparate outcomes have been attributed to multiple factors, including: i) the duration of the diabetic state at the time of experimentation (with *short-term* diabetes more typically associated with an apparent *reduced sensitivity* to I/R injury); ii) differences over time or among models in levels of insulin and fatty acids; and iii) the presence or absence of obesity.[22,104,105] The definitions of ‘short-term diabetes’, hyperinsulinemia, hyperlipidemia, etc., and precise

relationships of these factors with myocardial infarct size, are nebulous. However, it is important to emphasize: the translational relevance of experimental models characterized by a reduced sensitivity of the diabetic heart to I/R injury is considered to be questionable.[104]

4.2 Ischemic Conditioning in Models of Type-2 Diabetes: is Efficacy Maintained?

A PubMed search of the current literature reveals that, as a conservative estimate, there are >2,500 publications on ischemic pre-, post- and remote conditioning in which myocardial infarct size (the 'gold standard' of conditioning-induced cardioprotection) was among the primary endpoints. Remarkably, <0.5% of these papers have specifically investigated the *infarct-sparing effect* of *ischemic conditioning* in models of *type-2 diabetes* – a statistic that is extraordinary given the well-documented, profound consequences of diabetes on cardiac pathophysiology.

Among the small number of studies that have addressed this issue, a spectrum of rat and mouse models of type-2 diabetes have been utilized (Table 1-1). Nonetheless, irrespective of the model used and variations in study design, there is an emerging consensus: both pre- and post-conditioning either fail to reduce infarct size,[100,106-111] or the efficacy of conditioning is attenuated such that an amplified stimulus (i.e., an increased number of episodes of preconditioning ischemia) is required to evoke protection.[112-114]. Loss of conditioning-induced cardioprotection has been described in both lean and obese models type-2 diabetic rat models [106] and in obese rats before the onset of significant hyperglycemia[107]. Moreover, similar outcomes (a diminished responsiveness to the infarct-sparing effect of pre- and postconditioning) have also been reported in models of type-1 diabetes,[17,20-22,72,108,115-123] suggesting that the refractoriness of the diabetic heart to ischemic conditioning is not a simple consequence

Table 1–1: Effect of type-2 diabetes on infarct size reduction with ischemic conditioning

Author	Model	Reduction of infarct size?	Comments/mechanistic insights?
PRECONDITIONING			
Kristiansen[1]	Rat: Zucker Fatty	No	Protection lost in both lean and obese models.
	Rat: Goto-Kakizaki	No	No mechanism proposed.
Tsang[3]	Rat: Goto-Kakizaki	Attenuated	Efficacy attenuated; amplified preconditioning stimulus required to achieve protection. Impaired Akt phosphorylation.
Katakam[4]	Rat: Zucker Fatty	No	Protection lost before development of hyperglycemia.
			Impaired activation of mitochondrial KATP channels.
Hausenloy[5]	Rat: Goto-Kakizaki	Attenuated	Efficacy attenuated: amplified preconditioning stimulus required to achieve protection.
Whittington[6,7]	Rat: Goto-Kakizaki	Attenuated	Amplified preconditioning stimulus was protective in 3 and 8 month old rats.
		No	Complete loss in efficacy in 12 and 18 month old rats. Impaired Akt phosphorylation due to chronic up-regulation.
POSTCONDITIONING			
Wagner	Rat: WOKW	No	Impaired ERK, GSK-3-β phosphorylation
Bouhidel[8]	Mouse: ob/ob	No	Impaired Akt, ERK, p70S6 kinase, AMPK phosphorylation
Przyklenk[2]	Mouse: db/db	No	Impaired ERK phosphorylation
Zhu[9]	Mouse: db/db	No	Loss of protection associated with differential regulation of mitochondrial proteome
Oosterlinck[10]	Mouse: ob/ob	Attenuated	No mechanism proposed.
REMOTE PRECONDITIONING No studies published			

of hyperglycemia, hyperinsulinemia, hyperlipidemia, obesity or, in all likelihood, any single pathophysiological feature of the disease. Nascent mechanistic insight has, however, been obtained into the cellular mechanisms that may contribute to the compromised efficacy of ischemic conditioning in diabetic models. Perhaps not

surprisingly, the complete or partial failure of pre- and postconditioning to limit infarct size in models of type-2 (and type-1) diabetes has largely been attributed to defects in RISK and AMPK signaling, with desensitization or impaired activation of multiple kinases (including PI3 kinase/Akt, ERK, p70S6 kinase, and/or GSK3β), possibly due to augmented activities of MKPs and other phosphatases, all having been implicated to play a role (Table1-1)).[17,21,22,100,108-110,112,121] Potential diabetes-associated defects in mitochondrial end-effectors have also been identified, including impaired activation of mitochondrial K_{ATP} channels [107,111].

Finally, there is a notable and fundamental gap in our current knowledge of the efficacy of ischemic conditioning in models of type-2 diabetes. All previous discussion has focused exclusively on pre- and postconditioning; to date, no published studies have utilized remote conditioning as the cardioprotective trigger (Table1-1). There is, however, one piece of evidence that diabetes may have a complex, confounding effect on the production or release of the as-yet unidentified humoral factor(s) from the site of the conditioning stimulus[124]. A model of 'transferred' protection was used, in which the conditioning stimulus (brief repeated episodes of limb ischemia) was applied to diabetic and non-diabetic patient cohorts, serum was collected, dialyzed and administered to a remote target (isolated buffer-perfused rabbit hearts), and the hearts were then subjected to a sustained period of ischemia. For both cohorts, serum collected after the conditioning stimulus rendered the rabbit hearts resistant to infarction: i.e., type-2 diabetes *per se* did not preclude the infarct-sparing effect of remote conditioning. However, in the subset of diabetic subjects with peripheral neuropathy, the transferred serum failed to reduce infarct size in acceptor rabbit hearts, implicating the requisite involvement of a diabetes-sensitive neurogenic component in this model of humorally-

mediated remote conditioning.[124] The consequences of type-2 diabetes in standard *in vivo* models of remote conditioning, and the concept that persistent efficacy of remote conditioning in diabetic models may depend on the intact innervation of the effector organ, are topics of ongoing study by our group and others (Figure 1-3).

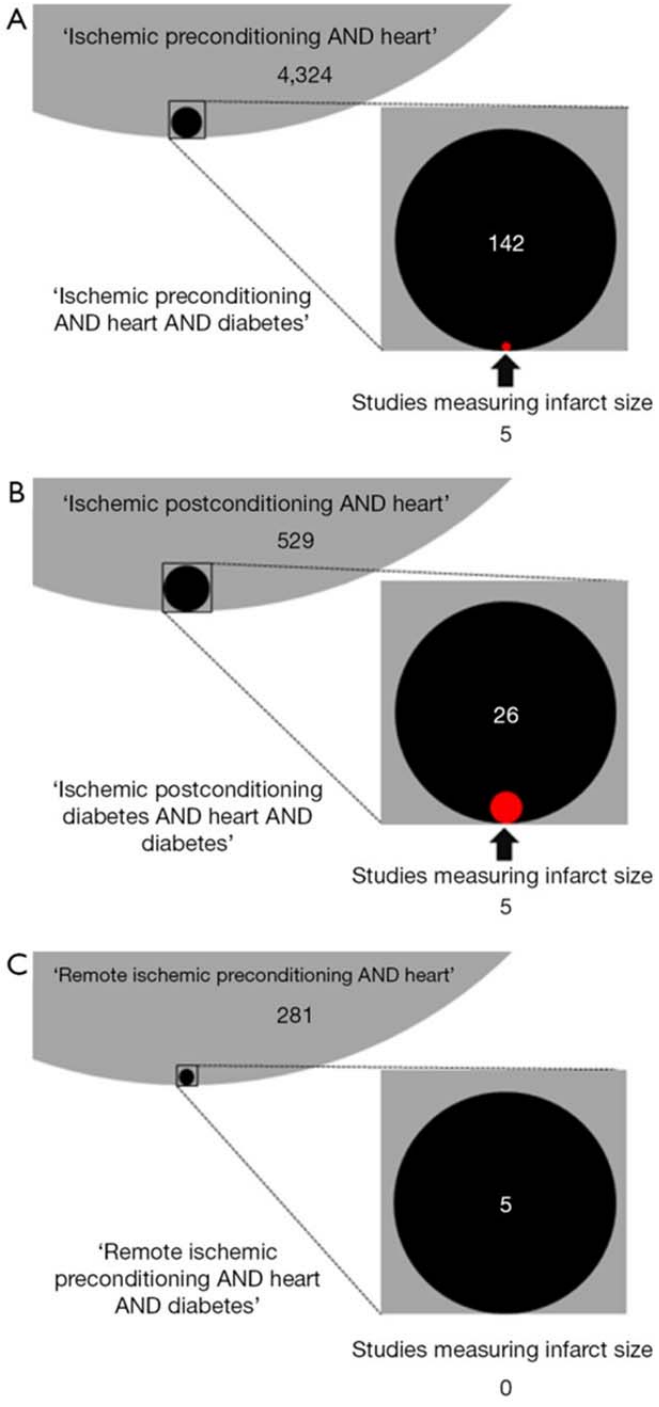


Figure 1–3: Schematic diagram underscoring the paucity of available data on the efficacy of ischemic conditioning in the setting of diabetes. PubMed queries were performed in September 2014 using the search terms 'ischemic preconditioning and heart' (A), 'postconditioning and heart' (B) and 'remote ischemic preconditioning and heart' (C), with and without the addition of the term 'diabetes'. Studies conducted in diabetic models in which infarct size (the gold standard of ischemic conditioning) was among the primary endpoints are highlighted.

4.3 Initial Insight: Ischemic Conditioning in Diabetic Patients

The wealth of preclinical evidence documenting reduction of infarct size with pre-, post- and remote conditioning has provided the groundwork and rationale for ongoing efforts to translate the concept of endogenous conditioning-induced cardioprotection for the clinical treatment of myocardial I/R injury.[8,10-12,125] As preconditioning is, by definition, a pretreatment – thereby limiting its potential for clinical use to planned ischemic events such as cardiac surgery and elective percutaneous intervention (PCI) – current attention is focused largely on postconditioning and remote conditioning. Results from ~40 Phase II clinical trials have been reported, and large Phase III trials are in progress (reviewed in references 8,125). Overall, the data have been mixed: ~60% of the studies observed significant reductions in cardiac enzyme release and other surrogate endpoints reflecting myocardial infarct size in conditioned cohorts *versus* controls, while the remainder reported either no effect or exacerbated outcomes.[8,125]. In addition, recent meta-analyses of pooled data from multiple trials underscored the variability among studies and concluded that, at present, there is borderline evidence, or no evidence, for cardioprotection with either postconditioning or remote conditioning.[126,127]

In addition to differences in patient demographics and enrollment criteria, protocol logistics (including the number and timing of the conditioning stimuli and duration of sustained ischemia), choice of endpoints, etc., extrapolation of the results obtained in preclinical models of type-2 diabetes suggest that two related factors – the confounding effects of diabetes, together with differing proportions of diabetic patients among studies – may also contribute to the aforementioned variability. Indeed, in an effort to mitigate this concern, some investigators have prospectively excluded the

enrollment of diabetic patients.[128-131] Initial evidence appears to support the concept that conditioning-induced cardioprotection may be impaired or lost in patients with diabetes. For example, in two clinical trials in which prospective subset analyses were performed and cardiac enzyme release served as the surrogate for infarct size, preconditioning (triggered by prodromal angina) had no beneficial effect, while postconditioning tended to exacerbate myocardial injury in diabetic cohorts.[132,133] In addition, in a third trial evaluating the efficacy of remote conditioning administered following elective PCI, the incidence of post-procedural MI was significantly increased in patients with *versus* without diabetes.[134] Finally, in an *ex vivo* analysis, preconditioning attenuated hypoxia-reoxygenation-induced cell death in human atrial samples harvested from non-diabetic patients at the time of cardiac surgery, but failed to render atrial tissue resistant to injury in samples obtained from patients with type-2 diabetes.[135]

5. Summary and Future Directions

Despite the paucity of studies conducted in preclinical models of type-2 diabetes in which myocardial infarct size was among the primary endpoints (Table1-1), a consensus is emerging: the diabetic rodent heart is refractory to the profound infarct-sparing effect of preconditioning, postconditioning and, possibly, remote conditioning. It could be argued these data may be of limited clinical relevance, given the overt simplicity of the mouse and rat models: the duration of diabetes is comparatively acute (on the order of weeks, rather than months-years), the models do not mimic the multiple comorbid conditions seen in substantial subsets of patients, and preclinical protocols typically do not incorporate the battery of pharmacologic agents that would be administered to patients as standard clinical care for both AMI and the management of hyperglycemia. Nonetheless, although far from conclusive, the initial clinical data

appear to corroborate the preclinical results.

Does ischemic conditioning have the potential to achieve the as-yet unmet clinical challenge of attenuating myocardial ischemia-reperfusion injury and improving outcome in patients post-MI? And, if so: is the efficacy of conditioning-induced cardioprotection compromised in diabetic patients? Definitive resolution of these issues will require the successful execution of Phase III clinical trials that are prospectively designed with sufficient statistical power to discern the presence *versus* absence of an infarct-sparing effect of ischemic conditioning in stratified subgroups of patients with and without type-2 diabetes.

CHAPTER 2: EXOSOMES IN REMOTE PRECONDITIONING

1. A Brief History of Exosomes

Exosomes are cell-derived, membrane particles associated with cell-to-cell communication. Emerging evidence has implicated them in health, disease and medicine, thus fueling a rapidly growing field. Recently, exosomes have been implicated in the humoral component of remote preconditioning, and investigating exosomes could contribute to our understanding of the communication mechanism of this cardioprotective phenomenon. The biology of exosomes will be discussed, with an emphasis on their history in treating AMI, and newly described role in remote preconditioning.

In 1967, Wolf *et al.* described lipid-rich particles, or "platelet dust" that promote hemostasis, present in the serum after removal of platelets.[136] In the mid-80s, two groups lead by Johnstone and Stahl described similar vesicles released when endosomes fuse with the plasma membrane in reticulocytes.[137,138] Pan *et al.* and Harding *et al.* observed internalization of transferrin receptors by endocytosis, followed by inward budding of the endosomal limiting membrane, resulting in formation of intraluminal vesicles. Endosomes containing vesicles (commonly known as multivesicular bodies) then fused with the plasma membrane releasing the intraluminal vesicles containing transferrin receptors into the extracellular space.[139,140] Externalization of these vesicles appeared to involve the reversal of the endocytic pathway where, instead of lysosomal delivery, they are exocytosed, thereby leading to the name *exosomes* (a term originally proposed to describe small plasma membrane derived vesicles in 1981).[141,142] As with the lysosomal pathway, exosomes were initially identified exclusively as a method of waste disposal; exfoliating unwanted membrane

proteins, such as reticulocyte transferrin receptors. More recently however, exosomes are being implicated in a growing list of physiological and pathophysiological processes. A PubMed search (February 2017) of the term “exosome” retrieved >5800 papers (Figure 2-1). We added qualifying terms to the query, such as “cancer” or “immune”, to roughly illustrate the context of exosomes research.

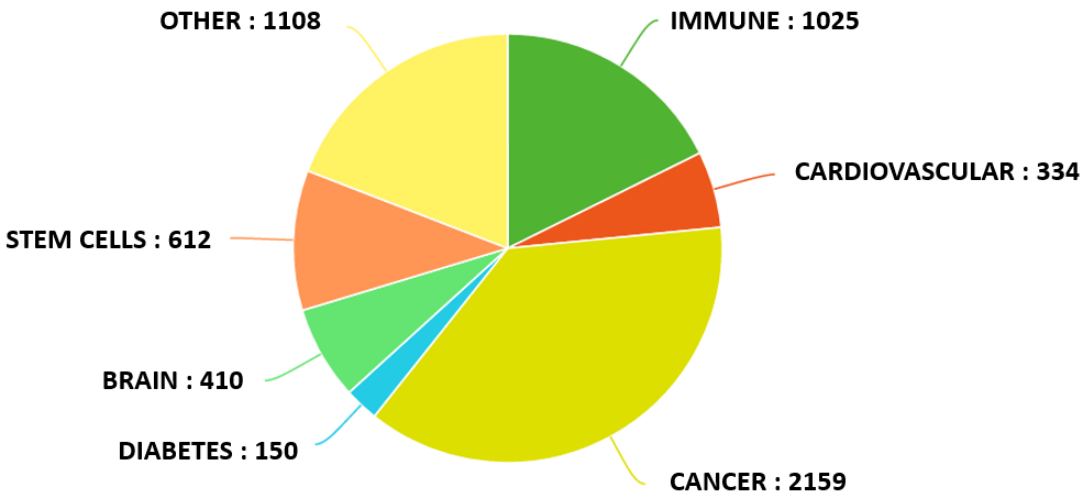


Figure 2–1: The focus of studies in exosome literature. A literature search of PubMed revealed the occurrence of the term “exosome” with another term. “Other” represents the number of studies that contain the term “exosome” without one of the terms listed above.

2. Exosomes in a Nutshell

Extracellular vesicles are categorized into multiple subgroups, of which the most widely studies are apoptotic bodies, microvesicles and exosomes. Extracellular vesicles are functionally and morphologically distinct and are ideally classified in terms of the mechanism of biosynthesis and physical characteristics, such as size. By definition the term exosome refers strictly to extracellular vesicles formed in endosomes and released by exocytosis. Experimentally, subclasses of extracellular vesicles are defined by the method of isolation. Exosomes are distinct from other extracellular vesicles in their biosynthesis, communication and physical characteristics, which will be

briefly introduced (Figure 2-2).

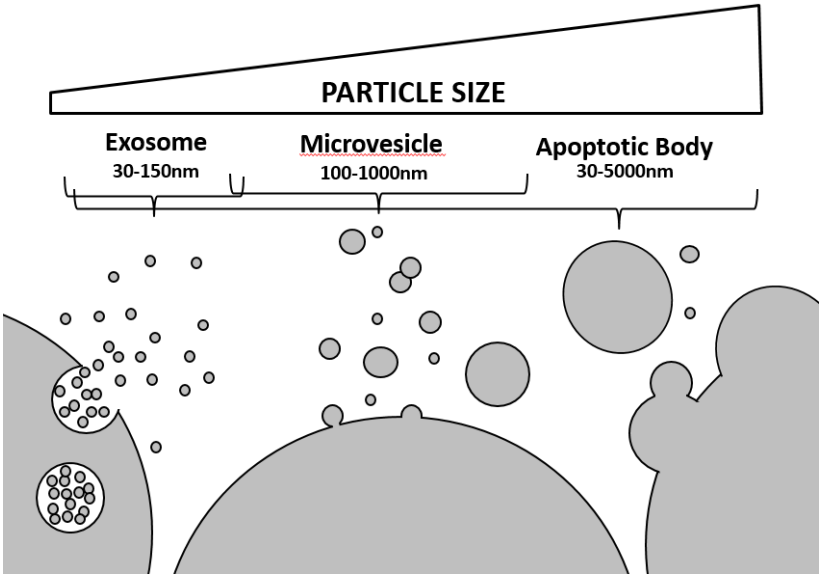


Figure 2–2: Extracellular vesicle size distribution and mechanism of release. Exosomes, microvesicles and apoptotic bodies are distinct vesicles found in the extracellular space. Although vesicles, particularly exosomes, have been defined by their size, it is becoming evident that there may be significant overlap.

2.1 Biosynthesis, Release and Uptake

Exosomes are formed as intraluminal vesicles in the endosomal pathway. Endosomes originate by budding off the *trans*-Golgi network or the plasma membrane into the cytoplasm. As these organelles mature, intraluminal vesicles are formed by inward budding of the endosomal limiting membrane and become exosomes when released by the cell.[143] The process of cargo-loading and vesiculation are regulated processes, resulting in formation of multivesicular bodies with discrete populations of intraluminal vesicles. Posttranslational modification of proteins (especially ubiquitination) and localizing sequences in RNA are reported to play a role in sorting cargo into the vesicles. Lipid raft formation in the endosomal limiting membrane by protein-mediated lipid modifications and lateral segregation of membrane components assist in the budding process of vesicle formation. Seminal studies demonstrated that endosomal sorting complex responsible for transport (ESCRT) protein complexes are central in sorting cargo, membrane budding/scission and membrane trafficking, though

subsequent studies have uncovered ESCRT-independent biosynthetic pathways.[144-147] Cluster of differentiation (CD) and small integral membrane protein of lysosomes and late endosomes (SIMPLE) proteins have been proposed as ESCRT-independent mediators of exosome biogenesis.[148-150] Additionally, cholesterol, ceramide and phosphatidic acid are integral components in lipid rafts which are considered necessary (and possibly sufficient) for forming intraluminal vesicles.[151,152]

Trafficking endosomal membranes is a constitutive and regulated process, which carries intraluminal vesicles to the plasma membrane for secretion or to lysosomes for degradation. Rabs are a family of small GTPases essential for endosomal membrane trafficking, regulating transport along actin filaments and microtubules by molecular motors (kinesin and dynein). Rabs have also been implicated in intraluminal vesicle budding and multivesicular body fusion with the plasma membrane. More than 70 Rab proteins regulate vesicular trafficking in eukaryotes, emphasizing the complexity of this process [Reviewed by Stenmark, 2009].[153] After translocation to the cell periphery, multivesicular bodies fuse with the plasma membrane resulting in externalization of intraluminal vesicles. Membrane fusion is a three step process extensively characterized in synapse vesicles and secretory granules. Initially, the donor membrane (multivesicular body) is (1) tethered to the target membrane (plasma membrane).[154,155] Tethering factors are bound to the inner leaflet of the target membrane and associate with factors on the donor membrane, which loosely and specifically juxtapose the membranes. After tethering, SNARE protein complexes on opposing membranes bind to one another and initiate membrane (2) docking, where the vesicular and plasma membranes become tightly associated.[156] Following docking, SNAREs catalyze (3) fusion of the two membranes. Although the process remains

largely enigmatic, fusion likely involves formation of a fusion pore by a “zipper” action facilitated by the SNAREs.[157]

The general paradigm for regulated uptake suggests that membrane signatures anchor vesicles to the target membrane on recipient cells, which subsequently internalize the exosome or its contents.[158,159] Membrane protein interactions have been illustrated to facilitate recruitment and uptake.[160,161] For example, tetraspanins are cell surface proteins involved in cell adhesion that have been implicated in exosome uptake.[162,163] The Zöller lab reported that the N-terminal of tetraspanin 8 is necessary for exosome uptake into endothelial cells, which may be involved in cancer-activated angiogenesis.[162,164] Proteoglycans, integrins and immunoglobulins are other common protein signatures implicated in exosome uptake.[165-167] Internalization of exosomes or

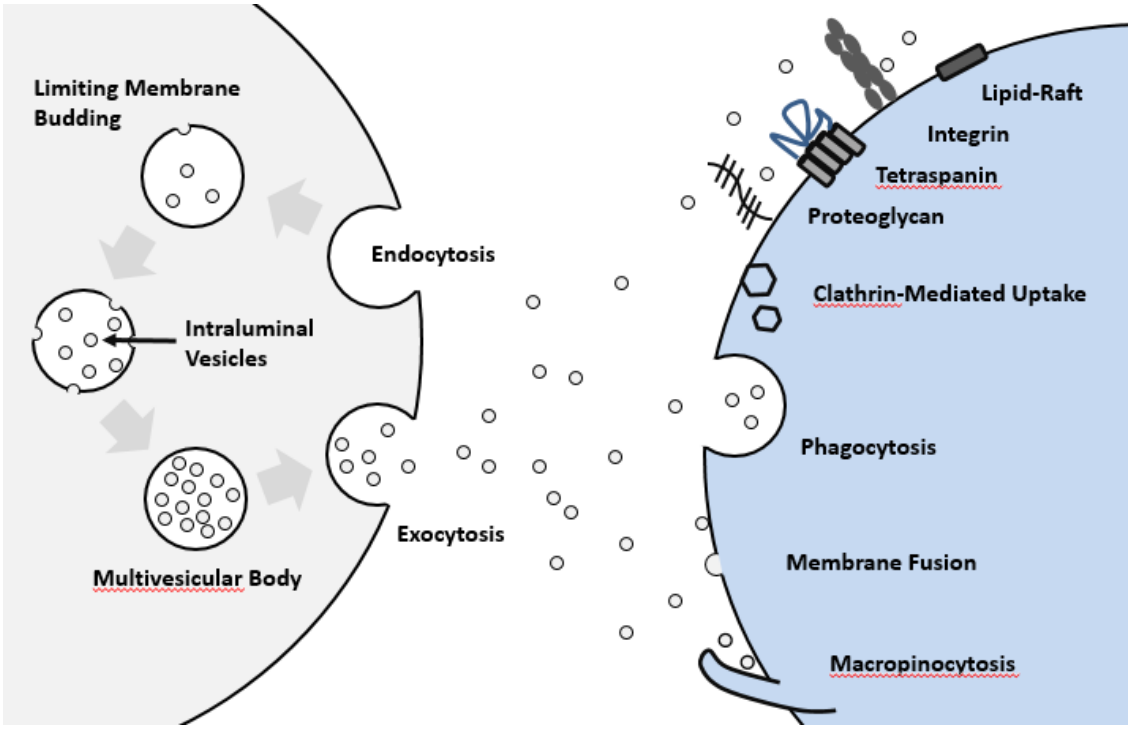


Figure 2–3: Exosome biosynthesis and uptake. Exosomes are formed in endosomes as intraluminal vesicles and subsequently secreted from the source cell by exocytosis into the extracellular space, illustrated in the cell on the left. There are multiple mechanisms involved with exosome uptake, illustrated in the cell on the right.

its contents can be mediated through multiple pathways, most of which are energy dependent, including lipid raft-mediated and clathrin-mediated endocytosis, phagocytosis and micropinocytosis[168-172] (see Figure 2-3).

Exosome biosynthesis, release and uptake are becoming recognized as highly regulated processes. The first evidence of regulation is arguably reported by Harding *et al.* and Pan *et al.* in their seminal studies with reticulocytes, reporting that transferrin receptors were selectively shed from the plasma membrane through exosome secretion.[137,138] Three decades later, there is convincing evidence of exosome regulation, which takes place at nearly every step and may be modified by a variety of stimuli, including extracellular signaling,[173] calcium concentration,[174,175] pH,[176] oxidative stress[177] and temperature.[178] Cellular uptake of exosomes can be cell type-selective or prioritized, a trait that is being explored for use in synthetic exosomes as homing signals.[163] Exosome characteristics may be altered in disease states, especially cancer.[179] Studies report that circulatory exosomes are increased by risk factors for cardiovascular disease including type-2 diabetes,[180] obesity,[181] and senescence.[182] Importantly, transient ischemia has been reported to effect exosome secretion, which will be discussed in section *4-2: Exosomes in Remote Preconditioning*.

2.2 Physical and Molecular Characteristics

*E*xosomes have diverse characteristics, particularly with respect to molecular composition. Exosome composition is determined by a variety of factors including their cellular origin (i.e., derived from platelets versus endothelial cells versus muscle cells, etc.), subcellular localization and cell activation. ExoCarta, a public bioinformatics platform, has compiled >22,000 proteins, RNAs and lipids (October 2016) discovered in exosomes. Ultimately, the diversity in composition and other physical features results in

a heterogeneous population of vesicles which are difficult to define. Moreover, many of these properties are not exclusive to exosomes. Nonetheless, exosomes are classified by a subset of common properties, introduced below.

Exosomes are most commonly classified by size (30 to 150nm), as typically measured with electron microscopy or nanoparticle tracking analysis. Early electron microscopy suggested that exosomes are cup shaped[183], however improved sample preparation revealed their innate, spherical structure.[184] Exosomes have two functional compartments; the vesicle membrane and the cytosolic lumen. The exosomal membrane is derived from the parent cell plasma membrane and although there are similarities in composition[142], the lipid and protein content is modified. Many studies report that exosome membranes have the properties of lipid rafts; high liquid-order[185], enrichment with cholesterol, sphingomyelin and ganglioside GM3[186,187] and markers for detergent resistant domains.[188,189] Additionally, exosomal membranes, unlike their parent cell membrane and other nano-vesicles, are not asymmetrical and phosphatidylethanolamine is distributed in both the inner and outer membrane leaflet.[190] Phosphatidylserine has also been reported in the outer leaflet allowing detection with annexin-5, however these findings are inconsistent and possibly a trait specific to parent cell-type.[191,192]

Exosomes are also regularly classified by marker proteins. Exosome formation is a distinct process; accordingly, the presence of proteins from the biosynthetic pathway (flotillin, Alix, TSG101) are considered to be hallmarks that distinguish exosomes from other extracellular vesicles. Other structural and transmembrane proteins such as the tetraspanins CD63, CD9 and CD81 are also common exosome markers. Intraluminal vesicles encapsulate cytosol during formation and consequently

exosomes contain small molecules present in the cytosol such as metabolites.[193] Nonetheless, there have been no signatures established by proteomic, metabolomics, lipidomic or transcriptomic studies that exclusively distinguish exosomes from other extracellular vesicles.

3. Methods for the Isolation and Characterization of Secreted Exosomes

A large variety of experimental techniques have been developed to address the demands in exosome research, however techniques (especially isolation) may affect experimental outcome. To promote the use of suitable techniques the field has been divided into three subcategories based on experimental objectives; discovery, diagnostics and preparative research.[13] In the interest of brevity, experimental techniques will be introduced, with a focus on techniques relevant to this study.

3.1 Methods and Techniques: Isolation

Exosomes have been isolated using multiple methods, with the goal of yielding a vesicular population of functional/intact exosomes without contamination (e.g. devoid of protein aggregates and non-exosome vesicles). Current techniques have been combined and refined in an attempt to improve isolation, however the process of isolating exosomes has limitations and no perfect method has been developed. The most frequently used method of isolation is differential centrifugation, which isolates particles by size, density and sedimentation distance. Exosomes are among the smallest extracellular vesicles, and thus require the highest force for sedimentation.[194] Large structures are sedimented by low force centrifugation (2000*xg* for whole cells followed by 10,000-20,000*xg* to sediment debris and large vesicles) and exosomes are subsequently pelleted by high force ultracentrifugation (100,000-120,000*xg* for 1-2 hours). Sucrose density gradients or cushions separate particles by buoyant density to

improve isolation specificity, however the purity may still be inadequate for certain applications. Exosomes can be isolated by size exclusion, where molecular weight cutoff ultrafiltration or chromatography is used to separate particles by size. Commercial isolation kits have been developed, utilizing polymeric precipitation and low force centrifugation to sediment exosomes. Membrane proteins can also be targeted for immunoaffinity capture to isolate exosomes with more specificity, which is valuable in optimizing the purity of the preparation.[194]

3.2 Methods and Techniques: Characterization

Exosomes are characterized based on morphology (size, shape, etc.), biogenesis, function and composition. In characterizing exosomes, the technique used to study the particles will depend on the characteristics of interest. Electron microscopy, dynamic light scattering, atomic force microscopy and nanoparticle tracking analysis are used to provide insight into exosome concentration, size distribution, and shape.[195] Electron microscopy is valuable in determining the size of exosomes, and has provided evidence of the mechanism of biogenesis, release and uptake of exosomes.[139] With regard to function, fluorescent lipid dyes and membrane permeant dyes have been used to monitor exosome uptake with confocal microscopy,[159] while proteomic, lipidomic and transcriptional analyses have been employed to reveal the molecular composition of vesicles.

Although there is an expanding repertoire of techniques, experimental limitations have impaired our understanding of exosomes, resulting in inconsistencies in the literature. The *International Society for Extracellular Vesicles* has called for technical requirements in an attempt to standardize experimentation and unite the extracellular vesicle field.[196] In the meantime, understanding the techniques used to isolate and

characterize a particular extracellular vesicle type should take precedence over how they are classified. The experimental limitations are discussed in 3.3 Experimenting with Exosomes: Technical Limitations.

3.3 Experimenting with Exosomes: Technical Limitations

In this chapter, we have presented “common properties” of exosomes, however experimental limitations may influence our general understanding of exosomes and how they are defined or classified. Understanding these limitations is important for designing studies, interpreting data and comparing results. Perhaps the most important experimental limitations involve isolation techniques, because inefficient collection of exosomes could affect subsequent characterization or quantification. Quality and yield are the priority when isolating exosomes; however no isolation technique is ideal.[2,3] For example, in this dissertation work we use differential ultracentrifugation, which is the most common isolation technique. Although ultracentrifugation reliably isolates exosomes, there are two major limitations; (1) Non-exosome particles contaminate the exosome rich pellet,[13,2] and (2) the traditional ultracentrifugation will not sediment exosomes entirely, resulting in a partial exosome preparation.[19] Characterization and quantification of exosomes is another process that is limited by experimental techniques. A commonly measured characteristic in exosome research is vesicle quantity or concentration. Quantifying exosome is difficult however, because often times contaminating proteins and non-exosome vesicles will obscure the true exosome measurements.[23] Additionally, exosome markers will provide qualitative assessment of exosome content, however because they are not exclusive to exosomes nor ubiquitously present in all exosomes, they cannot be used for accurate quantification.[24] In addition to these examples, there are many other techniques for studying exosomes

and their inherent limitations can be minimized by choosing the appropriate technique based on experimental objectives.

4. Exosomes: A Delivery Service for Remote Preconditioning

Exosomes have been purported to be cardioprotective in the context of treating ischemia reperfusion injury.[197-199] However, in 2014, Giricz *et al.* were the first to demonstrate that exosomes are involved in remote preconditioning.[200]

4.1 Treating Myocardial Infarction with Exosomes

Exosomes were recognized for cardioprotective capabilities before their implication in remote preconditioning. In the early 2000s, stem cells garnered attention for their potential in regenerating damaged tissue, including infarcted myocardium. Initial theories predicted that implanted stem cells would differentiate into cardiac cells, replace damaged tissue and improve function.[201-203] Indeed, stem cells have been shown in some studies to favorably modify post-infarction left ventricular remodeling, promote neo-vascularization and improve cardiac function. [204-207] However, subsequent studies reported that implanted stem cells do not differentiate into cardiac cells and that beneficial tissue regeneration that was reported previously can in all likelihood be attributed to paracrine signaling from the stem cells.[208-219] In fact, some studies suggest that paracrine signaling from stem cells functions through cardioprotective signaling (to improve inotropy, reduce reactive oxygen species formation and limit apoptosis by activating pro-survival signaling) rather than/in addition to reparative signaling.[220-222] In 2010, Lai *et al*. reported that exosomes are directly involved this component of stem cell therapy.[197,198] Arslan and colleagues corroborated these findings, reporting an infarct limiting effect of mesenchymal stem cell exosomes.[199] The cardioprotective capacity of stem cell derived exosomes continues to receive considerable attention.[223-225]

What evidence suggests that remote preconditioning can elicit cardioprotection via exosomes in the absence of stem cell therapy? In other words, does tissue that is typically subjected to ischemic conditioning (myocardium, skeletal muscle) secrete exosomes with cardioprotective qualities? Two lines of evidence support this concept. First, exosomes from non-stem cells have been implicated in communicating survival signals between cells. For example, Guitart *et al.* reported that exosomes from astrocytes are involved in conferring resistance against oxidative stress in cultured neurons,[226] while Song *et al.* illustrated that exosomes from cancer cells prevent monocyte apoptosis by activating the MAPK signaling cascade.[227] These same survival mechanisms are activated by remote preconditioning to prevent cardiomyocyte apoptosis. Importantly, cardiomyocytes have been shown to accept information from non-stem cell exosomes which mediate hypertrophy resistance following MI.[228] Second, hypoxia and ischemia (i.e. ischemic preconditioning stimulus) promote exosome secretion and modify exosome cargo, such as miRNA-30a[229], TNF-α[230], HSP20[231], HSP60[232]. Yamaguchi *et al.* reported that levels of insulin-like growth factor-1 and miR-29a in exosomes was increased by chronic remote preconditioning and may be involved with improved functional recovery and wound healing following MI.[233] Eldh *et al.* reported that exosomes secreted from mast cells subjected to an oxidative challenge are able to promote resistance against oxidative stress in target cells.[234] However, and in contrast, Li *et al*. found that miR-144 – a cardioprotective factor – was increased in the exosome-free (rather than exosome-rich) fraction of plasma following remote preconditioning, refuting the exosome theory.[235]

4.2 Exosomes in Remote Preconditioning

As discussed in the previous chapter, discovering the circulatory component

contributing to remote preconditioning, remains an important step in harnessing its cardioprotective power. In 2014, Giricz et al. reported that exosomes are necessary and sufficient for cardioprotection by remote preconditioning.[200] In this study, coronary perfusate was collected from *ex vivo* donor hearts after an ischemic conditioning protocol (3X 5-min episodes of global ischemia, interrupted by 5 min reperfusion). Recipient hearts were then pretreated for 30 min with donor coronary perfusate, or donor perfusate following exosome depletion (achieved by centrifugation), and subjected to a prolonged, 30 min period of sustained global ischemia. Pre-ischemic transfer of conditioned coronary perfusate was cardioprotective (i.e., reduced infarct size in the acceptor hearts when compared with controls); however, depletion of exosomes from the perfusate eliminated efficacy. These results strongly suggest that exosomes are requisite for remote preconditioning mediated cardioprotection against ischemia/reperfusion injury.

A limitation of the Giricz study is that no direct evidence was provided to show that the ischemic preconditioning stimulus causes the release of exosomes into the circulation. This issue was addressed in a subsequent protocol by Vicencio and colleagues who reported that remote preconditioning causes an increase in circulating exosomes, but, interestingly, concluded that exosomes per se provided protection regardless of whether a remote preconditioning stimulus had been applied.[236]

5. Summary and Future Directions

Interest in exosomes has surged in recent years, reflecting their seemingly ubiquitous role in biology and their potential in medical application. Exosomes have been implicated remote preconditioning and, although supporting evidence is limited and disputed, they may provide mechanistic insight into the humoral component of

cardioprotection.

The quest to identify the cardioprotective signal released by remote preconditioning is ongoing. Exploratory and functional studies have generated many candidates, but, to date, no unifying theme for the communication component of remote ischemic conditioning has been developed. Involvement of exosomes in humoral communication of remote preconditioning may facilitate the effort to elucidate the identity of the protective factors and promote successful clinical application. Theoretically, exosomes are ideal for protecting and transferring remote preconditioning signals between tissues. The first step is to verify the involvement of exosomes; future studies can focus on harnessing the therapeutic potential of exosomes.

CHAPTER 3: STUDY OVERVIEW

As discussed in Chapter 1, the prevalence of type-2 diabetes is increasing rapidly on a global scale. The diabetic population is especially susceptible to myocardial infarction and, despite significantly improved survival following a coronary event, the resulting morbidity and mortality remains unacceptably high. It is clear that type-2 diabetic patients are particularly dependent on development of novel cardioprotective strategies to treat AMI, and improve clinical outcome. Despite clinical appeal and promising experimental success, ischemic conditioning, a leading strategy for cardioprotection, appears to be ineffective in multiple comorbid states.

Remote preconditioning has had encouraging preclinical results, consistently showing potent infarct-sparing effects in many animal models, between species and in multiple tissues. Efficacy in human trials is less convincing however, which has impaired translation to clinical application – a fate consistent with every previous cardioprotective candidate. Nonetheless, preclinical success and compelling clinical advantages have prompted the effort to advance our understanding of remote preconditioning and strive to improve efficacy in the clinical setting. We argue that addressing two fundamental issues will help to facilitate translation of remote preconditioning; (I) evaluating the impact of comorbidities on therapeutic efficacy and (II) characterizing the mechanism that communicates protection across a spatial barrier. Given this background, my dissertation will address three objectives.

Objective 1: Evaluate the Efficacy of Remote Preconditioning in Type-2 Diabetes

As reviewed in Chapter 1, there is evidence that the efficacy of ischemic preconditioning and postconditioning is either attenuated or eliminated in animal models of type-2 diabetes. However, no studies to date have assessed whether the

cardioprotective effect of ***remote*** conditioning is lost or maintained in type-2 diabetic cohorts. Accordingly, the primary aim of this study was to evaluate the efficacy of remote preconditioning in the well-established Zucker rat model of type-2 diabetes.

Our goal was to focus on endpoints that have translational relevance. The priority was the measurement of infarct size, the primary determinant of clinical outcome following AMI and the traditional standard for quantifying cardioprotection. Hyperglycemia is a fundamental characteristic of diabetes, and although its effect on cardioprotection is debated, evidence suggests that it plays a role in the suppression of ischemic conditioning. We measured blood glucose to assess the effect of hyperglycemia on cardioprotection by remote preconditioning. Importantly, we utilized a model of early-stage diabetes to target moderate hyperglycemia – rather than the supra-clinical hyperglycemia often seen with the later stages in rodent models of diabetes. We hypothesize that *Remote Preconditioning is Ineffective in Early-stage Type-2 Diabetes In Vivo* (see Chapter 4).

Objective 2: Investigate the Role of Exosomes in Remote Preconditioning

As reviewed in Chapter 1, remote preconditioning transmits protection across a spatial barrier, with humoral signaling implicated in at least some models to play a role. Exosomes are extracellular particles found in the circulation which contribute to cell-to-cell communication. Although evidence is limited, these extracellular vesicles have been proposed to play a role in the humoral component of remote preconditioning. Our second objective was to investigate the role of exosomes in remote preconditioning by treating cultured cardiomyocytes with exosomes collected *in vivo* from ischemic conditioned serum. We hypothesized that *Exosomes Serve as a Transport Mechanism of Protection in Remote Preconditioning* (see Chapters 5 and 6).

Objective 3: Characterize the Defect in Remote Preconditioning Caused by Type-2 Diabetes

The final objective was to investigate the basis of the loss in cardioprotection associated with type-2 diabetes. The majority of studies have focused on the effect type-2 diabetes has on signaling cascades and end-effectors in cardiomyocytes, which may desensitize the myocardium to ischemic conditioning. Less understood is the effect of diabetes on the humoral component of protective signaling in remote preconditioning. Our final objective was to evaluate the consequences of type-2 diabetes on the humoral component (serum and circulatory exosomes) of remote preconditioning in rats. We hypothesized that *Type-2 Diabetes Inhibits Remote Preconditioning by Causing a Defect in Protective Signaling through the Serum and Exosomes* (see Chapter 6).

CHAPTER 4: HYPOTHESIS 1: REMOTE PRECONDITIONING IS INEFFECTIVE IN EARLY-STAGE TYPE-2 DIABETES *IN VIVO*

1. Rationale

Emerging evidence indicates that type-2 diabetes affects infarct size, a primary metric for AMI injury, potentially by (I) preventing cardioprotection from ischemic conditioning and/or (II) increasing the sensitivity of the myocardium to ischemia-reperfusion. While preclinical investigations have reported resistance to ischemic preconditioning and postconditioning in the setting of diabetes, evidence of the effect of type-2 diabetes on remote preconditioning is limited. Pre- and postconditioning have significant mechanistic overlap with remote conditioning (i.e. receptor-ligand interactions, intracellular survival kinase cascades and end-effectors: see Chapter 2) which suggests that remote conditioning may, similarly, be compromised in the setting of diabetes.

Accordingly, we hypothesize that the efficacy of infarct size reduction with remote preconditioning is reduced or eliminated in type-2 diabetes. Additionally, we hypothesize that type-2 diabetes sensitizes the myocardium to ischemia/reperfusion, resulting in larger infarct sizes when compared with normoglycemic controls. We investigated our hypothesis using an *in vivo* model of acute myocardial ischemia and reperfusion in a translationally relevant model of diabetes.

2. Materials

Male Zucker rats were purchased from Harlan Laboratories, Inc. (Indianapolis, IN). Unisperse blue pigment was a generous gift from Ciba Geigy Corp. (Hawthorne, NY). We used an Accu-Chek Aviva blood glucose meter and test strips from Roche Diagnostics (Indianapolis, IN), and 5-0 proline (12 inch with curved needle) and spooled 3-0 silk suture from Ethicon (Somerville, NJ). Triphenyltetrazolium chloride, syringe

filters (0.2μm), neutral buffered formalin (10%) and saline (0.9% NaCl) were purchased from Fisher Scientific International, Inc. (Pittsburgh, PA).

3. Methods

This study, including subsequent chapters that use animal protocols, was approved by the Institutional Animal Care and Use Committee of Wayne State University and strictly abided by the *Guide for the Care and Use of Laboratory Animals* from the Institute of Laboratory Animals Resources (NIH Publication Vol. 25 No. 28, revised 1996).

3.1 Zucker Rat Model of Type-2 Diabetes

The Zucker Diabetic Fatty (ZDF-Lepr^{fa}/Crl) rat is a genetic model of type-2 diabetes, which is the most common form of the disease in humans. The Zucker Diabetic Fatty rat was derived through selective inbreeding for hyperglycemia in the Zucker Fatty (*fa/fa)* rat[237], a strain with a mild diabetic phenotype caused by a missense mutation in the leptin receptor gene.[238] This homozygous mutation causes a predictable and gradual development of severe diabetes, characterized by progressive development of insulin resistance, hyperglycemia, obesity and hyperlipidemia. For clinical relevance, we have targeted the early stages of the progression to overt diabetes by using male rats between 10-12 weeks of age. Enrollment was limited to males, as female Zucker Fatty rats are not likely to develop hyperglycemia unless fed a high-fat diet.[237,239,240] In contrast, age-matched Zucker Lean rats (the genetic control strain) are homozygous or heterozygous for the wild-type gene, and are phenotypically normoglycemic and non-obese.[238] All animals were housed at room temperature on a 12-hour light/dark cycle and fed tap water and regular rodent chow, *ad libitum*.

3.2 *In Vivo* Model of Acute Myocardial Infarctin: Ischemia/Reperfusion

Zucker Diabetic Fatty rats and age matched normoglycemic Zucker Lean rats were subjected to left coronary artery occlusion and reperfusion using methods routinely employed by our laboratory and by others. Briefly: animals were anesthetized with pentobarbital sodium (40 mg/kg intraperitoneal, supplemented as required to maintain a deep surgical plane of anesthesia [on the order of 10 mg/kg/hour]). Rats were positioned on a temperature-controlled heating pad, a tracheostomy was performed and the lungs were ventilated with room air. The heart was exposed via a thoracotomy in the fourth intercostal space to expose the basal region of the heart, and the left coronary artery was ensnared by taking an intramyocardial stitch with a C-1 taper needle and 5-0 polypropylene suture from the atrioventricular groove to the region of the pulmonary cone. Additional sutures were tied to each arm of the stitch suture to facilitate later release of the occlusion knot.[241,242]

Myocardial ischemia was induced by tying a knot in the occlusion suture and maintained for 45 minutes, and was verified by tissue pallor, changes in the ECG tracing and arrhythmia. Reperfusion was initiated by pulling on the release sutures and verified by the return of tissue blush. Blood glucose concentration was measured immediately before the onset of coronary occlusion using the Accu-Chek monitor and test strips.

3.3 Remote Preconditioning Treatment Groups

Zucker Lean and Zucker Diabetic Fatty rats were randomly allocated into two treatment groups; sham operated controls and remote preconditioned. All animals underwent bilateral femoral artery dissection and isolation. This was followed by a 40-minute intervention period, during which rats underwent either; (1) Remote

preconditioning: animals were subjected to 4-5 minute cycles of bilateral femoral artery occlusion, interspersed with 5 minutes reperfusion, followed immediately by coronary occlusion, seen in Figure 4–1; or (2) A time-matched, 40-minute control period with no femoral occlusion, followed by coronary occlusion, Figure 4–1.

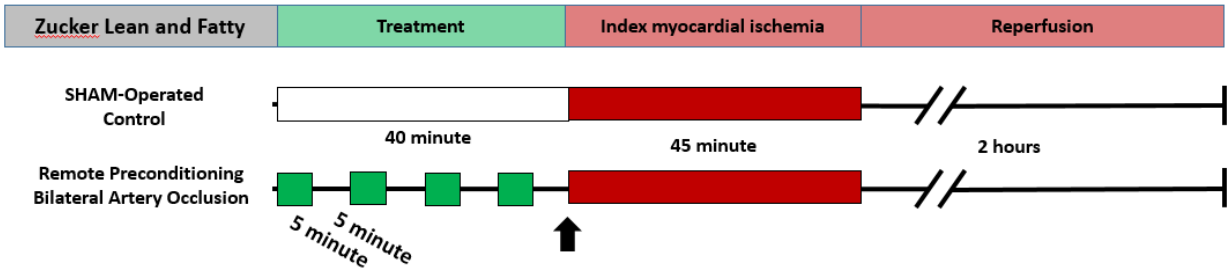


Figure 4–1: Treatment schematic. Zucker Lean and Zucker Diabetic Fatty rats received sham operation or remote preconditioning. The red box represents coronary artery occlusion, green boxes represent bilateral femoral artery occlusion, the white box represents matched time with no occlusions. Infarct size was assessed after 2 hours of coronary reperfusion. The arrow denotes timing of the blood glucose measurement.

3.4 Tissue Processing and Imaging

After 45 minutes of ischemia and 120 minutes of reperfusion, the occluding knot was re-tightened and an intravenous injection of 0.5 mL Unisperse Blue pigment was administered to delineate the area at risk (AR): i.e., the region of the heart distal to the occlusion and devoid of blue dye. Subsequently, the heart was arrested in diastole (by intravenous injection of saturated KCL) and excised, sectioned transversely and the right ventricle removed. Photographs were taken of the sections for later quantitation of AR. Immediately thereafter, the sections were incubated in 2,3,5-triphenyltetrazolium chloride (TTC) for 15 minutes at 37°C, followed by photographic documentation. Using this method, viable tissue is stained red while the necrosis remains unstained so appears pale, examples shown in Figure 4–2A. Area at risk and infarct size were analyzed in a blinded manner using image analysis software (SigmaScan Pro SPSS

Inc., Chicago IL) and measurements were corrected for tissue weight. Area at risk is reported as a % of the left ventricle and infarct size is reported as a % of the area at risk.

3.5 Data and Statistic Analysis

Data were analyzed with GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA). Endpoints were compared by student t-test (for 2-group comparisons) or one-way Analysis of Variance (ANOVA: for 4-group comparisons). If significant F-values were obtained by ANOVA, post-hoc pairwise comparisons between groups were made with the Newman-Keuls test. Results are presented as means ± SEM. For the cohorts that underwent remote preconditioning, linear regression was used to analyze the relationship between blood glucose and infarct size.

4. Results

4.1 Remote Preconditioning is ineffective in Early-stage Type-2 Diabetes

Area at risk (the primary determinant of infarct size in the rat model) did not differ among groups: mean values were 20 ± 2%, 18 ± 2%, 23 ± 1%, and 19 ± 2% of the total LV weight in the Control-Lean, RIPC-Lean, Control-Fatty and RIPC-Fatty cohorts, respectively (Figure 4–2B).

In untreated control animals, infarct size (i.e., area of necrosis, expressed as a % of the area at risk) was comparable in both strains, averaging 63 ± 6% in the Zucker Lean group and 60 ± 9% in diabetic Zucker Fatty rats. As expected, in the Zucker Lean cohort, remote preconditioning was cardioprotective: infarct size was significantly smaller in the RIPC-Lean group when compared with the Lean-Controls (10 ± 3%* versus 63 ± 6%, respectively, *$p<0.01$ vs Lean Controls, see Figure 4–2c). In contrast, remote preconditioning was ineffective in the diabetic strain: there was no difference in

infarct size between the RIPC-Fatty and the Control-Fatty groups (60 ± 9% vs 65 ± 4% respectively), Figure 4–2C.

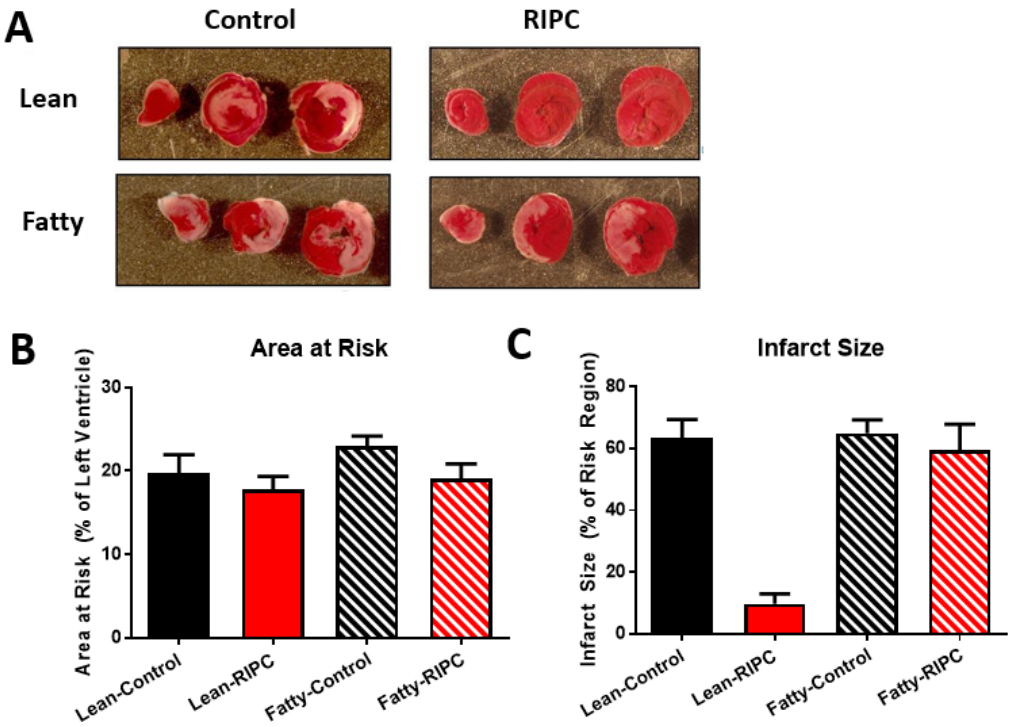


Figure 4–2: Efficacy of remote preconditioning is lost in type-2 diabetes. A. Original images of rat hearts from Zucker Lean and Zucker Fatty animals assigned to receive remote ischemic preconditioning (RIPC) or no intervention (Control). Hearts were cut into transverse slices and stained with triphenyltetrazolium chloride; viable tissue stains red whereas necrotic myocardium remains unstained and appears pale**. B.** Area at risk, expressed as a % of the total LV weight (mean ± SEM) **C.** Infarct size, expressed as a % of the risk region (mean ± SEM). * P<0.01 vs. Lean-Control, N= 6-9/group.

4.2 Hyperglycemia Alone Does Not Explain the Loss in Efficacy of Remote Preconditioning

As expected, the pooled cohort of diabetic Zucker Fatty rats (including both Control and RIPC groups) was characterized by moderate but significantly elevated non-fasting blood glucose values when compared with the Zucker Lean rats (278 ± 15 vs 150 ± 12 mg/dl, P<0.01); (Figure 4–3A). To obtain insight into whether the loss in efficacy of remote preconditioning in Zucker diabetic rats was simply due to hyperglycemia, infarct size was plotted as a function of blood glucose concentration for

all animals (both Zucker Fatty and Zucker Lean) treated with remote preconditioning (Figure 4–3B). If this concept were true, one would expect that all data points would fall along a single regression line: i.e., rats with the highest blood glucose values would develop the largest infarcts. This was not supported by the data; in rats treated with remote preconditioning, there was no association between blood glucose concentration and infarct size.

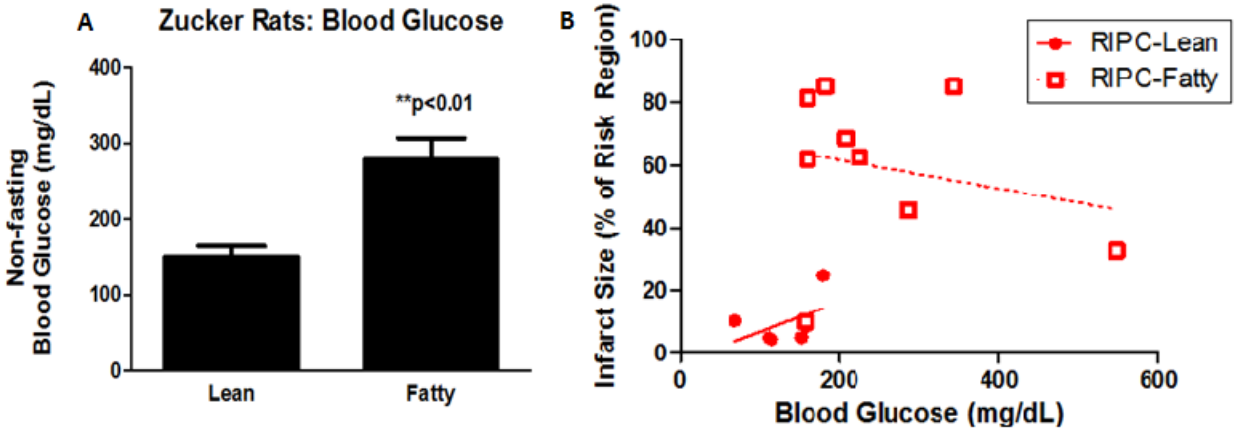


Figure 4–3: Failure of remote preconditioning to reduce infarct size in the diabetic Zucker cohort is not associated with hyperglycemia. A. Blood glucose for Zucker Lean and diabetic Zucker Fatty rats measured immediately before prolonged ischemia (mean ± SEM). B. Infarct size plotted as a function of blood glucose concentration for all rats (Zucker Lean and Zucker Fatty) treated with remote preconditioning. RIPC, remote preconditioning.

5. Summary

In summary, we report that, in this rat model of type-2 diabetes, there is no increase in the sensitivity of the myocardium to ischemia-reperfusion *per se*. This is supported by the observation that infarct size was similar (not increased) in Control Zucker Fatty animals when compared with Control Zucker Lean animals. Second, and most importantly, remote preconditioning is defective in the Zucker model of type-2 diabetes, providing no protection against lethal ischemia/reperfusion injury *in vivo*. This is demonstrated by our finding that, while remote preconditioning had the expected, potent infarct-sparing effect in Zucker Lean rats, this protective effect was absent in

diabetic Zucker Fatty rats. Finally, we report that the defect in remote preconditioning does not appear to be caused exclusively by hyperglycemia. Our data support this conclusion, as we show no overall association between blood glucose concentration and infarct size in RIPC-treated rats.

CHAPTER 5: HYPOTHESIS 2: THE TRADITIONAL ULTRACENTRIFUGATION TECHNIQUE FOR EXOSOME ISOLATION IS INADEQUATE TO ISOLATE PROTECTIVE FACTOR(S) FROM REMOTE PRECONDITIONING

1. Rationale

As discussed in Chapter 3, protection from remote preconditioning is communicated from the site of the ischemic conditioning stimulus to the at-risk myocardium. Convincing evidence indicates that a humoral factor(s) is, at least partially, involved with overcoming this spatial barrier; however the identity of the protective factor remains unknown.

Exosomes are nano-sized extracellular vesicles involved with intracellular communication (see Chapter 2). Exosomes have garnered recognition for their potential as a delivery system, providing protected and targeted transportation of both endogenous, exogenous[243,244] and synthetic[245] cargo. Recent evidence has implicated exosomes as the carrier mechanism for the protective humoral factor(s) involved in remote preconditioning; Giricz *et al.* proposed that exosomes are required for the reduction of infarct size achieved by treating naïve hearts with coronary perfusate from preconditioned, buffer-perfused hearts.[200] Vicencio *et al.* later reported that exosomes isolated from blood plasma are sufficient to elicit protection, regardless of whether samples were from sham or remote preconditioned subjects.[236] It is clear that exosomes have the potential to participate in cardioprotective signaling; however their role in remote preconditioning remains poorly characterized. Our objective was to investigate the mechanistic role of circulatory exosomes in remote preconditioning.

We hypothesized that protection from remote preconditioning could be transferred to cultured cardiomyocytes through blood serum, a phenomenon described previously.[53] Additionally, based on findings reported by Giricz *et al.*, we hypothesized

that the protective component in serum is transported by exosomes. We predicted that exosomes could be isolated from remote preconditioned serum and transported to cardiomyocytes to promote resistance against lethal hypoxia/reoxygenation injury. To test our hypothesis, we measured the protective effect of remote preconditioned serum, exosome-rich serum and exosome-depleted serum, with the latter two fractions obtained using the traditional ultracentrifugation technique for isolating exosomes.

2. Materials

Cultured HL-1 cardiomyocytes were a generous gift from Dr. William Claycomb (Louisiana State University Health Science Center, New Orleans, LA). Claycomb medium, 0.05% trypsin/EDTA, fetal bovine serum (FBS), ascorbic acid, penicillin/streptomycin, L-glutamine, norepinephrine, gelatin, fibronectin, HEPES, sodium-lactate, sodium bicarbonate, KCL, and Krebs-Henseleit (KH) buffer were purchased from Sigma-Aldrich, Inc (St Louis, MO). Sprague Dawley retired breeder rats were purchased from Harlan Laboratories Inc. (Indianapolis, IN). Mini-PROTEAN precast PAGE gels, sodium dodecyl sulfate (SDS), urea, dithiothreitol, Tris base, nitrocellulose membranes, Triton X-100 and Tween 20 were purchased from Bio-Rad Laboratories (Hercules, CA). Primary and secondary antibodies used for immunoblot analysis were purchased from Cell Signaling Technology (Danvers, MA).

3. Methods

3.1 Culturing HL-1 Cardiomyocytes

HL-1 cells are an immortalized mouse cardiomyocyte cell line, derived from a murine atrial tumor, that maintain their morphology and contractile function through repeated passaging.[246] Cells were maintained at 37°C in 5% CO_2 and 95% room air with ~95% humidity. Claycomb culture medium, supplemented with 10% FBS,

penicillin/streptomycin (respectively, 100 U/ml; 100 µg/ml), L-glutamine (2mM final) and norepinephrine (0.01mM final) was replaced daily and cells were sub-cultured when they reached 100% confluency, as previously described.[246] HL-1 cells were passaged as directed by the protocol (Multi Channel Systems, Reutlingen, Germany). Briefly: flasks were washed twice with trypsin (0.05% with EDTA) and incubated with trypsin until cells were dislodged. Cells were collected from the flask and washed with culture medium (contains trypsin inhibitor). The cells were then pelleted by centrifugation at 500xG for 5 minutes, resuspended in culture medium and re-plated in flasks pre-coated with gelatin/fibronectin.

3.2 Hypoxia/Reoxygenation

HL-1 cells grown in non-coated, tissue culture TC-treated 60 mm culture dishes, were subjected to 2.5 hours hypoxia and 20 hours of reoxygenation when they reached ~90% confluency. Hypoxia was established by replacing culture medium with hypoxia buffer (mM: 125 NaCl, 8 KCl, 1.2 KH2PO4, 1.25 MgSO4, 1.2 CaCl2, 6.25 NaHCO3, 20 HEPES, 5.5 glucose, 20 2-deoxy-D-glucose, 5 Na-lactate; preheated to 37°C and adjusted to pH 6.6), sealing culture dishes in a hermetic chamber with GasPak EZ Gas Generating Sachets (GasPakTM EZ, BD Biosciences, San Jose, CA) and incubating the chamber at 37°C. Cells were reoxygenated by removing dishes from the hypoxia chamber, replacing the hypoxia buffer with serum-free Claycomb media (preheated to 37°C), and incubating per usual in room air at 37°C.

3.3 Trypan Blue Cell Death Assay

Twenty hours after hypoxia/reoxygenation, all HL-1 cells were collected, including adherent live and dead cells (by trypsinization) and non-adherent cells. The cells were pelleted by centrifugation (500g for 5 minutes) and resuspended in 2 mL

Claycomb media to yield 2-3 million cells per mL). The cell suspension was stained with 0.4% Trypan Blue dye (1:1 ratio of cell suspension:dye) to distinguish viable from dead cells. Trypan blue dye is membrane impermeable and thus cannot penetrate viable cells with intact plasma membrane while, in contrast, dead and dying cells have damaged plasma membranes, allowing trypan blue to accumulate within the cell. Stained cell suspensions were visualized with phase contrast microscopy at 10x magnification (inverted microscope: Fisher Scientific, Pittsburgh, PA); viable and dead cells (appearing unstained and blue, respectively) were counted using a standard hemocytometer. Percent cell death was calculated as:

$$\text{Cell death (\%)} = (\text{dead cells} / (\text{dead cells} + \text{live cells})) \times 100$$

3.4 Remote Preconditioning and Serum Collection

The goal of the dissertation is to assess the exosome hypothesis of remote conditioning in Zucker Lean and diabetic Zucker Fatty rats. However, as a first step (and as described in this Chapter), Sprague Dawley rats were used for preliminary studies to validate the protocol for serum transfer to HL-1 cells and proof-of-concept for exosome isolation and exosome transfer to HL-1 cells.

Rats were anesthetized with pentobarbital sodium (40 mg/kg intraperitoneal), ventilated with room air and maintained on a temperature-controlled heating pad. All animals underwent femoral artery isolation with blunt dissection as described in Chapter 4, and were subsequently allocated into two treatment groups; remote preconditioned animals received brief repeated episodes of femoral artery occlusion (4x 5-minute ischemia and 5-minute reperfusion) and the sham-operated group received 40 minutes without femoral artery occlusion (see Figure 5–1). Following remote preconditioning or the time-matched sham period, a thoracotomy was performed and 6-8 mL of blood was

rapidly collected from the left ventricle via cardiac puncture with an 18-guage needle. Blood was allowed to coagulate at room temperature for 20 minutes and serum was isolated by centrifugation at 2,000xG for 20 minutes at 4°C.

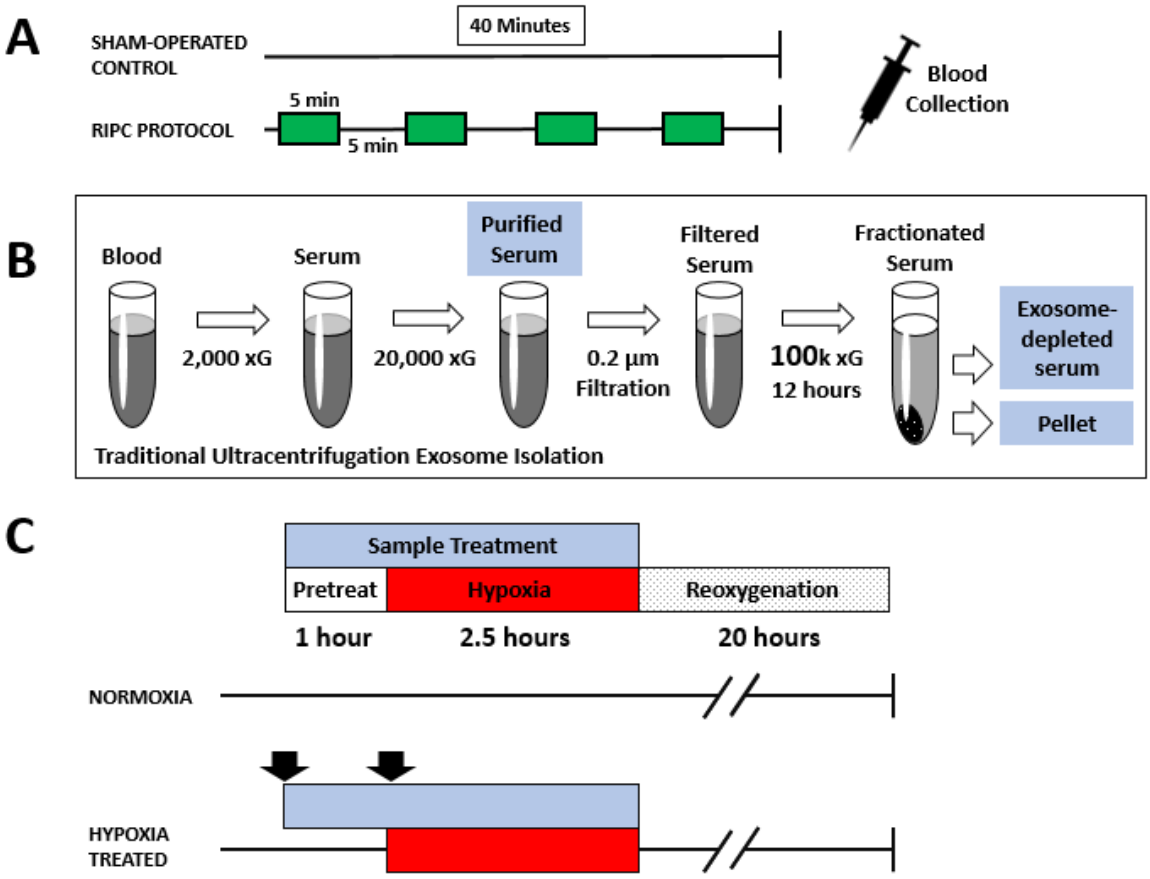


Figure 5–1: Methods schematic for transferring protection from remote preconditioning to HL-1 cardiomyocytes. A. Sprague Dawley rats underwent remote preconditioning or sham-operation, followed by blood collection. **B.** Blood was processed by traditional ultracentrifugation to obtain purified serum, exosome-depleted serum and an exosome-rich pellet. **C.** HL-1 cells underwent normoxia or hypoxia. Hypoxic cells were treated with buffer (control), purified serum, exosome-depleted serum or the exosome-rich-pellet resuspended in buffer, administered at 1 hour before the onset of hypoxia, re-administered at the onset of hypoxia (black arrows) and maintained throughout 2.5 hours of hypoxia. Cell death was measured 20 hours after reoxygenation. Green boxes represent bilateral femoral artery occlusion. Blue boxes represent sample treatments for HL-1 cells. White arrows denote centrifugation or filtration steps. RIPC, remote preconditioning.

3.5 Traditional Ultracentrifugation for Exosome Isolation from Serum

Exosomes were isolated from serum using the traditional ultracentrifugation technique.[194] First, serum was centrifuged at 20,000xG for 45 minutes to sediment cell

debris and contaminants. Next, 2 mL of purified serum was filtered through a 0.2µm syringe filter to remove larger extracellular vesicles and protein aggregates, while the remaining serum was aliquoted into storage tubes. The filtered serum was then centrifuged at 120,000xG for 2 hours, yielding a presumably exosome-rich pellet and exosome-depleted supernatant. The pellet was resuspended in 2 mL (equivalent to the starting volume of serum) of 1x Krebs Henseleit buffer. During exosome isolation, all samples were kept at 4°C or on ice. The same ultracentrifuge, ultracentrifuge tubes (ThermoFisher Scientific, Pittsburgh) and rotor (fixed angle S110AT-0019: Hitachi Koki Co., Takeda, Hitachiaka City) were used for the preparation of all samples, and the sample volume (2 mL) was kept constant (see Figure 5–1).

3.6 Serum and Exosome Transfer: Treating HL-1 Cardiomyocytes

HL-1 cells were treated with purified serum, the resuspended pellet and exosome-depleted serum isolated by traditional exosome isolation. Treatment was initiated at 1 hour before the onset of hypoxia by replacing the medium in each culture dish with 3 mL of fresh Claycomb medium supplemented with purified serum or serum fractions (see treatment details below). Treatment was continued during hypoxia by replacing the Claycomb medium with 3 ml hypoxia buffer again supplemented with purified serum or serum fractions.

3.7 HL-1 Cardiomyocyte Treatment Groups

To establish the model and optimize the dose, HL-1 cells were initially treated with 0%, 1%, 2%, 4% and 8% serum/medium, by volume. In these preliminary experiments, we found comparable results (attenuation of HL-1 cell death) with 4% and 8% serum/medium (data not shown). Accordingly, in subsequent serum transfer experiments, the lowest effective dose, 4% serum/medium, was applied. In exosome

transfer experiments, HL-1 cells were treated with either 100μL buffer alone (control), 100μL exosome-depleted supernatant, or 10μL, 50μL or 100μL of the presumably exosome-rich (resuspended pellet) sample.

3.8 Gel Electrophoresis Immunoblotting

To confirm the presence of HSP60[232] (an exosome marker) in the exosome-rich pellet, samples were probed by standard immunoblotting. Samples were prepared with lysis buffer (8M urea, 5mM dithiothreitol, 2% SDS and 25mM Tris Cl, pH 8) and incubated for 30 minutes at room temperature, then treated with Lamelli buffer (without heating). Samples were resolved on 4-15% gradient gels at 15-50 μg/well and protein was transferred from gel to nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in 0.1% Tween 20 Tris-buffered saline (TTBS) for 1 hour at room temperature and probed overnight at 4°C with primary antibody (diluted 1:1000 in 5% milk in TTBS). Horseradish peroxidase-conjugated secondary antibodies (1:10,000 in 5% milk in TTBS) were incubated for 1 hour at room temperature and immunoreactive proteins were visualized by adding enhanced chemiluminescence substrate and exposure to X-ray film (ThermoFisher Scientific, Pittsburgh, PA)

3.9 Statistical Analysis

Data were analyzed with GraphPad Prism software, GraphPad, Inc. (La Jolla, CA). Endpoints were compared among groups by one-way ANOVA. If significant F-values were obtained by ANOVA, Tukey's multiple comparisons test was used for subsequent pairwise comparisons or Dunnett's multiple comparison test was used to compare treated groups to control. Results are reported as means ± SEM.

4. Results

4.1 Serum from Remote Preconditioned Sprague Dawley Rats Protects HL-1 Cardiomyocytes from Hypoxia/Reoxygenation

As expected, in HL-1 cardiomyocytes treated with buffer, hypoxia/reoxygenation caused significant cell death when compared with the normoxic controls: 45±1% versus 10±2% of the total cell count, respectively (*p<0.001 vs Normoxia, see Figure 5–2A). Treatment with serum from remote preconditioned animals reduced cell death to 31 ± 2% (*p<0.05 vs Buffer-treated, see Figure 5–2A). However, treatment with serum from sham-operated Sprague Dawley rats did not have a significant effect on cell death in HL-1 cells subjected to hypoxia/reoxygenation (Figure 5–2B).

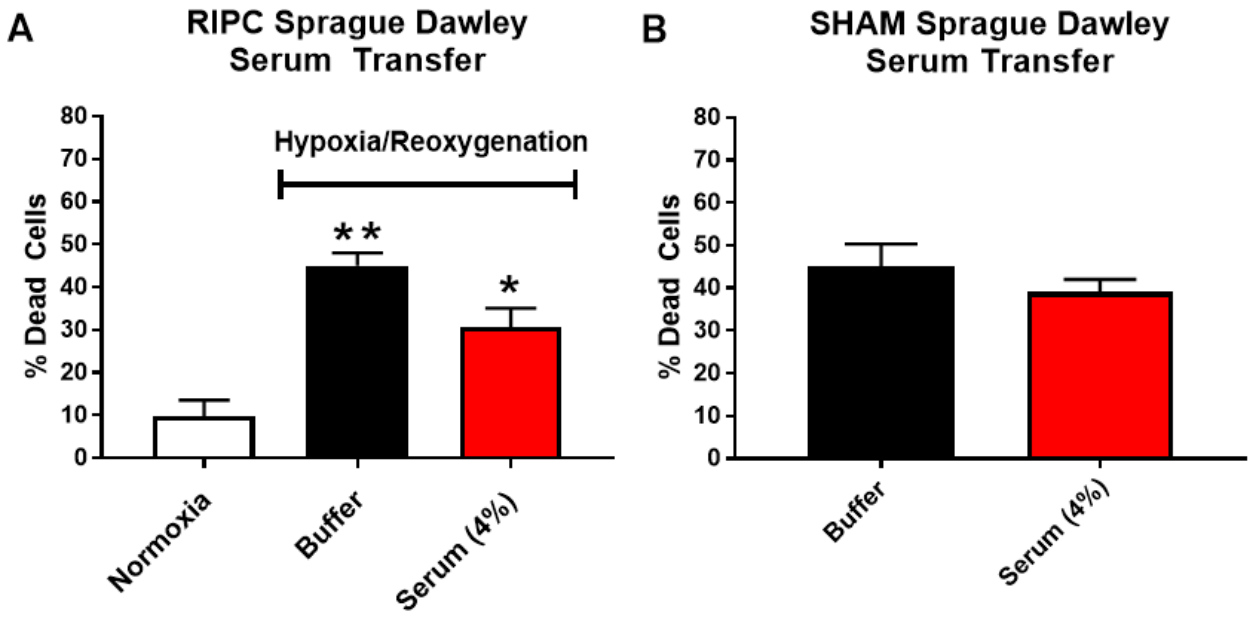


Figure 5–2: HL-1 cells are protected by treatment with remote preconditioned serum. Cell death was assayed with trypan blue and reported as a % of the total cell count. RIPC, remote preconditioning. n=5/group, *p<0.05 versus Buffer-treated, **p<0.001 versus Normoxia.

4.2 Treating HL-1 Cells by Exosome Transfer: Traditional Ultracentrifugation Isolation

The traditional ultracentrifugation technique (100,000xG for 2 hours) fractionated serum into two phases, a pellet and a supernatant. The pellet contained exosome markers, demonstrated by qualitative immunoreactivity for HSP-60 (Figure 5–3). In apparent contrast to our hypothesis, HL-1 cell death caused by hypoxia/reoxygenation was not significantly changed by treatment with 10μL, 50μL, 100μL of resuspended pellet (presumably exosome-rich, EXO: 37 ± 5%, 38 ± 6%, 39 ± 5% cell death, respectively) compared with the buffer-treated control group (39 ± 5% cell death, see Figure 5–3). Rather, cell death was significantly reduced by treatment with the supernatant (presumably exosome-depleted; $p<0.05$ vs KH buffer treated, $p<0.05$ vs EXO treated groups, see Figure 5–3).

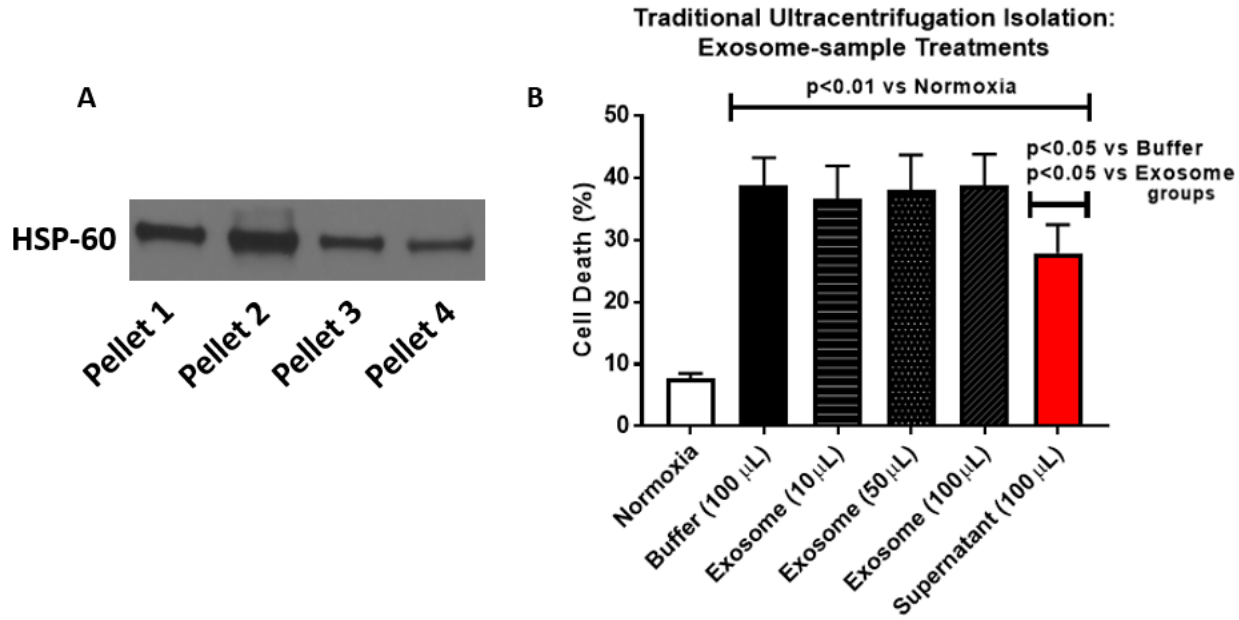


Figure 5–3: HL-1 cells are protected by the supernatant from the traditional ultracentrifugation technique. **A.** Immunoreactivity for HSP-60 in pellets obtained from individual serum samples. **B.** HL-1 cells were treated with varying doses of exosome-rich sample or supernatant. Cell death was assayed with trypan blue and reported as a % of the total cell count. n=5/group.

5. Summary

In summary, we report that the protection from remote preconditioning can be transferred through the serum and render cultured HL-1 cardiomyocytes resistant to hypoxia/reoxygenation. This is supported by the observation that treatment with serum collected from remote preconditioned Sprague Dawley rats reduced HL-1 cell death caused by hypoxia/reoxygenation, while serum from non-remote preconditioned rats did not protect cells. Secondly, we found that the traditional ultracentrifugation isolation of exosomes fractionates serum into a supernatant and an exosome rich pellet, demonstrated by immunoreactivity for exosome markers. However, and contrary to our hypothesis, we report that protection from remote preconditioning was not transferred in the presumably exosomes-rich pellet that was isolated by the traditional ultracentrifugation technique. Rather, we found that protection was transferred by the presumably exosome-depleted supernatant. This conclusion is supported by our observation that treatment with the pellet sample did not affect HL-1 cell death caused by hypoxia/reoxygenation, while the supernatant significantly reduced cell death.

CHAPTER 6: HYPOTHESIS 3: ENHANCED ULTRACENTRIFUGATION TECHNIQUE FOR EXOSOME ISOLATION SEQUESTERS A PROTECTIVE FRACTION OF SERUM

1. Rationale

In Chapter 5, we investigated the role of exosomes in the humoral component of remote preconditioning. Contrary to our hypothesis (and a previous report[200]), we found that the exosome-rich pellet isolated with the traditional centrifugation technique did not transmit protection to HL-1 cardiomyocytes. Instead, the exosome-depleted serum rendered cells resistant to hypoxia/reoxygenation (See Figure 5–3). These results may be interpreted as evidence that; 1) exosomes do not transmit the protective factor(s) from remote preconditioning. Alternatively, it is possible that 2) exosomes do transmit protection, however the traditional ultracentrifugation isolation technique is insufficient to adequately isolate exosomes, resulting in a supernatant that contains the protective exosomes.

Ultracentrifugation isolation of exosomes is ultimately a method of differential centrifugation, which isolates particles/structures at different rates, depending on physical attributes of the solute (diameter, density) and the solution (viscosity). Theoretically, as a particle with finite characteristics, exosomes can be specifically sedimented from a solution. In Chapter 5, exosomes were isolated using the traditional ultracentrifugation technique, the most commonly used method for isolating exosomes. It is becoming clear, however, that this protocol is inefficient, achieving partial exosome sedimentation.[19,247,248] This may be particularly problematic for viscous solutions, such as serum. To accurately test our hypothesis that exosomes are responsible for transmitting protective signals, the isolation technique must produce a high yield, exosome-rich sample and an exosome-depleted sample. Our objective was to enhance

the ultracentrifugation procedure in order to augment sedimentation and improve depletion of exosomes in the serum. Accordingly, we aimed to enhance the ultracentrifugation technique by sequentially increasing the centrifugation force in an effort to increase the exosome yield and improve the depletion of exosomes from the serum. First, we hypothesized that sequentially increasing ultracentrifugation force would reveal the optimal ultracentrifugation protocol, i.e. improved exosome sedimentation. Second, we hypothesized that the exosome-rich samples isolated with the enhanced ultracentrifugation technique would confer the protection of remote preconditioning, whereas exosome-depleted serum will not protect cells.

2. Materials

Refer to Chapter 5, section 2.

3. Methods

3.1 Culturing H-1 Cardiomyocytes

Refer to Chapter 5, section 3.1.

3.2 Hypoxia/Reoxygenation

Refer to Chapter 5, section 3.2

3.3 Trypan Blue Cell Death Assay

Refer to Chapter 5, section 3.3

3.4 Remote Preconditioning and Serum Collection

Refer to Chapter 5, section 3.4

3.5 Sequential Ultracentrifugation to Maximize Exosome Isolation

Evidence suggests that sedimentation of exosomes with the traditional ultracentrifugation technique (used in Chapter 5) is not efficient.[247] To identify a protocol that maximizes exosome sedimentation, we sequentially increased the radial

force of ultracentrifugation and monitored exosome-marker content. Serum was collected from Sprague Dawley rats and filter purified as described in Chapter 4. Serum was then centrifuged, beginning with the traditional exosome isolation force (100,000xG), and increased stepwise by 50,000xG increments. The pellet was collected after each step and resuspended in 1x Krebs Henseleit (KH) buffer. After the terminal spin at 300,000xG, the pellet and supernatant were collected for exosome analysis. All spin cycles were 2 hours, except the last step, which lasted 12 hours. See Figure 6–1A for protocol.

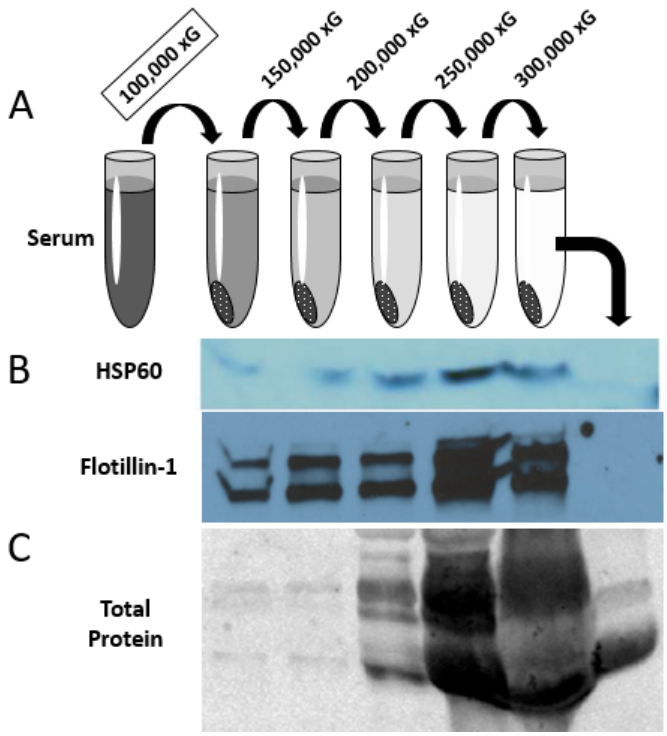


Figure 6–1: Sequential ultracentrifugation for isolation of exosomes from serum. A. Protocol schematic for sequential ultracentrifugation. The box denotes the traditional ultracentrifugation force. **B.** Immunoblotting was used to detect exosome markers in the pellet from each step and the supernatant from the terminal step. **C.** Sypro Ruby gel stain was used to detect protein content in each lane.

3.6 Enhanced Ultracentrifugation Isolation of Exosomes from Serum

Based on results obtained with sequential ultracentrifugation, an enhanced ultracentrifugation protocol was established; for optimal exosome sedimentation, filtered serum was centrifuged at 300,000xG for 12 hours. This robust isolation protocol fractionated the serum into a pellet and a biphasic supernatant, a top phase and bottom

phase (see Figure 6–2B). The pellet was resuspended in 2 mL (equivalent to the starting volume of serum) of 1x KH buffer. After initial coagulation of blood at room temperature for serum collection, all samples were kept at 4°C or on ice.

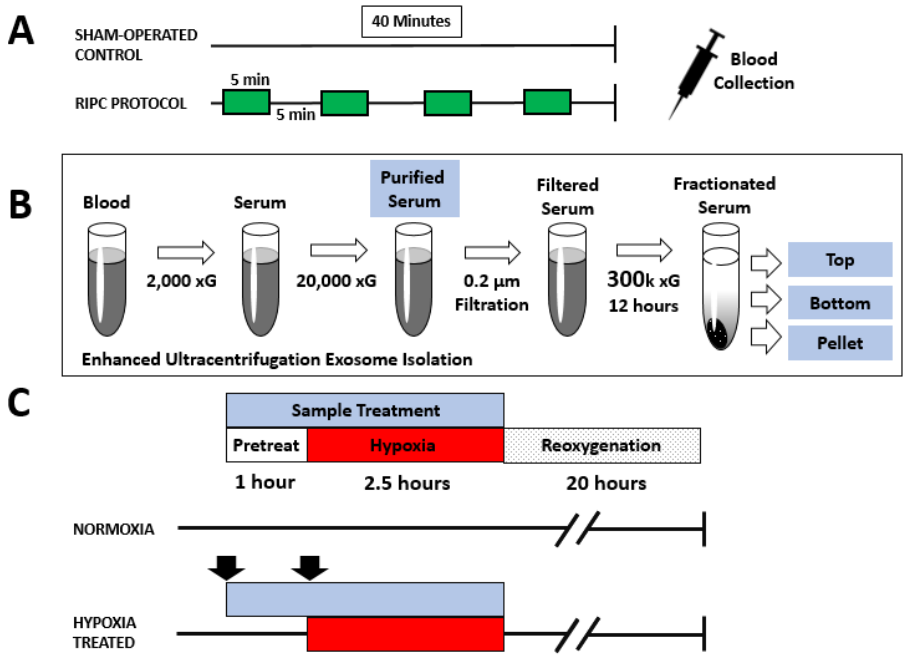


Figure 6–2: Protocol schematic for transferring fractionated serum to HL-1 cardiomyocytes. A. Sprague Dawley rats underwent sham-operation or remote preconditioning, followed by blood collection. Green boxes represent bilateral femoral artery occlusion. **B.** Blood is processed by the *enhanced ultracentrifugation isolation technique* to obtain samples to treat HL-1 cells; purified serum, pellet, top and bottom phases of the supernatant. White arrows denote centrifugation or filtration steps. **C.** HL-1 cells were subjected to normoxia or hypoxia/reoxygenation. Cells were untreated or treated with buffer (buffer) or processed blood samples (3.5 hour treatment beginning 1 hour prior to hypoxia). Cell death was measured 20 hours after reoxygenation. Blue boxes represent sample treatments for HL-1 cells. Black arrows denote administration of buffer, serum or serum fractions. RIPC, remote preconditioning.

3.7 Sample Transfer and HL-1 Cardiomyocyte Treatment Groups

The HL-1 cell treatment technique was the same as described in Chapter 5 section 3.7, and the treatment regimen is depicted in Figure 6–2. HL-1 cells were treated with buffer, whole unfractionated serum (4%, as in Chapter 5) or fractions of serum harvested after the enhanced exosome isolation protocol (described below). For all serum fractions, the treatment volumes were adjusted using KH buffer to match the

volume of unfractionated serum applied to the cells.

3.8 Detecting Exosome Markers and Total Protein

Each successive pellet from the sequential ultracentrifugation protocol was resuspended in 0.5 mL KH buffer, 25% of the starting volume, to facilitate exosome-marker detection. The pellet isolated by the last step (300,000xG) was highly concentrated and, thus, was diluted in 4 mL KH buffer (two-fold dilution from the starting volume of serum) to prevent overloading the gel. Exosome content was evaluated as described in Chapter 5 Section 3.6, by separating proteins by SDS-PAGE, transferring proteins to nitrocellulose membranes and immunoblotting for exosome markers (HSP60,[232] flotillin-1[188] and GAPDH[249]). Our objective was to qualitatively determine the exosome content of the samples to assess the efficacy of each sequential ultracentrifugation step. As such, we were interested in the presence/absence and relative concentration of exosomes rather than the ratio of exosomes to protein, so samples were loaded into gel wells in equal volumes, regardless of protein concentration. Total protein separation was visualized with SYPRO Ruby stain on a separate gel, as per the manufacturer's instructions (Invitrogen). Briefly, the SDS-acrylamide gel was fixed (50% methanol, 7% acetic acid) for 30 minutes, stained overnight with SYPRO Ruby and washed (10% methanol, 7% acetic acid) for 1 hour. Red fluorescence was photographed with a VersaDoc MP 4000 imaging system (Bio-Rad, Hercules, CA). Immunoblots and protein stain images were converted to gray scale.

3.9 Statistical Analysis

Data were analyzed with GraphPad Prism software, GraphPad, Inc. (La Jolla, CA). Data are presented as means ± SEM. Endpoints were compared among groups

by one-way ANOVA as appropriate. If significant F-values were obtained by ANOVA, Dunnett's multiple comparisons test was used to compare treated groups to control.

4. Results

4.1 Sequential Ultracentrifugation and the Enhanced Protocol Maximize Exosome Sedimentation

Exosomes were detected in the pellet after every ultracentrifugation step by immunoblotting for HSP60, flotillin-1 and GAPDH (Figure 6–1B). The supernatant collected after the last step of ultracentrifugation (300,000xG for 12 hours) did not contain exosomes markers (see Figure 6–1B). Protein content of the pellet was proportional to the ultracentrifugation force, with the highest content in the pellet collected after the 300,000xG step.

The simplified protocol for enhanced ultracentrifugation isolation of exosomes (300,000xG for 12 hours) separated serum into three fractions; the pellet, the bottom

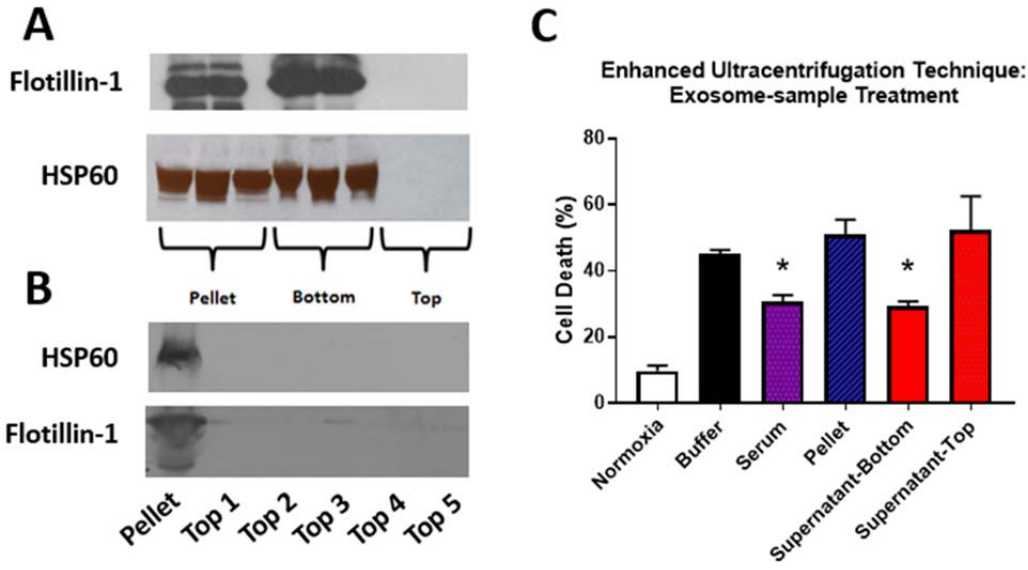


Figure 6–3: Exosome content and HL-1 cell death following treatment with serum or serum fractions isolated by enhanced ultracentrifugation. A, B. Exosome content was qualitatively assayed by immunoblotting for exosome markers. **C.** Cell death was assayed with trypan blue to assess viability in each treatment condition. Cell death is reported as a % of the total cell count. RIPC, remote preconditioning. n=5/group, *P=<0.01.

phase of the supernatant (directly adjacent to the pellet, containing exosome markers) and the top phase of the supernatant (devoid of exosome makers, as detected by qualitative immunoblotting analysis; see Figure 6 – 3A).

4.2 Treating HL-1 Cells with Samples from the Enhanced Ultracentrifugation Technique

Percent HL-1 cell death was not significantly changed by treatment with either the resuspended pellet (51 ± 4%) or and top fraction of the supernatant (52 ± 10%) when compared to buffer treatment (45 ± 1%; Figure 6 – 3B). In contrast, treatment with the bottom fraction of the supernatant reduced HL-1 cell death when compared with buffer treatment: 29 ± 2% vs 45 ± 1, respectively ($p<0.01$, see Figure 6 – 3B).

5. Summary

In summary, we report that the traditional ultracentrifugation technique does not efficiently sediment exosomes from serum, resulting in a partial isolation of exosomes and residual exosomes in the supernatant. These conclusions are supported by qualitative immunoblot analysis detecting exosome markers in pellets formed by ultracentrifugation forces exceeding the traditional ultracentrifugation protocol (up to 300,000xG). We report that ultracentrifugation at 300,000xG for 12 hours fractionates serum into three distinct phases; the pellet (as expected, expressing exosome markers), the bottom phase of the supernatant (which was also enriched with exosome markers) and the top phase of the supernatant (which is depleted of exosome markers). Finally, we report that exosomes may play a role the humoral component of remote preconditioning, however they are not sufficient for protection. This is illustrated by the reduction in cell death from hypoxia/reoxygenation by treatment with the exosome marker-rich bottom phase, but not the exosome marker-rich pellet.

CHAPTER 7: HYPOTHESIS 4: THE HUMORAL COMPONENT OF REMOTE PRECONDITIONING IS DEFECTIVE IN TYPE-2 DIABETES

1. Rationale

As reported in Chapter 4, we have provided the first evidence that, in agreement with our hypothesis, remote preconditioning does not reduce infarct size in the Zucker rat model of type-2 diabetes. These results are consistent with previous studies reporting that protection with ischemic preconditioning and postconditioning is attenuated or lost in the setting of diabetes; however the mechanism of this dysfunction remains unclear.

The majority of previous studies have focused on the concept that the diabetic myocardium becomes refractory to protection from ischemic conditioning, potentially as a result of changes in protein expression, activity of signaling cascades and/or activation of end effectors in cardiomyocytes.[100,108,110-112,250-256] In the setting of remote conditioning, there is a possible alternative explanation for the failed protection: as discussed in Chapter 1, Jensen *et al.* investigated the humoral component of remote preconditioning in diabetes, rather than the effect of diabetes on the myocardium. This study reported that plasma collected from diabetic patients subjected to a remote preconditioning stimulus was protective (i.e. reduced infarct size when administered to buffer-perfused rabbit hearts), while plasma from diabetic volunteers with peripheral neuropathy did not maintain this protective capacity.[124] The objective of this chapter is to pursue this concept and assess the effect of type-2 diabetes on the humoral component of remote preconditioning.

We hypothesize that diabetes causes a defect in the humoral signaling of remote preconditioning, which prevents transfer of protection. We tested our hypothesis with

an *in vitro* analysis of the protective efficacy of serum and serum fractions harvested from hyperglycemic Zucker Fatty and normoglycemic Zucker Lean rats and isolated using our enhanced ultracentrifugation technique described in Chapter 6. HL-1 cardiomyocytes were treated with serum and serum fractions from remote preconditioned rats from both strains and subjected to hypoxia/reoxygenation. As in our previous experiments, the primary endpoint was HL-1 cell viability following reoxygenation. We proposed that serum and supernatant-bottom fractions obtained from Zucker Lean rats subjected to remote conditioning will provide protection against hypoxia/reoxygenation, while samples from Zucker Fatty rats will not reduce cell death. We also predict that the supernatant-top fraction will not affect cell death caused by hypoxia/reoxygenation in either Zucker cohort.

2. Materials

See Materials sections from Chapter 4 and 5.

3. Methods

3.1 Culturing HL-1 Cardiomyocytes

Refer to Chapter 5, section 3.1.

3.2 Hypoxia/Reoxgenation

Refer to Chapter 5, section 3.2

3.3 Trypan Blue Cell Death Assay

Refer to Chapter 5, section 3.3

3.4 Serum and Exosome Transfer: Treating HL-1 Cardiomyocytes

In Chapter 5 and Chapter 6, Sprague Dawley rats were used in preliminary studies to validate the protocol for serum transfer to HL-1 cells and proof-of-concept for exosome isolation and exosome transfer to HL-1 cells. Here we addressed our primary

objective; we assess the hypothesized role of exosomes in the humoral transfer of protection in remote preconditioning in the setting of type-2 diabetes.

Zucker Fatty rats were used as a model of early-stage type-2 diabetes, as introduced in Chapter 4, while Zucker Lean rats served as the normoglycemic control strain. Remote preconditioning, blood collection and processing, exosome isolation and

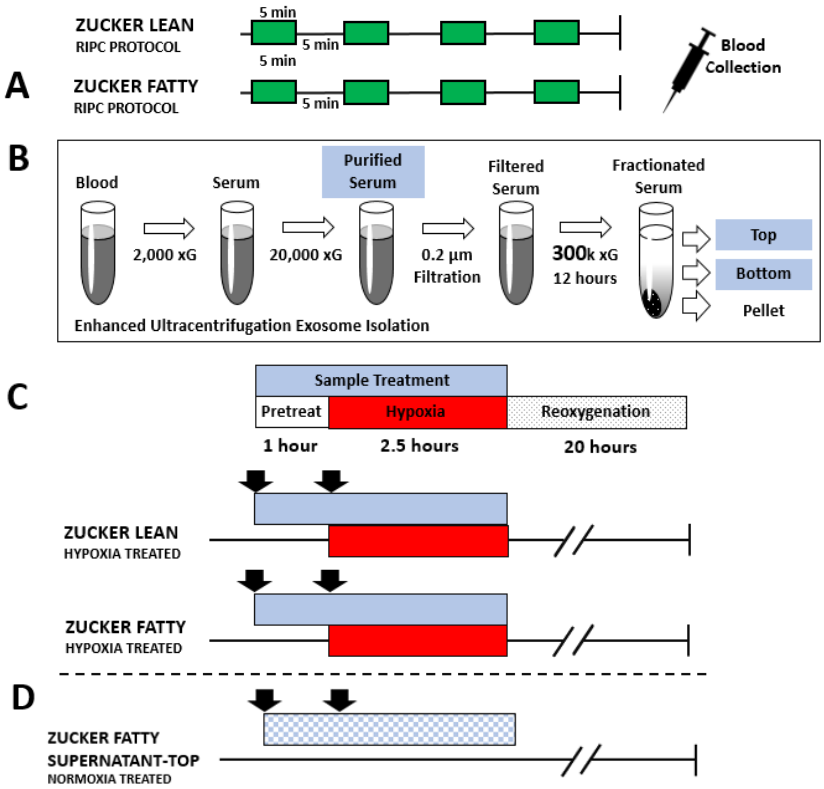


Figure 7–1: Methods schematic for transferring protection from remote preconditioning to HL-1 cardiomyocytes in the setting of type-2 diabetes. A. Zucker Lean and Zucker Fatty rats underwent remote preconditioning followed by blood collection. **B.** Blood was processed using the enhanced ultracentrifugation technique to obtain purified serum, supernatant-top and supernatant-bottom serum fractions. **C.** HL-1 cells were subjected to hypoxia and were treated with serum samples collected from Zucker Lean and Zucker Fatty rats. HL-1 cells were treated with buffer (control), purified serum, supernatant-bottom or supernatant-top fractions, administered at 1 hour before the onset of hypoxia, re-administered at the onset of hypoxia (black arrows) and maintained throughout 2.5 hours of hypoxia. Cell death was measured 20 hours after reoxygenation. **D.** In additional experiments, HL-1 cells were treated with the supernatant-top fraction under normoxic conditions. Green boxes represent bilateral femoral artery occlusion. Blue boxes represent sample treatments for HL-1 cells. Checked blue box represents treatment with supernatant-top fraction only. White arrows denote centrifugation or filtration steps. RIPC, remote preconditioning.

HL-1 cell treatment were the same as in Chapter 6. Briefly, Zucker Lean and Zucker Fatty rats underwent the standard remote preconditioning stimulus, consisting of bilateral femoral artery occlusion (4x 5-minute ischemic and 5-minute reperfusion). Immediately following the last period of femoral artery reperfusion, blood was collected centrifuged to isolate the serum, see Figure 7-1A. Serum was purified by centrifugation at 20,000 xG and filtered (0.2μm). The enhanced ultracentrifugation exosome isolation technique described in Chapter 6, section 3.6 was used to fractionate the serum into three phases; pellet, supernatant-bottom and supernatant-top, see Figure 7-1B. HL-1 cardiomyocytes were treated with purified serum, supernatant-top and supernatant-bottom, see Figure 7-1C. The pellet was excluded from analysis based on our previous evidence that this fraction had no protective effect against hypoxia/reoxygenation in Sprague Dawley experiments.

3.5 Supplemental Experiments: Treatment with the Supernatant-top Fraction Under Normoxic Conditions

In order to pursue an unexpected observation made in the protocol described above, supplemental experiments were performed in which we investigated the effect of treatment with supernatant-top fraction obtained from Zucker Lean and Zucker Fatty rats on cardiomyocytes under *normoxic* conditions. HL-1 cells were treated with the supernatant-top fraction in a manner that was designed to mimic the timing and conditions of the hypoxia-reoxygenation protocol (see Figure 7 – 1D); treatment was initiated by replacing the medium in each culture dish with 3 mL of fresh Claycomb medium supplemented with samples of the supernatant-top fraction for 1 hour, and was continued by replacing Claycomb culture media with 3 mL serum-free media supplemented with the top fraction. After 2.5 hours, the culture media was replaced

with 3 mL serum-free media without supplementation. Cell viability was assayed with trypan blue 20 hours later, as described in Chapter 5, section 3.3.

3.6 Statistical Analysis

Data were analyzed with GraphPad Prism software, GraphPad, Inc. (La Jolla, CA). Data are presented as means ± SEM. Endpoints were compared among groups by one-way ANOVA. If significant F-values were obtained by ANOVA, Newman-Keuls multiple comparisons test was used to compare treated groups to control.

4. Results

4.1 Zucker Lean Rats: Serum and the Supernatant-bottom Fraction Protect HL-1 Cells from Hypoxia/Reoxygenation

As expected, treatment with purified serum and the supernatant-bottom fraction from remote preconditioned Zucker Lean rats significantly reduced hypoxia/reoxygenation-induced cell death (38 ± 5%, 39 ± 6%, respectively) when compared with buffer-treated control cells (48 ± 6% cell death, $p<0.05$; Figure 7–2). Consistent with our hypothesis, treatment with the supernatant-top fraction of serum

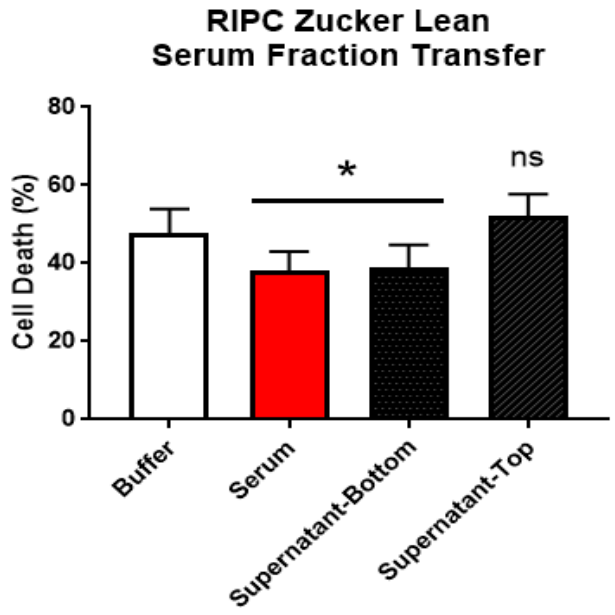


Figure 7–2: HL-1 cells are protected by treatment with serum and supernatant-bottom fraction from Zucker Lean rats. HL-1 cells subjected to hypoxia/reoxygenation were treated buffer or with serum samples from remote preconditioned Zucker Lean rats. RIPC, remote preconditioning. ns, not significant vs buffer-treated. n=6/group, *$p<0.05$ versus Buffer-treated.

from remote preconditioned Zucker Lean rats did not have a significant effect on % cell death in HL-1 cells subjected to hypoxia/reoxygenation (52 ± 10%, see Figure 7–2).

4.2 Zucker Fatty Rats: Serum and the Supernatant-bottom Fraction are not Protective – the Supernatant-top Fraction Exacerbates Cell Death Caused by Hypoxia/Reoxygenation

In contrast to data obtained in Zucker Lean and Sprague Dawley rats (and as expected from our hypothesis), neither the serum nor the supernatant-bottom fraction of serum from Zucker Fatty rats had a significant protective effect: mean hypoxia/reoxygenation-induced HL-1 cell death was 53 ± 4% and 45 ± 5%, respectively, *versus* 53 ± 4% in buffer-treated cells; see Figure 7 – 3). Unexpectedly, and contrary to our observations made with samples from the other rat strains, treatment with the supernatant-top fraction isolated from Zucker Fatty rats caused a significant ***increase*** in cell death following hypoxia/reoxygenation.

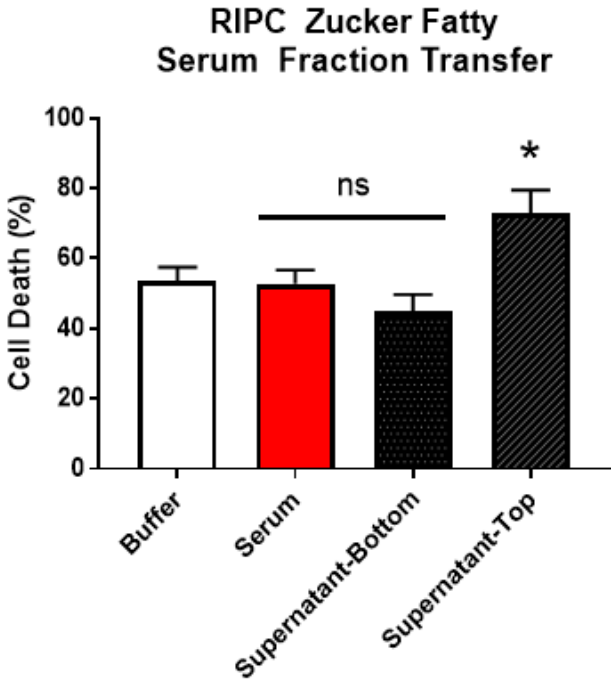


Figure 7–3: HL-1 cells are not protected by treatment with serum and supernatant-bottom fraction from Zucker Fatty rats. HL-1 cells subjected to hypoxia/reoxygenation were treated with buffer or serum samples from remote preconditioned Zucker Fatty rats. RIPC, remote preconditioning. ns, not significant vs Buffer-treated. n=6/group, *p<0.05 versus Buffer-treated.

4.3 Zucker Fatty Rats: the Supernatant-top Fraction of Serum does not Exacerbate Cell Death under Normoxic Conditions

We report that under normoxic conditions, treatment with the supernatant-top fraction from Zucker Lean rats and Zucker Fatty rats did not have a significant effect on cell viability when compared to buffer-treated HL-1 cardiomyocytes. Cell death averaged 15 ± 2%, 17 ± 3% and 10 ± 2%, respectively; $p>0.05$ by ANOVA, Figure 7–4).

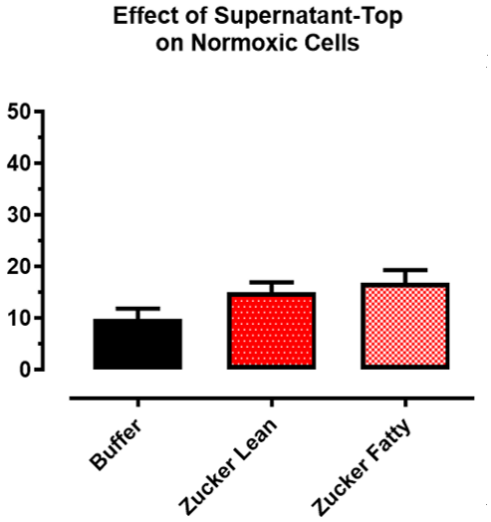


Figure 7–4: The supernatant-top fraction of serum does not exacerbate cell death in HL-1 cells under normoxic conditions. Not significant by ANOVA. n=4-7/group.

5. Summary

In summary, we report that, consistent with our predictions, the humoral component of remote preconditioning is defective in the Zucker rat model of type-2 diabetes. This conclusion is supported by the observation that, while treatment with serum and the supernatant-bottom fraction collected from remote preconditioned Zucker Lean rats rendered HL-1 cardiomyocytes resistant to hypoxia/reoxygenation, treatment with serum and the supernatant-bottom fraction collected from remote preconditioned Zucker Fatty rats did not have a significant effect on cell death.

We suggest that the defect in the humoral mediated protection may be, at least partially, caused by a cytotoxic component in the diabetic circulation, present in the supernatant-top fraction of serum collected from Zucker Fatty rats. This conclusion is

supported by the observation that the supernatant-top fraction from Zucker Lean animals had no effect on HL-1 cell death, while the supernatant-top fraction from Zucker Fatty rats significantly exacerbated cell death caused by hypoxia/reoxygenation. Moreover, we report that the toxic effect of the supernatant-top fraction only occurs in the setting of hypoxia/reoxygenation, illustrated by the modest, non-significant effect of supernatant-top fraction treatment of HL-1 cells under normoxic conditions.

CHAPTER 8: HYPOTHESIS-GENERATING ANALYSIS: INVESTIGATING THE COMPONENTS OF BIOACTIVE SERUM FRACTIONS

1. Rationale

This dissertation has largely focused on investigating the humoral component of remote preconditioning and its function in the setting of type-2 diabetes. The humoral theory suggests that the remote preconditioning stimulus causes the release of protective factor(s) into the blood, which are dispersed throughout the circulation. The appeal of identifying an endogenous molecule that can evoke robust cardioprotection is obviously compelling; however the humoral factor(s) of remote preconditioning remains unknown.

In general, two strategies have been used to search for the elusive protective factor(s) of remote preconditioning; (1) direct identification of the protective factor(s) using proteomic or transcriptomic techniques (including 2-dimensional differential gel electrophoresis, mass spectrometry or microarray analysis, or by monitor changes in protein/RNA abundance in the circulation before and after ischemic conditioning[235,257-264]), or (2) characterization of the protective factor(s) by evaluating the ability of sub-fractions of serum/plasma or tissue effluent to reduce infarct size or attenuate cardiomyocyte death.[51-53] For example, Shimizu *et al.* processed plasma from remote preconditioned rabbits and humans by dialysis (12-14kDa cut-off membrane) and reverse phase chromatography with a C18 Sep Pak column, and provided evidence that protection is afforded by a hydrophobic molecule(s) smaller than 15kDa.

In Chapters 5, 6 and 7, we used filtration followed by ultracentrifugation techniques to isolate the protective component of serum from remote preconditioned rats. Based on our hypothesis that the protective molecule(s) are contained within

exosomes, we devised an exosome ultracentrifugation isolation technique which sequestered an exosome-rich sub-fraction of the serum that transferred protection to HL-1 cardiomyocytes. Unexpectedly, we also isolated a component of serum from diabetic rats that exacerbates cell death caused by hypoxia/reoxygenation. As such, the objective of this hypothesis-generating chapter is two-fold. Our first aim was to interrogate the particle content (including, but not limited to, exosomes) of serum fractions produced by the enhanced ultracentrifugation technique with nanoparticle tracking analysis (conducted by Systems Biosciences Inc. Palo Alto, CA), and obtain preliminary insight into the effect of strain (Sprague Dawley, Zucker Lean, Zucker Fatty) and treatment (RIPC versus sham) on particle concentration and size. We hypothesize that our enhanced ultracentrifugation technique will facilitate the isolation of differential exosome populations involved with communicating protection. More specifically, we speculate that: (1) the supernatant-top fraction, presumed to be exosome-poor by immunoblot analysis of classic exosome markers (Chapter 6), will be depleted of particles in all samples irrespective of strain or treatment; (2) the particle concentration will be greater in the protective fractions (serum and supernatant-bottom) obtained from remote preconditioned Sprague Dawley and Zucker Lean rats versus strain-matched shams, while, in contrast, (3) particle concentrations will be comparable in serum and supernatant-bottom fractions obtained from remote preconditioned versus sham-operated Zucker Fatty rats. In our second aim, we propose that the high-force ultracentrifugation used in our protocols may facilitate the detection of differential protein expression by mass spectrometry and, thus, the discovery of novel protective candidates. Thus, in collaboration with Dr. Xuequn Chen, we conducted exploratory mass spectrometry of the serum fractions, with the goal of revealing possible

differences in abundance of proteins between treatment groups.

2. Materials

See Materials sections from Chapter 4 and 5.

3. Methods

3.1 Remote Preconditioning and Serum Collection

Refer to Chapters 5 section 3.4.

3.2 Samples Analyzed

Nanoparticle tracking analysis and mass spectrometry were used to compare particle content and protein profiles between treated and untreated groups in Sprague Dawley and Zucker cohorts (see Table 8-1). As discussed in Chapter 6, serum samples were obtained following centrifugation at 20,000 xG, while the top and bottom fractions were collected after filtration through a 200 nm membrane and ultracentrifugation at 300,000 xG.

Table 8–1: Analysis Sample List

Nanoparticle Tracking Analysis	SHAM	RIPC
Sprague Dawley	Serum Supernatant-Top Supernatant-Bottom	Serum (n=2) Supernatant-top (n=2) Supernatant-bottom (n=2)
Zucker Lean	Supernatant-Top Supernatant-Bottom	Supernatant-Top Supernatant-Bottom
Zucker Fatty	Supernatant-Top Supernatant-Bottom	Supernatant-Top Supernatant-Bottom
Tandem Mass Spectrometry	**SHAM**	**RIPC**
Sprague Dawley	Serum Supernatant-Top Supernatant-Bottom	Serum Supernatant-Top Supernatant-Bottom
Zucker Lean		Supernatant-Top (n=3) Supernatant-Bottom (n=2)
Zucker Fatty		Supernatant-Top (n=2) Supernatant-Bottom (n=2)

Samples were analyzed by Nanoparticle Tracking Analysis and tandem mass spectrometry. n=1/group, unless indicated otherwise.

3.3 Nanoparticle Tracking Analysis

Particle populations were characterized by System Biosciences, Inc. (Palo Alto, CA) with nanoparticle tracking analysis using a NanoSight LM10 (NanoSight, Amesbury, United Kingdom). The Nanosight visualizes particle movement in solution with an ultramicroscope, which detects scattered light. Nanoparticle tracking analysis calculates the diameter of individual particles which is proportional to the random movement of the particle in solution, otherwise known as Brownian motion.[265] Diameter measurements were tabulated in 1-nanometer increments, for each size increment, the particle concentration (number/mL) was quantified. All samples were analyzed in triplicate. Given our focus on exosomes, analysis was limited to particles within the size range of exosomes (0-200 nm). **Importantly, however, nanoparticle tracking analysis detects particles in solution without discrimination, and thus includes non-exosome vesicles and protein aggregates:** i.e., data reflect particle (rather than exosome) sizes and concentrations.

3.4 Sample Preparation for Mass Spectrometry

Samples were processed and analyzed by mass spectrometry as described previously.[266] Serum and supernatant-bottom samples were first depleted of abundant plasma proteins using the Pierce Albumin/IgG Removal Kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocol. Using this method, Blue dye and Protein A, which are conjugated to agarose resin in the stationary phase of the column, bind and sequester albumin and immunoglobulin G, respectively. Serum and supernatant-bottom samples were then separated by SDS-PAGE.

Samples were prepared for mass spectrometry by Dr. Xuequn Chen. Briefly, the gels were stained with GelCode Blue stain (ThermoFisher Scientific, Waltham, MA),

washed with ammonium bicarbonate, reduced in 10 mM DTT and alkylated with 55mM iodoacetamide. Proteins were digested overnight with four proteases: Lys-C, trypsin, Glu-C, and chymotrypsin. Supernatant-top fraction samples had low protein concentration and required concentration by overnight acetone precipitation. Pellets were resuspended in 50 mM ammonium bicarbonate, reduced in 5 mM Tris(2-carboxyethyl)phosphine and alkylated with 25 mM methyl methanethiosulfonate. Protein was digested overnight with trypsin.

3.5 Mass Spectrometry

Mass spectrometric analysis using nanoLC-MS/MS was kindly conducted by Dr. Chen. Peptides were separated with reverse-phase chromatography through a C18 column with the Dionex Ultimate HPLC system (ThermoFisher Scientific, Waltham, MA). MS and MS/MS spectra were acquired on an Applied Biosystems QSTAR XL mass analyzer, MASCOT 2.4.0 (Matrix Science, Inc. Boston, MA) was used to search the National Center for Biotechnology Information non-redundant database, and the outcomes were analyzed using Scaffold 4.6.1 software (Proteome Software, Inc. Portland, OR). The minimum requirement for identifying a peptide was set at 90% accuracy and two unique peptides were required to identify a protein. Minimum requirement for protein identification was set at 50% accuracy (threshold was set low to increase the output of identified proteins). Protein abundance was reported as total spectrum count, and comparisons were made between groups and reported as fold change (see below). In the instance that the initial value was zero and the initial value was larger than zero, fold change was reported as the final value.

3.6 Statistical Analysis

For each cohort and fraction, nanoparticle size is reported as the mode of the

replicates ± SEM, while particle concentration (number/mL) is reported as both a frequency distribution (mean values ± SEM for particles ranging from 0-200 nm) and mean total concentration ± SEM.

Given the small number of independent samples (n=1-2 per group) probed in these exploratory experiments, statistical comparisons among cohorts and between serum fractions were not performed. However, qualitative comparisons of nanoparticle concentration and protein abundance between groups were made by calculating fold change, defined as:

Fold Change = (Value 2 – Value 1)/Value 1

4. Results

4.1 Nanoparticle Tracking Analysis: Characterizing the Particle Population in Sprague Dawley Samples

As expected, nanoparticles were detected in the serum and supernatant-bottom samples from Sprague Dawley rats (Figure 8-1). In addition, the concentration of particles was ~1.0 to 1.4-fold higher in samples from rats that underwent RIPC when compared with shams: for serum, mean concentrations were 2.0 $x10^{12}$ versus 1.0 x 10^{12} particles/mL (a 1.0-fold increase, data not shown) and, for the supernatant-bottom fractions, concentrations were 2.2 x 10^{12} versus 9.0 x 10^{11} particles/mL (a 1.4-fold increase (see Figure 8–1 B, D). Particle size in the supernatant-bottom fraction was comparable in the two cohorts, with modes of 60 and 58 nm.

Contrary to our hypothesis, the supernatant-top fractions (presumed to be devoid of exosomes based on immunoblot analysis) contained particles, with mean concentrations of 1.4 x 10^{11} particles/mL in samples from sham-operated animals versus 1.3 x 10^{12} particles/mL in samples from remote preconditioned rats (8.3-fold

larger concentration, see Figure 8–1B, D). The mode of the particle size in this fraction ranged from 60 nm to 52 nm, see Figure 8–B, D.

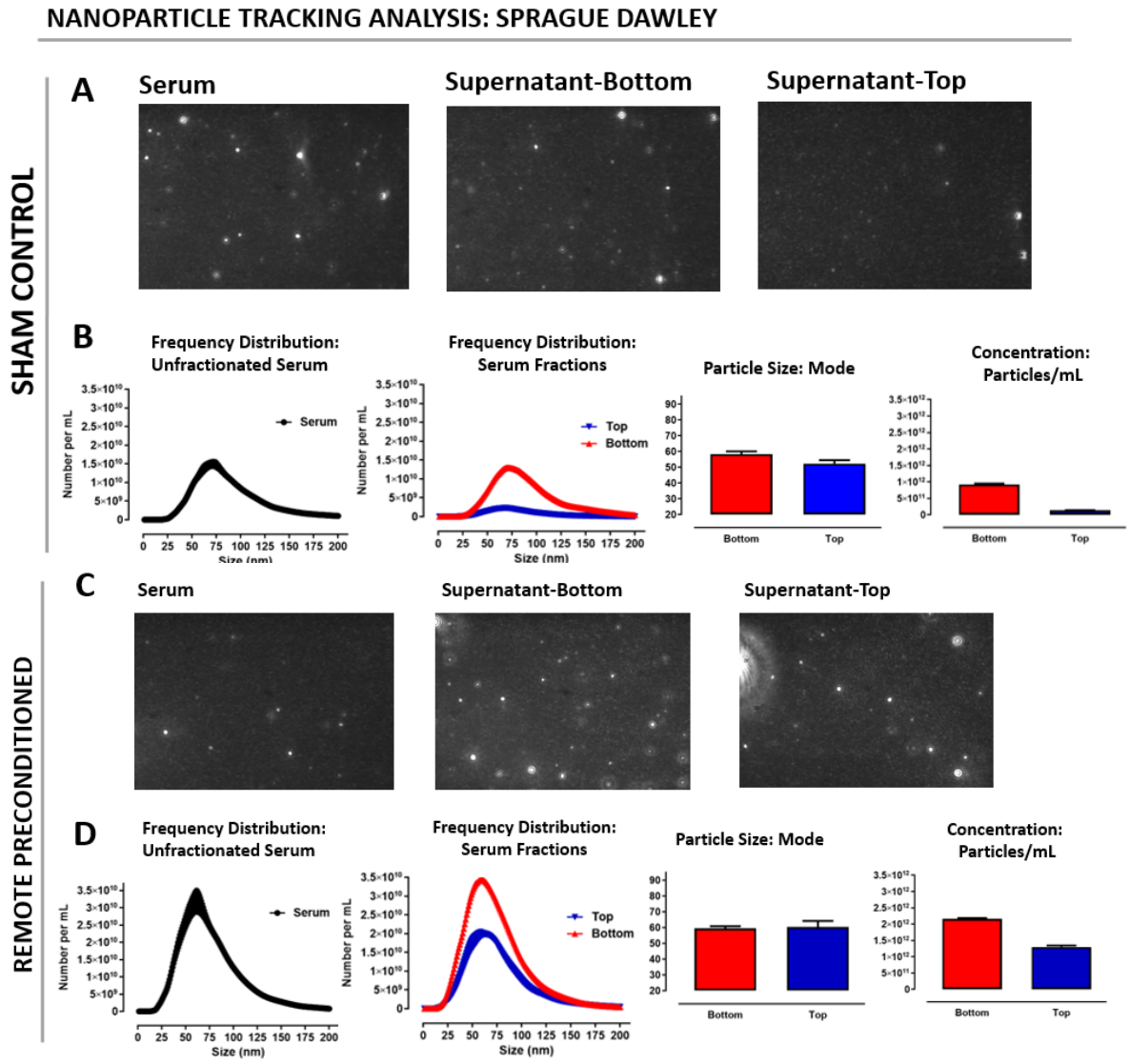


Figure 8–1. Nanoparticle tracking analysis of samples from Sprague Dawley rats. Serum and the supernatant-top and -bottom were analyzed with nanoparticle tracking analysis to count and measure the size of particles in solution. **A.** Screenshot from nanosight video and **B.** analysis of sham samples. **C.** Screenshot from nanosight video and **D.** analysis of samples from remote preconditioned samples. 3 replicates per sample, n=1 independent samples/group.

4.2 Nanoparticle Tracking Analysis: Characterizing the Particle Population in the Zucker Lean Fractions

For samples obtained from the Zucker Lean cohort, nanoparticle concentrations were of a comparable magnitude to those observed in samples from Sprague Dawley

rats. However, the 1.1 to 8.3-fold increase in nanoparticle concentration seen with RIPC in Sprague Dawley rats was not observed in fractions harvested from Zucker Lean rats. Rather, nanoparticle concentrations in serum, the supernatant-bottom fractions and the supernatant-top fractions were 0.15-fold lower, 0.9-fold lower (see Figure 8–2), and equivalent (0.04-fold higher, see Figure 8-2) in RIPC samples versus

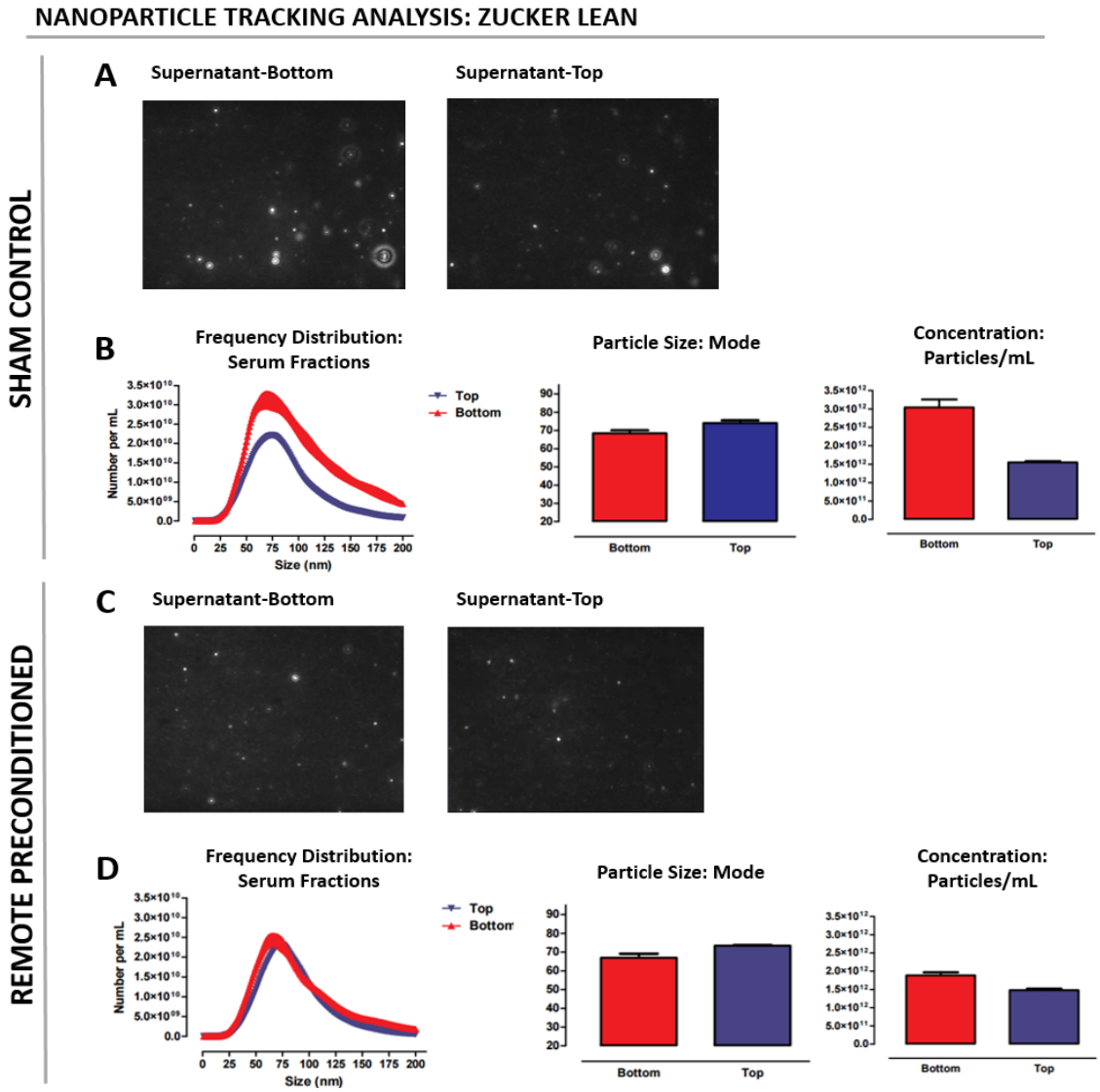


Figure 8–2: Nanoparticle tracking analysis of samples from Zucker Lean rats. The top and bottom fractions of serum were analyzed with nanoparticle tracking analysis to count and measure the size of particles in solution. **A.** Screenshot from nanosight video and **B.** analysis of sham samples. **C.** Screenshot from nanosight video and **D.** analysis of samples from remote preconditioned samples. 3 replicates per sample, n=1 independent samples/group.

shams. Particle sizes were qualitatively larger in the Zucker Lean cohort, with modes ranging from 67-74 nm (Figure 8–2).

4.3 Nanoparticle Tracking Analysis: Characterizing the Particle Population in the Zucker Fatty Fractions

In Zucker Fatty rats, nanoparticle concentrations in the serum-bottom fractions were similar in magnitude to those observed in the other two strains, with a 0.17-fold lower concentration of particles present following remote preconditioning when compared with sham-controls. In contrast, nanoparticle concentration in the supernatant-top fraction of sham-control rats was orders of magnitude higher than seen in all other samples from all cohorts (3.9×10^{13} particles/mL), while the mean concentration in the remote preconditioned top fraction was 14-fold lower at 2.6×10^{12} particles/mL. Particle sizes in the supernatant-top fraction were 63 nm in both groups (comparable to values observed in Sprague Dawley cohorts), but were qualitatively larger in the supernatant bottom fractions (modes of 75-88 nm, Figure 8-3).

4.4 Mass Spectrometry: Effect of Remote Preconditioning on Serum Protein Expression in Sprague Dawley and Zucker Rats

For Sprague Dawley rats, MS/MS analysis revealed increases in abundance of 20 proteins in serum and 23 proteins in the protective supernatant-bottom fraction, harvested following RIPC when compared with sham controls. Of the twenty serum proteins, four proteins also had increased abundance in the supernatant-bottom fraction, listed in Table 8–2A, B. These proteins include: Apolipoprotein E, which was increased 1-fold in the remote preconditioned serum and 1.3-fold in the supernatant-bottom fraction compared to sham control samples. Hemoglobin subunit beta-1 was increased in both the serum and supernatant-bottom fraction by 0.3-fold. Carboxylesterase C1 abundance was greater in the serum and supernatant-bottom

fraction by 0.2-fold and 0.1-fold, respectively. Ceruloplasmin was increased by 0.1-fold and 0.2-fold in the serum and supernatant-bottom, respectively. To further interrogate the involvement of these proteins with remote preconditioning, we compared the abundance of these protective candidates in the supernatant-bottom fraction from

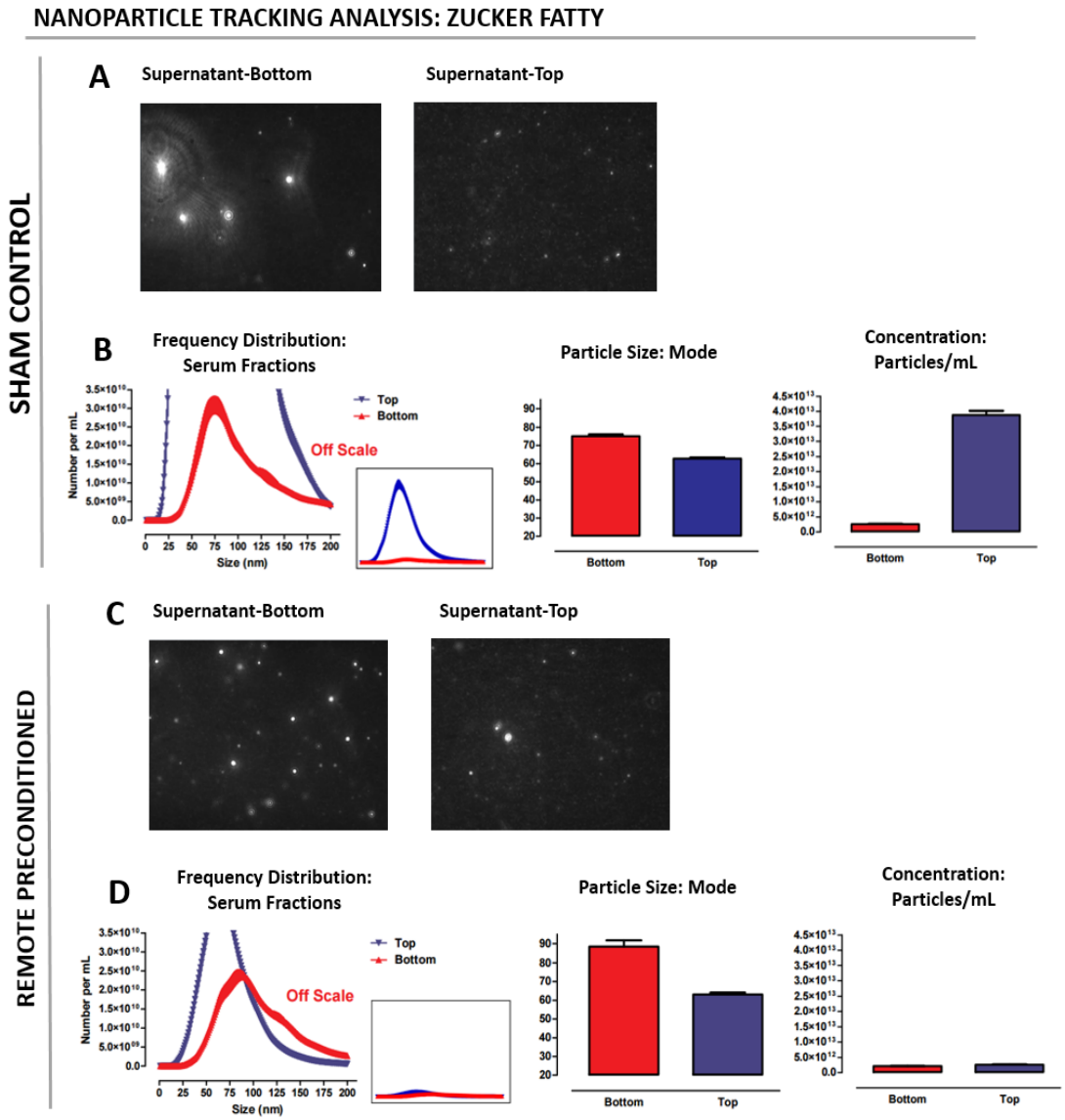


Figure 8–3: Nanoparticle tracking analysis of samples from Zucker Fatty rats. The top and bottom fractions of serum were analyzed with nanoparticle tracking analysis to count and measure the size of particles in solution. **A.** Screenshot from nanosight video and **B.** analysis of sham samples. Inset graph is a larger scale representation of the Frequency distribution. **C.** Screenshot from nanosight video and **D.** analysis of samples from remote preconditioned samples. Inset graph is a larger scale representation of the Frequency distribution.3 replicates per sample, n=1 independent samples/group.

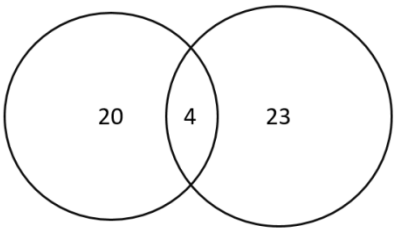
Zucker Lean and Zucker Fatty rats, Table 8–2C, D, respectively. Consistent with the concept that protective proteins will be depleted or deficient in diabetic serum, all four proteins have lower expression in the supernatant-bottom sample from Zucker Fatty rats compared to Zucker Lean rats; Apolipoprotein-E abundance is decreased from 4 to 3 (~0.3-fold decrease), hemoglobin subunit beta-1 is decreased from 4 to 0.5 (~0.9-fold decrease). Carboxylesterase C1 abundance is decreased from 12 to 6 (0.5-fold decrease) and ceruloplasmin is decreased from 15.5 to 14.5 (0.1-fold decrease).

Table 8–2: Mass spectroscopy reveals differential abundance of proteins

A

	Sprague Dawley Remote Preconditioned versus Sham	
	Fold Change	
	Serum	Supernatant-Bottom
Inter-alpha-trypsin inhibitor heavy chain H3	7.0	
Gelsolin	2.0	
Apolipoprotein E	1.5	1.3
Keratin, type II cytoskeletal 6A	1.0	
Histidine-rich glycoprotein	1.0	
Hemoglobin subunit alpha-1/2	1.0	
Ig lambda-2 chain C region	1.0	
Plasminogen	1.0	
Clusterin	0.5	
Complement C3	0.5	
Hemoglobin subunit beta-1	0.3	0.3
Fetuin-B	0.3	
Carboxylesterase 1C	0.2	0.1
Alpha-1-antiproteinase	0.2	
Ceruloplasmin	0.1	0.2
Serum albumin	0.1	
Apolipoprotein A-1	0.1	
Alpha-1-inhibitor 3	0.1	
Murinoglobulin-1	0.1	
Serotransferrin	0.1	

B



C

Zucker *Lean*: Remote Preconditioned	
	Supernatant-Bottom **Average Abundance**
Apolipoprotein E	4
Hemoglobin subunit beta-1	4
Carboxylesterase 1C	12
Ceruloplasmin	15.5

D

Zucker *Fatty*: Remote Preconditioned	
	Supernatant-Bottom **Average Abundance**
Apolipoprotein E	3
Hemoglobin subunit beta-1	0.5
Carboxylesterase 1C	6
Ceruloplasmin	14.5

A list demonstrating proteins with an increased abundance in *serum* from remote preconditioned Sprague Dawley compared to sham controls. Also listed are proteins with increased abundance in the *supernatant-bottom* fraction that also overlap with serum proteins. **B.** Venn diagram demonstrating the overlap between the proteins that had increased abundance in both the serum and supernatant-bottom from remote preconditioning in Sprague Dawley rats. Additionally, the abundance of the four proteins that were commonly increased by remote preconditioning were listed for the supernatant-bottom fraction from **C.** Zucker Lean rats and **D.** Zucker Fatty rats. n=1/group for Sprague Dawley samples. n=2-3/group for Zucker samples.

Finally, with the interest of identifying proteins that may be involved with the toxic effect of the supernatant-top sample from Zucker Fatty rats, we compared the differences in protein abundance in the supernatant-top fraction between the two Zucker

strains. Initially we uncovered 86 proteins that had an increased (42) or decreased (44) abundance in the Zucker Fatty rats compared to the Zucker Leans. We eliminated 15 proteins from this list that were identified as protective candidates from the Sprague Dawley samples. Of the remaining 71, ten proteins with the largest increase in abundance and ten with the largest decrease in abundance are tabulated in Table 8–3.

Table 8–3: Potential toxic candidates in the supernatant-top sample from Zucker Fatty rats.

Supernatant-Top Samples: Zucker Fatty versus Zucker Lean			
	Fold Change: Increase		Fold Change: Decrease
Protein Z-dependent protease inhibitor	9.5	Tropomyosin beta chain	-1
Hemopexin	3.5	Keratin, type I cytoskeletal 42	-1
Beta-2 glycoprotein 1	2.5	Serine protease inhibitor Kazal-type 1-like	-1
Alpha-1-acid glycoprotein	2	Profilin-1	-0.7
Apolipoprotein A-II	1.6	Myosin light chain 1/3, skeletal mus iso	-0.7
Glutathione peroxidase 3	1.5	Polyadenylate-binding protein 1	-0.7
T-kininogen-1	1.5	Thioredoxin	-0.7
Apolipoprotein A-IV	1.1	Keratin, type II cytoskeletal 75	-0.7
Beta-2-microglobulin	1	Serine protease inhibitor A3K	-0.6
Coagulation factor X	1	Keratin, type II cytoskeletal 1	-0.6

The list demonstrates proteins with the largest differential abundance in the supernatant-top fraction from Zucker Fatty rats compared to Zucker Lean. n=2-3/group for Zucker samples.

5. Summary

In summary, we report qualitative results on the size and concentration of nanoparticles (under 200 nm diameter) in serum samples from Sprague Dawley, Zucker Lean and Zucker Fatty rats. Despite the exploratory nature of these experiments, three notable observations were made. First, contrary to our hypothesis, nanoparticle tracking analysis demonstrated that high concentrations of exosome-sized particles were present in the supernatant-top fractions obtained from all cohorts. This was unexpected, based on the absence of classic exosome markers as detected by immunoblotting, in this fraction (see Chapter 6). Second, although RIPC was associated with an increase in nanoparticle concentrations in Sprague Dawley rats, this

was not a consistent finding. In our preliminary analysis, RIPC was not accompanied by an increase in nanoparticle concentration in serum and serum fractions obtained from Zucker Lean rats – a strain in which RIPC was cardioprotective. Finally, hyperglycemic Zucker Fatty rats appear to be characterized by nanoparticle concentrations that are orders of magnitude higher than those observed in the normoglycemic strains, an observation that appears to be consistent with previously published findings.[267] Our preliminary MS/MS analysis demonstrates that there are proteins (4) common between the serum and supernatant-bottom samples which may be involved with cardioprotection through upregulation by remote preconditioning. Data in the Zucker cohorts supports these findings, suggesting that abundance of these proteins is greater in supernatant-bottom fraction from Zucker Lean rats compared to Zucker Fattys. Additionally, we have interrogated the supernatant-top fraction to identify potential toxic factors in the serum from Zucker Fatty rats by comparing the proteomic profile to the supernatant-top fraction from Zucker Lean rats.

CHAPTER 9: DISCUSSION

1. Summary of Results

The present study tested three hypotheses with two complementary models; analysis of remote preconditioning *in vivo* and *in vitro.* We made four novel observations:

i. Remote preconditioning, normally protective against myocardial ischemia/reperfusion injury, is ineffective in the Zucker Fatty rat model of early-stage type-2 diabetes.

ii. Exosomes isolated with the traditional ultracentrifugation technique do not transmit protection. An enhanced ultracentrifugation technique is necessary to sequester a protective sub-fraction from remote preconditioned serum.

iii. Exosomes may be involved with remote preconditioning, but are not sufficient for remote preconditioning.

iv. The failure of remote preconditioning is, at least partially, caused by a defect in the humoral component of cardioprotective signaling.

2. Remote Preconditioning in Type-2 Diabetes: Current Knowledge and New Contributions

Remote preconditioning is a candidate treatment for acute myocardial infarction. Despite overwhelming experimental support and obvious clinical advantages, translation of remote preconditioning has been unsuccessful. As discussed in Chapter 1, diabetes may be a primary cause of attenuated clinical efficacy.

Although the evidence in type 1 diabetes is mixed, ischemic preconditioning and postconditioning are consistently attenuated or lost in rodent models of type-2 diabetes. Additionally, clinical evidence supports that diabetes causes resistance to cardioprotection by ischemic conditioning.[132,133] Remote preconditioning has

considerable mechanistic overlap with ischemic pre- and post-conditioning in terms of protective trigger, signaling and intracellular effectors, which might suggest that there is a similar defect in remote preconditioning the setting of diabetes.

2.1 Infarct Reduction by Remote Preconditioning *In Vivo*

In Chapter 2, we evaluate the efficacy of remote preconditioning in the setting of type-2 diabetes *in vivo.* Of the innumerable studies in the field of ischemic conditioning, ten have investigated the cardioprotective efficacy in the setting of type-2 diabetes, five focusing on ischemic preconditioning and five on ischemic postconditioning (Table 1 – 1). These investigations have reported a common finding: that ischemic conditioning is impaired or eliminated in models of type-2 diabetes. Accordingly, we hypothesized that the cardioprotective efficacy of remote preconditioning is reduced a model of type-2 diabetes.

In the current study, using the Zucker rat model of type-2 diabetes and an *in vivo* model of myocardial ischemia/reperfusion, we demonstrated that the cardioprotective efficacy of remote preconditioning is eliminated in type-2 diabetes. This is the first study investigating remote preconditioning in the setting of type-2 diabetes *in vivo*. These results are consistent with Kristiansen *et al.* and Katakam *et al.*, who reported that ischemic preconditioning (as opposed to remote preconditioning) did not reduce infarct size in Zucker Fatty rats.[106,107] Our results are also supported by studies in other models of type-2 diabetes including Goto Kakizaki rats,[106,110,112,113] db/db mice,[107,108] ob/ob mice[100,111,114] and WOWK rats with metabolic syndrome[109] that report impaired or abolished cardioprotection. Moreover, our data is consistent with two studies that evaluated remote *POST*-conditioning[123] and remote *PER*-conditioning[268] in the streptozotocin-induced model of type 1 diabetes.

2.2 Defect of Remote Preconditioning: Hyperglycemia and Ischemic/Reperfusion Sensitivity

The reason that cardioprotection is impaired in the setting of type-2 diabetes is unclear. A number of preclinical studies have implicated hyperglycemia and sensitization to ischemia/reperfusion, however this evidence is inconsistent. Hyperglycemia, the primary clinical manifestation of type-2 diabetes, may negatively impact the efficacy of ischemic preconditioning[269-272], postconditioning[108] and remote preconditioning.[273] Additionally, clinical evidence suggests that the myocardium is sensitized to ischemia/reperfusion in the setting of diabetes,[81,82] however evidence from preclinical studies is less convincing.[22] Hyperglycemia upon admission for AMI treatment is correlated with mortality,[274,275] which is correlated with infarct size. Lastly, there is evidence that hyperglycemia is correlated with increased area at risk [276] which may cause resistance to ischemic conditioning.[277] Accordingly, we hypothesized that hyperglycemia sensitizes the myocardium to ischemia/reperfusion and reduces the efficacy of remote preconditioning.

Our results did not support this hypothesis. First, we found that infarct size between the control groups from Zucker Fatty and Zucker Lean cohorts was comparable, suggesting that neither type-2 diabetes, nor hyperglycemia *per se*, increase the sensitivity of the myocardium to ischemia/reperfusion. Second, infarct size in the remote preconditioned groups from Zucker Fatty and Zucker Lean rats did not correlate with blood glucose, which we would have expected if hyperglycemia attenuated protection. Lastly, area at risk was comparable between all groups.

The effect hyperglycemia and type-2 diabetes have on resistance to ischemic

conditioning in preclinical models of diabetes is controversial. Kersten *et al*. illustrated that the severity of hyperglycemia plays a critical role in the attenuation of ischemic preconditioning, however Gu *et al*.[278] and Wagner *et al*.[109] describe impaired ischemic conditioning even with mild-moderate hyperglycemia. Bayanyai et al. report that acute hyperglycemia in healthy (non-diabetic) rats impaired remote preconditioning, suggesting that hyperglycemia *per se* may cause resistance to cardioprotection in the setting of diabetes.[273] Nonetheless, we report that remote preconditioning is impaired in type-2 diabetes, despite: (i) no evidence of exacerbation of ischemic/reperfusion injury, (ii) mild hyperglycemia and (iii) treatment with a robust conditioning stimulus.

3. Diabetes and the Humoral Component of Remote Preconditioning: Current Knowledge and New Contributions

By definition, remote preconditioning is capable of overcoming a spatial barrier to evoke protection. Currently, humoral signaling is regarded as one of two central components theorized as a mediator of cardioprotection, although the mechanism has not been fully elucidated. Exosomes are extracellular vesicles recognized for their role in cell-to-cell communication and could hypothetically play an important role in the humoral component of remote preconditioning

The humoral component is supported by convincing evidence that protection against ischemia/reperfusion can be transferred in the blood from preconditioned animals (or perfusion buffer from preconditioned tissues) to naïve animals or tissues. Theoretically, the remote preconditioning stimulus releases protective factors into the circulation, which are delivered to distant, at-risk tissues. The exact mechanisms involved are unclear, however a better understanding will surely help improve protective efficacy and clinical translation. Exosomes are an obvious candidate for carrying

protective signals through the circulation; capable of protecting bioactive cargo in the harsh extracellular environment and targeted delivery of bioactive molecules.[163] Indeed, exosomes have received attention for cardioprotective capabilities, however evidence of this function are based on studies in stem cell treatment of myocardial infarction. Only recently have exosomes been implicated in remote preconditioning and evidence of their involvement is limited[200] and nebulous.[236] We propose that exosomes are an integral factor in the humoral component of remote preconditioning. Moreover, we hypothesize that this mechanism is compromised in the setting of type-2 diabetes, which contributes to impaired cardioprotection.

3.1 Exosome Isolation: Traditional and Enhanced Ultracentrifugation Isolation Techniques

In 2014, Giricz *et al.* reported the first evidence that exosomes are involved in the transfer mechanism of remote preconditioning.[200] In this seminal study, vesicles were obtained from the coronary effluent of ischemic preconditioned, buffer perfused hearts using the traditional ultracentrifugation protocol for isolating exosomes, which resulted in an exosome-marker rich pellet and an exosome-depleted coronary effluent. Treatment with the exosome-rich sample was sufficient to evoke protection against ischemia/reperfusion in buffer perfused hearts, while the exosome-depleted effluent had no effect. Our aim was to investigate the role of exosomes in the cardioprotective humoral signaling of remote preconditioning.

In preliminary experiments, we used the traditional ultracentrifugation technique to collect serum exosomes. In contrast to the Giricz study, we found that the exosome-rich pellet was not protective, whereas the exosome-depleted serum provided significant protection. To explain this disparity, we hypothesized that the traditional

ultracentrifugation protocol does not adequately sediment cardioprotective exosomes from serum, a concept supported by previous studies.[19,247] We modified the isolation protocol by increasing radial force and duration to optimize exosome sedimentation and facilitate depletion of exosomes from serum.[248,279] Ultimately, the enhanced ultracentrifugation protocol fractionated serum into three phases; a pellet (rich with exosome markers) and a biphasic supernatant. The top half of the supernatant was devoid of exosome markers (supernatant-top) and the bottom half was rich with exosome markers (supernatant-bottom).

The next step was to test the protective capacity of each sample as a treatment against hypoxia/reoxygenation injury in cardiomyocytes. First, we report that transfer of the exosome-rich supernatant-bottom fraction to HL-1 cardiomyocytes attenuated cell death, suggesting that, consistent with the Giricz study, exosomes play a role in remote preconditioning. Second, regardless of exosome content, the remaining fractions did not provided protection, suggesting that exosomes are not sufficient for protection. These results indicate that enhanced ultracentrifugation technique may separate functionally distinct populations of exosomes. As discussed in Chapter 2 however, it is important to note that ultracentrifugation isolation sediments protin aggregates and other particles in addition to exosomes and based on these results we cannot unequivocally conclude that exosomes are responsible for protection.[13,280]

3.2 Protection with the Supernatant-bottom Fraction of Sham Serum

We report that the supernatant-bottom fraction collected from sham operated Sprague Dawley rats transmitted modest but significant protection against hypoxia/reoxygenation-induced cell death in HL-1 cardiomyocytes. These unexpected results may be an example of “remote preconditioning of trauma” described by Ren *et*

al., who reported that an abdominal incision caused significant cardioprotection, which was not additive with traditional/ischemic remote preconditioning.[281,282] Accordingly, in our experimental protocol, the femoral artery isolation surgical procedure may initiate "remote preconditioning of trauma". The finding that the supernatant-bottom sample – and not unfractionated serum – evoked protection suggests that exosomes are involved with the signaling component of "remote preconditioning of trauma". Alternatively, previous studies have reported cardioprotective effects of exosomes in the absence of a remote-ischemic or traumatic preconditioning stimulus.[197-199,236] For example, Vicencio *et al.* reported that, for reasons that were not elucidated, exosomes collected from sham subjects had the same protective capacity as those collected from remote preconditioned subjects.[236]

3.3 The Effect of Type-2 Diabetes on the Humoral Component of Remote Preconditioning

The infarct sparing efficacy of ischemic preconditioning and postconditioning is exclusively attenuated or eliminated in animal models of type-2 diabetes. The majority of these studies have focused on the concept that the defect in ischemic conditioning is caused by the effect that diabetes has on the myocardium.[100,108,110-112,250-256] A 2012 study by Jensen and colleagues, was the first to investigated the effect of type-2 diabetes on the humoral component of remote preconditioning. Using serum harvested from human subjects and applied to buffer-perfused rabbit hearts, the authors reported that the diabetes-associated defect in transferred protection was only apparent in in serum from diabetic patients with peripheral neuropathy.[124] The same group also reported that, through humoral mediated signaling, diabetes reduces the susceptibility of healthy myocardial tissue to ischemia/reperfusion, however this phenomenon

occurred independent of remote preconditioning.[283] We postulated that a defect in the humoral component contributes to the failure of remote preconditioning in the setting of type-2 diabetes. To test this hypothesis, we treated cardiomyocytes with serum and fractionated serum samples collected from diabetic Zucker Fatty rats subjected to remote preconditioning. We report two primary observations:

Consistent with our hypothesis, (1) *treatment with serum and the supernatant-bottom fraction obtained from remote preconditioned Zucker Fatty rats did not communicate protection to cardiomyocytes subjected to hypoxia/reoxygenation*. Ours is the first study providing evidence that a defect in humoral signaling contributes to attenuated cardioprotection by remote preconditioning. Jensen et al. reported that attenuated protection is dependent on peripheral neuropathy. Zucker Fatty rats progressively develop peripheral neuropathy,[284] however we used a model of early-stage diabetes, which suggests that the defect is independent of this pathology. A subsequent study by the same group observed improved myocardial functional recovery following ischemia/reperfusion after treatment with plasma dialysate from sham and remote preconditioned diabetic subjects.[283]

Remarkably, (2) *we also report that the supernatant-top fraction of serum from Zucker Fatty rats exacerbated cardiomyocyte death caused by hypoxia/reoxygenation*. Furthermore, this toxicity is dependent on hypoxia/reoxygenation, as the supernatant-top fraction had no effect on cells in a normoxic environment. These findings are unexpected, considering that in Zucker Fatty rats (i) the ischemia/reperfusion injury is not exacerbated and (ii) the unfractionated serum has no effect on cell viability. In other words, the toxic factors present in the diabetic serum only become apparent when the serum is fractionated. Diabetes is an inflammatory disease, with increased expression

of circulating cytokines, some of which have been implicated in cardiomyocyte apoptosis including interleukin-1β[285] and tumor necrosis factor-α[286], which may contribute to the injurious effects of the supernatant-top fraction. Hyperlipidemia is commonly associated with type-2 diabetes and models of type-2 diabetes, including Zucker Fatty rats; studies have demonstrated that a hyperlipidemic diet sensitize the myocardium to ischemia/reperfusion[287,288] or encourage resistance to ischemic preconditioning.[289] Importantly, there is evidence that certain fatty acids promote apoptosis in cultured cardiomyocytes.[290]

3.4 Nanoparticle Tracking Analysis

Interrogation of the particle content of our samples was critical, particularly because we used a novel isolation technique that has not been characterized. We used nanoparticle tracking analysis, a common technology used for detecting exosomes, to measure particle size and concentration. Based on our data, we were able to make one important conclusion; that exosome-sized particles were present in all of the fractionated serum samples – including the presumably exosome-poor top fraction - from all rat strains. This finding corroborates our previous conclusions that (1) exosomes may be necessary for remote preconditioning and that (2) exosomes are not necessarily sufficient for protection. Lastly, these results suggests that (3) the enhanced ultracentrifugation isolation technique does not sediment all particles from the serum.

Vicencio *et al*. reported that exosome release is stimulated by a remote preconditioning stimulus and that particle concentration correlates with protection.[236] Giricz *et al*. also report indirect evidence that exosome concentration is increased by an ischemic conditioning stimulus.[200] Based on our observations that particle

concentration was increased in Sprague Dawley and reduced in Zucker Lean rats, it is unclear how remote preconditioning effects exosome release. Moreover, in our experiments, particle concentration does not correlate with protection, an observation supported by the lack of protection from treatment with the supernatant-top fraction and pellet, both of which contain particles. Importantly, nanoparticle tracking analysis cannot not distinguish exosomes from non-exosome particles. Accordingly, we predict that the particles present in this fraction are non-exosome particles, based on immunoblotting evidence that this sample is depleted of classic exosome markers. Interestingly, very-low density lipoprotein particles are less dense than exosomes (1.006 g/mL[291] for very-low density lipoprotein versus 1.13-1.19 g/mL for exosomes[292]) and may remain in the supernatant following ultracentrifugation.

4. Qualitative Proteomic Characterization: Targeting the Humoral Component of Remote Preconditioning

Currently, the identity – and molecular nature – of the protective humoral factor(s) in remote preconditioning remains unknown. Hepponstal *et al*.[257,263], Pang *et al*.[258], Hibert *et al*.[260,261], Helgeland *et al*.[262] Lang *et al*.[259] have explored the circulation for proteins that are differentially expressed in response to ischemic conditioning. Using functional techniques to assay for protection (by treating naïve tissues with serum or perfusion buffer collected from ischemic conditioned tissue that has been filtered) the humoral factor has been described as being small (between 3.5 and 15 kilo Daltons) and hydrophobic.[51-53]

Similar to previous reports, we used mass spectroscopy to detect changes in abundance of circulating proteins. In contrast however, we used our novel method of prolonged ultracentrifucation to isolate a protective fraction of the serum, and theorized

that this would facilitate detection of changes in abundance of candidate protective protein(s). After initial trouble-shooting and analysis of serum and serum fractions obtained from Sprague Dawley rats, we compared the changes in expression between Zucker Lean rats, that have intact protective humoral signaling, and Zucker Fatty rats where the humoral component is defective. Lastly, with the interest of identifying toxic circulating factors, we have compared the protein abundance in the supernatant-top fraction of Zucker Fatty and Zucker Lean rats.

Beginning with the Sprague Dawley cohort, we found 20 proteins in the unfractionated serum and 23 proteins in the supernatant-bottom fraction that were upregulated. Four of these candidate proteins were commonly upregulated in both the serum and supernatant-bottom fraction and, furthermore, all 4 proteins had increased expression in the Zucker Lean rats compared to Zucker Fattys; apolipoprotein-E, hemoglobin subunit beta-1, carboxylesterase 1C and ceruloplasmin. There is evidence that ceruloplasmin may protect against ischemia/reperfusion induced ventricular fibrillation, and apolipoprotein-E[293] and its mimetics have been demonstrated protection against brain ischemia.[294] Additionally, some evidence suggests that apolipoproteins-E is present in exosomes secreted from astrocytes and upregulated in response to hypoxia.[226] Ceruloplasmin has been discovered in urinary exosomes, in the setting of kidney disease. However, there is currently no evidence to suggest that carboxylesterase 1C or hemoglobin subunit beta-1 are involved with protective signaling. In the supernatant-top fraction, 86 proteins were upregulated or downregulated in Zucker Fatty rats compare to Zucker Lean rats. After removing protective candidates, identified above, 71 proteins were implicated in the toxic component of diabetic serum. It is also worth noting that apolipoprotein B-100,

apolipoprotein C-I, III, VI and apolipoprotein E – all common proteins in very low density lipoprotein – are more abundant in the supernatant-top fraction compared to the bottom fraction in nearly all rat strains, data not shown. This supports the hypothesis that the particles present in the supernatant-top fraction are very low density lipoprotein. Interestingly, previous data suggest that very low density lipoprotein may exacerbate the injury caused by ischemia[295] and hypoxia.[296] Moreover, type-2 diabetes is associated with an increase in circulating VLDL[297], which may explain the increased particle concentration in the supernatant-top fraction collected from Zucker Fatty rats and could contribute to its toxic effect.

As with all mass spectrometry studies in remote preconditioning, our proteomic data are hypothesis generating. While the proteins identified as protective or toxic may be involved with remote preconditioning, further investigation will be required to implicate the candidates in cardioprotection or toxicity. Importantly, our data do not suggest that proteins are exclusively responsible for protection or toxicity. It is possible that other molecule types, such as nucleic acids or lipids, may contribute to cell fate . Additionally, this technique of mass spectrometry does not detect modification of proteins such as phosphorylation or oxidation, which may also contribute to protection or toxicity.

5. Technical Limitations and Merits

A primary limitation in this study is the inadequacy of experimental techniques, such as isolation and quantification methods, in the exosome field, which are inherent and well documented.[13] To our credit, we enhanced the standard exosome isolation technique based on Svedberg kinetics (the forces involved with sedimentation) to improve yield and completeness, which resulted in the discovery of two functionally

distinct fractions of the serum. Nonetheless, the enhanced ultracentrifugation technique probably sacrifices purity for yield, resulting in exosome preparations contaminated with non-exosome particles, such as protein aggregates or lipoproteins. A second limitation is that, in our model, it is not possible to apply the remote preconditioning stimulus and blood withdrawal without tissue manipulation and this possibly stimulating ancillary protective signaling such as remote preconditioning of trauma. While our results obtained in supernatant-bottom fractions obtained from sham cohorts suggests that preconditioning of trauma may have occurred, our results did provide evidence that this protective phenomenon communicates through the exosome-rich bottom fraction. The third limitation is the use of a cell culture model, where it is unclear how closely hypoxia/reoxygenation *in vitro* mimics AMI *in vivo*, and, more specifically HL-1 cells (an immortal cell line) rather than primary cardiomyocytes. Nonetheless, we have found that HL-1 cells are sensitive to acute hypoxia/reoxygenation and cell death can be easily quantified. Importantly, to investigate the humoral component of remote preconditioning it is necessary to have naïve (unconditioned) tissue, and using immortalized cardiomyocytes reduces the demand for whole animals. Additionally, treating cultured cells requires considerably less sample volume, compared with other naïve-tissue treatment options (transfer to whole animal or buffer-perfused heart). Follow-up studies are required to corroborate our findings with intact biological systems.

6. Conclusions and Future Directions

In consideration of our *in vivo* and *in vitro* results, we theorize that there is a balance of bioactive components in the serum that modulate the severity of ischemia/reperfusion. Convincing evidence has demonstrated that remote preconditioning causes the release of beneficial factors, tipping the balance to favor

cardioprotection. We further propose that, in the setting of type-2 diabetes, remote preconditioning is also associated with the release or activation of factors than may increase sensitivity of the myocardium to ischemia/reperfusion: i.e., we demonstrated that a sub-fraction of serum harvested from diabetic Zucker Fatty rats exacerbated hypoxia-reoxygenation-induced cell death *in vitro*. This may be mediated by specific macromolecules, such as proteins or nucleic acids, or by the post-translational modifications, such as oxidation or phosphorylation, of one or more of these macromolecules. Despite a cytotoxic component of serum from Zucker Fatty rats, neither *in vivo* remote preconditioning in this cohort nor application of serum to HL-1 cells exacerbated cardiomyocyte death – an observation that reinforces the aforementioned notion of a balance among bioactive components in the intact serum.

The major conclusion of this dissertation study is that remote preconditioning is ineffective in the Zucker Fatty model of type-2 diabetes due to a defect in the protective humoral signaling. Giricz *et al*. illustrated that exosomes are a critical component in cardioprotection by remote preconditioning in *ex vivo* rat hearts.[200] Our data expand on this concept, reporting that while exosomes may be necessary, they are not sufficient to evoke cardioprotection. Moreover, the defect in the humoral component of remote preconditioning may relate to the balance of bioactive factors in the circulation. The possibility that a toxic factor is present in circulation – and increased in diabetes – may provide a foundation for future studies that could advance ischemic conditioning technology.

APPENDIX A

IACUC Protocol Approval Letter

WAYNE STATE UNIVERSITY

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A3310-01

PROTOCOL # A 02-01-14

Protocol Effective Period: March 5, 2014 – February 28, 2017

TO: Dr. Karin Przyklenk
CVRI/Physiology
1107.4 Elliman Clinical Research Building

FROM: Lisa Anne Polin, Ph.D.
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 02-01-14
"Mechanisms of Remote Ischemic Conditioning"

DATE: March 5, 2014

Your animal research protocol has been reviewed by the Wayne State University Institutional Animal Care and Use Committee, and given final approval for the period effective **March 5, 2014** through **February 28, 2017**. The listed source of funding for the protocol is **Start-up, Indirect cost account, NIH**. The species and number of animals approved for the duration of this protocol are listed below.

Species	Strain	Qty.	USDA Cat.
RATS	Sprague-Dawley, female, retired breeders (approx 250-350 grams)	70	D
DOGS	Class A dogs, either sex, 10-20 kg	14	D

Be advised that this protocol must be reviewed by the IACUC on an annual basis to remain active. Any change in procedures, change in lab personnel, change in species, or additional numbers of animals requires prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol form and full committee review.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

APPENDIX B

Permission from publisher for Chapter 1

Permission form

Requestor Information:

Name: Joseph Wider

Affiliation: Wayne State University

Address: 540 E. Canfield, Detroit MI, 48201

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Tell Us About Your Publication:

Authors/Editors: Joseph Wider, Karin Przyklerk

Title of Publication: Ischemic conditionin: the challenge of protecting the diabetic heart

Article Title (if applicable):

Projected Date of Publication: Oct 2014

Publisher: AME Publishing Company

Purpose of Publication (Educational, Commercial, etc.): Educational Review

What Publication Material Would You Like To Use/Adapt?

Authors: Joseph Wider, Karin Przyklenk

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It is understood that full credit will be given to the author and publisher, either as a footnote, or as a reference within the text, or both.

Date: 27 Feb. 2017

Signature: Joseph Wider

Permission is granted for use of the said material as stipulated.

Stephen Wang

2017.3.6

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REFERENCES

1. Go AS, Mozaffarian D, Roger VL, et al. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation.* 2014;129(3):e28-e292.
2. Wang J, Yao Y, Wu J, Li G. Identification and analysis of exosomes secreted from macrophages extracted by different methods. *International journal of clinical and experimental pathology.* 2015;8(6):6135-6142.
3. Van Deun J, Mestdagh P, Sormunen R, et al. The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling. *Journal of extracellular vesicles.* 2014;3.
4. Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? *The Journal of clinical investigation.* 1985;76(5):1713-1719.
5. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *The New England journal of medicine.* 2007;357(11):1121-1135.
6. Sanada S, Komuro I, Kitakaze M. Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures. *American journal of physiology Heart and circulatory physiology.* 2011;301(5):H1723-1741.
7. Przyklenk K. Reduction of myocardial infarct size with ischemic "conditioning": physiologic and technical considerations. *Anesthesia and analgesia.* 2013;117(4):891-901.
8. Heusch G. Cardioprotection: chances and challenges of its translation to the clinic. *Lancet.* 2013;381(9861):166-175.
9. Thompson PL, Fletcher EE, Katavatis V. Enzymatic indices of myocardial

necrosis: influence on short- and long-term prognosis after myocardial infarction. *Circulation.* 1979;59(1):113-119.

10. Lefer DJ, Bolli R. Development of an NIH consortium for preclinicAl AssESsment of CARdioprotective therapies (CAESAR): a paradigm shift in studies of infarct size limitation. *Journal of cardiovascular pharmacology and therapeutics.* 2011;16(3-4):332-339.
11. Longacre LS, Kloner RA, Arai AE, et al. New Horizons in Cardioprotection Recommendations From the 2010 National Heart, Lung, and Blood Institute Workshop. *Circulation.* 2011;124(10):1172-1179.
12. Hausenloy DJ, Erik Botker H, Condorelli G, et al. Translating cardioprotection for patient benefit: position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. *Cardiovascular research.* 2013;98(1):7-27.
13. Witwer KW, Buzas EI, Bemis LT, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles.* 2013;2.
14. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation.* 1986;74(5):1124-1136.
15. Zhao ZQ, Corvera JS, Halkos ME, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *American journal of physiology Heart and circulatory physiology.* 2003;285(2):H579-588.
16. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained

coronary occlusion. *Circulation.* 1993;87(3):893-899.

17. Przyklenk K. Efficacy of cardioprotective 'conditioning' strategies in aging and diabetic cohorts: the co-morbidity conundrum. *Drugs Aging.* 2011;28(5):331-343.
18. Selvin E, Parrinello CM, Sacks DB, Coresh J. Trends in prevalence and control of diabetes in the United States, 1988-1994 and 1999-2010. *Annals of internal medicine.* 2014;160(8):517-525.
19. Momen-Heravi F, Balaj L, Alian S, et al. Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles. *Front Physiol.* 2012;3:162.
20. 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.
21. Vinten-Johansen J, Granfeldt A, Mykytenko J, Undyala VV, Dong Y, Przyklenk K. The multidimensional physiological responses to postconditioning. *Antioxidants & redox signaling.* 2011;14(5):791-810.
22. Miki T, Itoh T, Sunaga D, Miura T. Effects of diabetes on myocardial infarct size and cardioprotection by preconditioning and postconditioning. *Cardiovasc Diabetol.* 2012;11:67.
23. Gyorgy B, Modos K, Pallinger E, et al. Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters. *Blood.* 2011;117(4):e39-48.
24. Kreimer S, Belov AM, Ghiran I, Murthy SK, Frank DA, Ivanov AR. Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics. *J Proteome Res.* 2015;14(6):2367-2384.
25. Kharbanda RK, Mortensen UM, White PA, et al. Transient limb ischemia induces

remote ischemic preconditioning *in vivo*. *Circulation.* 2002;106(23):2881-2883.

26. Przyklenk K, Kloner RA. Ischemic preconditioning: exploring the paradox. *Progress in cardiovascular diseases.* 1998;40(6):517-547.

27. Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiological reviews.* 2003;83(4):1113-1151.

28. Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiological reviews.* 2008;88(2):581-609.

29. Cohen MV, Downey JM. Is it time to translate ischemic preconditioning's mechanism of cardioprotection into clinical practice? *Journal of cardiovascular pharmacology and therapeutics.* 2011;16(3-4):273-280.

30. Heusch G, Boengler K, Schulz R. Cardioprotection: nitric oxide, protein kinases, and mitochondria. *Circulation.* 2008;118(19):1915-1919.

31. Liu GS, Thornton J, Van Winkle DM, Stanley AW, Olsson RA, Downey JM. Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. *Circulation.* 1991;84(1):350-356.

32. Cohen MV, Downey JM. Adenosine: trigger and mediator of cardioprotection. *Basic Res Cardiol.* 2008;103(3):203-215.

33. Goto M, Liu Y, Yang XM, Ardell JL, Cohen MV, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. *Circulation research.* 1995;77(3):611-621.

34. Schultz JE, Hsu AK, Gross GJ. Ischemic preconditioning in the intact rat heart is mediated by delta1- but not mu- or kappa-opioid receptors. *Circulation.* 1998;97(13):1282-1289.

35. Cohen MV, Yang XM, Liu GS, Heusch G, Downey JM. Acetylcholine, bradykinin,

opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K(ATP) channels. *Circulation research.* 2001;89(3):273-278.

36. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. *Journal of molecular and cellular cardiology.* 2002;34(5):509-518.

37. Ytrehus K, Liu Y, Downey JM. Preconditioning protects ischemic rabbit heart by protein kinase C activation. *The American journal of physiology.* 1994;266(3 Pt 2):H1145-1152.

38. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovascular research.* 2004;61(3):448-460.

39. Lacerda L, Somers S, Opie LH, Lecour S. Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. *Cardiovascular research.* 2009;84(2):201-208.

40. Suleiman MS, Halestrap AP, Griffiths EJ. Mitochondria: a target for myocardial protection. *Pharmacology & therapeutics.* 2001;89(1):29-46.

41. Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovascular research.* 2002;55(3):534-543.

42. Ruiz-Meana M, Rodriguez-Sinovas A, Cabestrero A, Boengler K, Heusch G, Garcia-Dorado D. Mitochondrial connexin43 as a new player in the pathophysiology of myocardial ischaemia-reperfusion injury. *Cardiovascular*

research. 2008;77(2):325-333.

43. Murphy E, Steenbergen C. What makes the mitochondria a killer? Can we condition them to be less destructive? *Biochimica et biophysica acta.* 2011;1813(7):1302-1308.

44. Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D, Ovize M. Postconditioning inhibits mitochondrial permeability transition. *Circulation.* 2005;111(2):194-197.

45. Hausenloy DJ, Lecour S, Yellon DM. Reperfusion injury salvage kinase and survivor activating factor enhancement prosurvival signaling pathways in ischemic postconditioning: two sides of the same coin. *Antioxidants & redox signaling.* 2011;14(5):893-907.

46. Vinten-Johansen J, Shi W. Perconditioning and postconditioning: current knowledge, knowledge gaps, barriers to adoption, and future directions. *Journal of cardiovascular pharmacology and therapeutics.* 2011;16(3-4):260-266.

47. Przyklenk K, Whittaker P. Remote ischemic preconditioning: current knowledge, unresolved questions, and future priorities. *Journal of cardiovascular pharmacology and therapeutics.* 2011;16(3-4):255-259.

48. Przyklenk K, Whittaker P. Genesis of remote conditioning: action at a distance - *'hypotheses non fingo'*? *J Cardiovasc Med.* 2013;14:179-185.

49. Hausenloy DJ, Yellon DM. Remote ischaemic preconditioning: underlying mechanisms and clinical application. *Cardiovascular research.* 2008;79(3):377-386.

50. Saxena P, Newman MA, Shehatha JS, Redington AN, Konstantinov IE. Remote ischemic conditioning: evolution of the concept, mechanisms, and clinical

application. *J Card Surg.* 2010;25(1):127-134.

51. Dickson EW, Blehar DJ, Carraway RE, Heard SO, Steinberg G, Przyklenk K. Naloxone blocks transferred preconditioning in isolated rabbit hearts. *J Mol Cell Cardiol.* 2001;33(9):1751-1756.

52. Serejo FC, Rodrigues LF, Jr., da Silva Tavares KC, de Carvalho AC, Nascimento JH. Cardioprotective properties of humoral factors released from rat hearts subject to ischemic preconditioning. *J Cardiovasc Pharmacol.* 2007;49(4):214-220.

53. Shimizu M, Tropak M, Diaz RJ, et al. Transient limb ischaemia remotely preconditions through a humoral mechanism acting directly on the myocardium: evidence suggesting cross-species protection. *Clin Sci (Lond).* 2009;117(5):191-200.

54. Donato M, Buchholz B, Rodriguez M, et al. Role of the parasympathetic nervous system in cardioprotection by remote hindlimb ischaemic preconditioning. *Experimental physiology.* 2013;98(2):425-434.

55. Gourine A, Gourine AV. Neural mechanisms of cardioprotection. *Physiology (Bethesda).* 2014;29(2):133-140.

56. Lim SY, Yellon DM, Hausenloy DJ. The neural and humoral pathways in remote limb ischemic preconditioning. *Basic Res Cardiol.* 2010;105(5):651-655.

57. Redington KL, Disenhouse T, Strantzas SC, et al. Remote cardioprotection by direct peripheral nerve stimulation and topical capsaicin is mediated by circulating humoral factors. *Basic Res Cardiol.* 2012;107(2):241.

58. Bertolotto C, Maulon L, Filippa N, Baier G, Auberger P. Protein kinase C theta and epsilon promote T-cell survival by a rsk-dependent phosphorylation and

inactivation of BAD. *The Journal of biological chemistry.* 2000;275(47):37246-37250.

59. Penna C, Rastaldo R, Mancardi D, et al. Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K+ channel and protein kinase C activation. *Basic Res Cardiol.* 2006;101(2):180-189.

60. Philipp S, Yang XM, Cui L, Davis AM, Downey JM, Cohen MV. Postconditioning protects rabbit hearts through a protein kinase C-adenosine A2b receptor cascade. *Cardiovascular research.* 2006;70(2):308-314.

61. Weinbrenner C, Nelles M, Herzog N, Sarvary L, Strasser RH. Remote preconditioning by infrarenal occlusion of the aorta protects the heart from infarction: a newly identified non-neuronal but PKC-dependent pathway. *Cardiovascular research.* 2002;55(3):590-601.

62. Simkhovich BZ, Przyklenk K, Kloner RA. Role of protein kinase C in ischemic "conditioning": from first evidence to current perspectives. *Journal of cardiovascular pharmacology and therapeutics.* 2013;18(6):525-532.

63. Skyschally A, Schulz R, Gres P, Korth HG, Heusch G. Attenuation of ischemic preconditioning in pigs by scavenging of free oxyradicals with ascorbic acid. *American journal of physiology Heart and circulatory physiology.* 2003;284(2):H698-703.

64. Hausenloy D, Wynne A, Duchen M, Yellon D. Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. *Circulation.* 2004;109(14):1714-1717.

65. Jennings RB. Historical perspective on the pathology of myocardial

ischemia/reperfusion injury. *Circulation research.* 2013;113(4):428-438.

66. Reimer KA, Murry CE, Yamasawa I, Hill ML, Jennings RB. Four brief periods of myocardial ischemia cause no cumulative ATP loss or necrosis. *The American journal of physiology.* 1986;251(6 Pt 2):H1306-1315.

67. Murry CE, Richard VJ, Reimer KA, Jennings RB. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circulation research.* 1990;66(4):913-931.

68. Murry CE, Richard VJ, Jennings RB, Reimer KA. Myocardial protection is lost before contractile function recovers from ischemic preconditioning. *The American journal of physiology.* 1991;260(3 Pt 2):H796-804.

69. Yoshiyama M, Merkle H, Garwood M, et al. Transmural distribution of 2-deoxyglucose uptake in normal and post-ischemic canine myocardium. *NMR in biomedicine.* 1995;8(1):9-18.

70. Tong H, Chen W, London RE, Murphy E, Steenbergen C. Preconditioning enhanced glucose uptake is mediated by p38 MAP kinase not by phosphatidylinositol 3-kinase. *The Journal of biological chemistry.* 2000;275(16):11981-11986.

71. Yabe K, Tanonaka K, Koshimizu M, Katsuno T, Takeo S. A role of PKC in the improvement of energy metabolism in preconditioned heart. *Basic Res Cardiol.* 2000;95(3):215-227.

72. Ji L, Zhang X, Liu W, et al. AMPK-regulated and Akt-dependent enhancement of glucose uptake is essential in ischemic preconditioning-alleviated reperfusion injury. *PloS one.* 2013;8(7):e69910.

73. Nederlof R, Eerbeek O, Hollmann MW, Southworth R, Zuurbier CJ. Targeting

hexokinase II to mitochondria to modulate energy metabolism and reduce ischaemia-reperfusion injury in heart. *British journal of pharmacology.* 2014;171(8):2067-2079.

74. Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nature reviews Molecular cell biology.* 2007;8(10):774-785.

75. Gurel E, Smeele KM, Eerbeek O, et al. Ischemic preconditioning affects hexokinase activity and HKII in different subcellular compartments throughout cardiac ischemia-reperfusion. *J Appl Physiol (1985).* 2009;106(6):1909-1916.

76. Pasdois P, Parker JE, Halestrap AP. Extent of mitochondrial hexokinase II dissociation during ischemia correlates with mitochondrial cytochrome c release, reactive oxygen species production, and infarct size on reperfusion. *Journal of the American Heart Association.* 2013;2(1):e005645.

77. Hermann R, Marina Prendes MG, Torresin ME, Velez D, Savino EA, Varela A. Effects of the AMP-activated protein kinase inhibitor compound C on the postconditioned rat heart. *The journal of physiological sciences : JPS.* 2012;62(4):333-341.

78. Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Mortality from coronary heart disease in subjects with type-2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *The New England journal of medicine.* 1998;339(4):229-234.

79. Sarwar N, Gao P, Seshasai SR, et al. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. *Lancet.* 2010;375(9733):2215-2222.

80. Krempf M, Parhofer KG, Steg PG, et al. Cardiovascular event rates in diabetic

and nondiabetic individuals with and without established atherothrombosis (from the REduction of Atherothrombosis for Continued Health [REACH] Registry). *The American journal of cardiology.* 2010;105(5):667-671.

81. Alegria JR, Miller TD, Gibbons RJ, Yi QL, Yusuf S, Collaborative Organization of RheothRx Evaluation Trial I. Infarct size, ejection fraction, and mortality in diabetic patients with acute myocardial infarction treated with thrombolytic therapy. *Am Heart J.* 2007;154(4):743-750.
82. Marso SP, Miller T, Rutherford BD, et al. Comparison of myocardial reperfusion in patients undergoing percutaneous coronary intervention in ST-segment elevation acute myocardial infarction with versus without diabetes mellitus (from the EMERALD Trial). *Am J Cardiol.* 2007;100(2):206-210.
83. Mukamal KJ, Nesto RW, Cohen MC, et al. Impact of diabetes on long-term survival after acute myocardial infarction: comparability of risk with prior myocardial infarction. *Diabetes care.* 2001;24(8):1422-1427.
84. Murcia AM, Hennekens CH, Lamas GA, et al. Impact of diabetes on mortality in patients with myocardial infarction and left ventricular dysfunction. *Archives of internal medicine.* 2004;164(20):2273-2279.
85. Zia MI, Ghugre NR, Roifman I, et al. Comparison of the frequencies of myocardial edema determined by cardiac magnetic resonance in diabetic versus nondiabetic patients having percutaneous coronary intervention for ST elevation myocardial infarction. *The American journal of cardiology.* 2014;113(4):607-612.
86. Chowdhry MF, Vohra HA, Galinanes M. Diabetes increases apoptosis and necrosis in both ischemic and nonischemic human myocardium: role of caspases and poly-adenosine diphosphate-ribose polymerase. *The Journal of thoracic and*

cardiovascular surgery. 2007;134(1):124-131, 131 e121-123.

87. Frustaci A, Kajstura J, Chimenti C, et al. Myocardial cell death in human diabetes. *Circulation research.* 2000;87(12):1123-1132.

88. Gu K, Cowie CC, Harris MI. Diabetes and decline in heart disease mortality in US adults. *JAMA : the journal of the American Medical Association.* 1999;281(14):1291-1297.

89. Donahoe SM, Stewart GC, McCabe CH, et al. Diabetes and mortality following acute coronary syndromes. *JAMA : the journal of the American Medical Association.* 2007;298(7):765-775.

90. Whiting DR, Guariguata L, Weil C, Shaw J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes research and clinical practice.* 2011;94(3):311-321.

91. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes care.* 2004;27(5):1047-1053.

92. Gregg EW, Zhuo X, Cheng YL, Albright AL, Narayan KMV, Thompson TJ. Trends in lifetime risk and years of life lost due to diabetes in the USA, 1985—2011: a modelling study. *The Lancet Diabetes & Endocrinology.* 2014;14:70161-70165.

93. Abel ED, O'Shea KM, Ramasamy R. Insulin resistance: metabolic mechanisms and consequences in the heart. *Arteriosclerosis, thrombosis, and vascular biology.* 2012;32(9):2068-2076.

94. Hafstad AD, Solevag GH, Severson DL, Larsen TS, Aasum E. Perfused hearts from Type-2 diabetic (db/db) mice show metabolic responsiveness to insulin.

American journal of physiology Heart and circulatory physiology. 2006;290(5):H1763-1769.

95. Carley AN, Atkinson LL, Bonen A, et al. Mechanisms responsible for enhanced fatty acid utilization by perfused hearts from type-2 diabetic db/db mice. *Archives of physiology and biochemistry.* 2007;113(2):65-75.

96. Desrois M, Sidell RJ, Gauguier D, King LM, Radda GK, Clarke K. Initial steps of insulin signaling and glucose transport are defective in the type-2 diabetic rat heart. *Cardiovascular research.* 2004;61(2):288-296.

97. Huisamen B. Protein kinase B in the diabetic heart. *Molecular and cellular biochemistry.* 2003;249(1-2):31-38.

98. Mocanu MM, Field DC, Yellon DM. A potential role for PTEN in the diabetic heart. *Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy.* 2006;20(4):319-321.

99. Miki T, Miura T, Hotta H, et al. Endoplasmic reticulum stress in diabetic hearts abolishes erythropoietin-induced myocardial protection by impairment of phospho-glycogen synthase kinase-3beta-mediated suppression of mitochondrial permeability transition. *Diabetes.* 2009;58(12):2863-2872.

100. Bouhidel O, Pons S, Souktani R, Zini R, Berdeaux A, Ghaleh B. Myocardial ischemic postconditioning against ischemia-reperfusion is impaired in ob/ob mice. *Am J Physiol Heart Circ Physiol.* 2008;295(4):H1580-1586.

101. Wang B, Raedschelders K, Shravah J, et al. Differences in myocardial PTEN expression and Akt signalling in type-2 diabetic and nondiabetic patients undergoing coronary bypass surgery. *Clinical endocrinology.* 2011;74(6):705-713.

102. Anderson EJ, Rodriguez E, Anderson CA, Thayne K, Chitwood WR, Kypson AP. Increased propensity for cell death in diabetic human heart is mediated by mitochondrial-dependent pathways. *American journal of physiology Heart and circulatory physiology.* 2011;300(1):H118-124.

103. Liu Y, Thornton JD, Cohen MV, Downey JM, Schaffer SW. Streptozotocin-induced non-insulin-dependent diabetes protects the heart from infarction. *Circulation.* 1993;88(3):1273-1278.

104. Paulson DJ. The diabetic heart is more sensitive to ischemic injury. *Cardiovascular research.* 1997;34(1):104-112.

105. Balakumar P, Sharma NK. Healing the diabetic heart: does myocardial preconditioning work? *Cellular signalling.* 2012;24(1):53-59.

106. Kristiansen SB, Lofgren B, Stottrup NB, et al. Ischaemic preconditioning does not protect the heart in obese and lean animal models of type-2 diabetes. *Diabetologia.* 2004;47(10):1716-1721.

107. Katakam PV, Jordan JE, Snipes JA, Tulbert CD, Miller AW, Busija DW. Myocardial preconditioning against ischemia-reperfusion injury is abolished in Zucker obese rats with insulin resistance. *Am J Physiol Regul Integr Comp Physiol.* 2007;292(2):R920-926.

108. Przyklenk K, Maynard M, Greiner DL, Whittaker P. Cardioprotection with postconditioning: loss of efficacy in murine models of type-2 and type-1 diabetes. *Antioxid Redox Signal.* 2011;14(5):781-790.

109. Wagner C, Kloeting I, Strasser RH, Weinbrenner C. Cardioprotection by postconditioning is lost in WOKW rats with metabolic syndrome: role of glycogen synthase kinase 3beta. *J Cardiovasc Pharmacol.* 2008;52(5):430-437.

110. Whittington HJ, Harding I, Stephenson CI, et al. Cardioprotection in the aging, diabetic heart: the loss of protective Akt signalling. *Cardiovasc Res.* 2013;99(4):694-704.

111. Zhu SG, Xi L, Kukreja RC. Type-2 diabetic obese db/db mice are refractory to myocardial ischaemic post-conditioning *in vivo*: potential role for Hsp20, F1-ATPase delta and Echs1. *J Cell Mol Med.* 2012;16(4):950-958.

112. Tsang A, Hausenloy DJ, Mocanu MM, Carr RD, Yellon DM. Preconditioning the diabetic heart: the importance of Akt phosphorylation. *Diabetes.* 2005;54(8):2360-2364.

113. Hausenloy DJ, Wynne AM, Mocanu MM, Yellon DM. Glimepiride treatment facilitates ischemic preconditioning in the diabetic heart. *J Cardiovasc Pharmacol Ther.* 2013;18(3):263-269.

114. Oosterlinck W, Dresselaers T, Geldhof V, et al. Diabetes mellitus and the metabolic syndrome do not abolish, but might reduce, the cardioprotective effect of ischemic postconditioning. *J Thorac Cardiovasc Surg.* 2013;145(6):1595-1602.

115. Kersten JR, Toller WG, Gross ER, Pagel PS, Warltier DC. Diabetes abolishes ischemic preconditioning: role of glucose, insulin, and osmolality. *Am J Physiol Heart Circ Physiol.* 2000;278(4):H1218-1224.

116. Nieszner E, Posa I, Kocsis E, Pogatsa G, Preda I, Koltai MZ. Influence of diabetic state and that of different sulfonylureas on the size of myocardial infarction with and without ischemic preconditioning in rabbits. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association.* 2002;110(5):212-218.

117. Yadav HN, Singh M, Sharma PL. Involvement of GSK-3beta in attenuation of the

cardioprotective effect of ischemic preconditioning in diabetic rat heart. *Molecular and cellular biochemistry.* 2010;343(1-2):75-81.

118. Drenger B, Ostrovsky IA, Barak M, Nechemia-Arbely Y, Ziv E, Axelrod JH. Diabetes blockade of sevoflurane postconditioning is not restored by insulin in the rat heart: phosphorylated signal transducer and activator of transcription 3- and phosphatidylinositol 3-kinase-mediated inhibition. *Anesthesiology.* 2011;114(6):1364-1372.

119. Fan Y, Yang S, Zhang X, Cao Y, Huang Y. Comparison of cardioprotective efficacy resulting from a combination of atorvastatin and ischaemic post-conditioning in diabetic and non-diabetic rats. *Clinical and experimental pharmacology & physiology.* 2012;39(11):938-943.

120. Badalzadeh R, Mohammadi M, Najafi M, Ahmadiasl N, Farajnia S, Ebrahimi H. The additive effects of ischemic postconditioning and cyclosporine-A on nitric oxide activity and functions of diabetic myocardium injured by ischemia/reperfusion. *Journal of cardiovascular pharmacology and therapeutics.* 2012;17(2):181-189.

121. Liu M, Zhou B, Xia ZY, et al. Hyperglycemia-induced inhibition of DJ-1 expression compromised the effectiveness of ischemic postconditioning cardioprotection in rats. *Oxidative medicine and cellular longevity.* 2013;2013:564902.

122. Potier L, Waeckel L, Vincent MP, et al. Selective kinin receptor agonists as cardioprotective agents in myocardial ischemia and diabetes. *The Journal of pharmacology and experimental therapeutics.* 2013;346(1):23-30.

123. Han Z, Cao J, Song D, et al. Autophagy is involved in the cardioprotection effect

of remote limb ischemic postconditioning on myocardial ischemia/reperfusion injury in normal mice, but not diabetic mice. *PLoS One.* 2014;9(1):e86838.

124. Jensen RV, Stottrup NB, Kristiansen SB, Botker HE. Release of a humoral circulating cardioprotective factor by remote ischemic preconditioning is dependent on preserved neural pathways in diabetic patients. *Basic Res Cardiol.* 2012;107(5):285.

125. Ovize M, Thibault H, Przyklenk K. Myocardial conditioning: opportunities for clinical translation. *Circulation research.* 2013;113(4):439-450.

126. Schevchuck A, Laskey WK. Ischemic conditioning as an adjunct to percutaneous coronary intervention. *Circulation Cardiovascular interventions.* 2013;6(4):484-492.

127. Healy DA, Khan WA, Wong CS, et al. Remote preconditioning and major clinical complications following adult cardiovascular surgery: Systematic review and meta-analysis. *International journal of cardiology.* 2014;176(1):20-31.

128. Venugopal V, Hausenloy DJ, Ludman A, et al. Remote ischaemic preconditioning reduces myocardial injury in patients undergoing cardiac surgery with cold-blood cardioplegia: a randomised controlled trial. *Heart.* 2009;95(19):1567-1571.

129. Thielmann M, Kottenberg E, Boengler K, et al. Remote ischemic preconditioning reduces myocardial injury after coronary artery bypass surgery with crystalloid cardioplegic arrest. *Basic Res Cardiol.* 2010;105(5):657-664.

130. Heusch G, Musiolik J, Kottenberg E, Peters J, Jakob H, Thielmann M. STAT5 activation and cardioprotection by remote ischemic preconditioning in humans: short communication. *Circulation research.* 2012;110(1):111-115.

131. Kottenberg E, Thielmann M, Bergmann L, et al. Protection by remote ischemic

preconditioning during coronary artery bypass graft surgery with isoflurane but not propofol - a clinical trial. *Acta anaesthesiologica Scandinavica.* 2012;56(1):30-38.

132. Ishihara M, Inoue I, Kawagoe T, et al. Diabetes mellitus prevents ischemic preconditioning in patients with a first acute anterior wall myocardial infarction. *J Am Coll Cardiol.* 2001;38(4):1007-1011.

133. Yetgin T, Magro M, Manintveld OC, et al. Impact of multiple balloon inflations during primary percutaneous coronary intervention on infarct size and long-term clinical outcomes in ST-segment elevation myocardial infarction: real-world postconditioning. *Basic Res Cardiol.* 2014;109(2):403.

134. Carrasco-Chinchilla F, Munoz-Garcia AJ, Dominguez-Franco A, et al. Remote ischaemic postconditioning: does it protect against ischaemic damage in percutaneous coronary revascularisation? Randomised placebo-controlled clinical trial. *Heart.* 2013;99(19):1431-1437.

135. Hassouna A, Loubani M, Matata BM, Fowler A, Standen NB, Galinanes M. Mitochondrial dysfunction as the cause of the failure to precondition the diabetic human myocardium. *Cardiovasc Res.* 2006;69(2):450-458.

136. Wolf P. The nature and significance of platelet products in human plasma. *British journal of haematology.* 1967;13(3):269-288.

137. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro*: selective externalization of the receptor. *Cell.* 1983;33(3):967-978.

138. Harding C, Stahl P. Transferrin recycling in reticulocytes: pH and iron are important determinants of ligand binding and processing. *Biochemical and*

biophysical research communications. 1983;113(2):650-658.

139. Pan BT, Teng K, Wu C, Adam M, Johnstone RM. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *The Journal of cell biology.* 1985;101(3):942-948.

140. Johnstone RM, Adam M, Pan BT. The fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro*. *Canadian journal of biochemistry and cell biology = Revue canadienne de biochimie et biologie cellulaire.* 1984;62(11):1246-1254.

141. Trams EG, Lauter CJ, Salem N, Jr., Heine U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochim Biophys Acta.* 1981;645(1):63-70.

142. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *The Journal of biological chemistry.* 1987;262(19):9412-9420.

143. Gould GW, Lippincott-Schwartz J. New roles for endosomes: from vesicular carriers to multi-purpose platforms. *Nature reviews Molecular cell biology.* 2009;10(4):287-292.

144. Babst M, Katzmann DJ, Estepa-Sabal EJ, Meerloo T, Emr SD. Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting. *Developmental cell.* 2002;3(2):271-282.

145. Katzmann DJ, Babst M, Emr SD. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell.* 2001;106(2):145-155.

146. Stuffers S, Sem Wegner C, Stenmark H, Brech A. Multivesicular endosome biogenesis in the absence of ESCRTs. *Traffic.* 2009;10(7):925-937.

147. Babst M, Katzmann DJ, Snyder WB, Wendland B, Emr SD. Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body. *Developmental cell.* 2002;3(2):283-289.

148. van Niel G, Charrin S, Simoes S, et al. The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. *Developmental cell.* 2011;21(4):708-721.

149. Zhu H, Guariglia S, Yu RY, et al. Mutation of SIMPLE in Charcot-Marie-Tooth 1C alters production of exosomes. *Molecular biology of the cell.* 2013;24(11):1619-1637, S1611-1613.

150. Perez-Hernandez D, Gutierrez-Vazquez C, Jorge I, et al. The intracellular interactome of tetraspanin-enriched microdomains reveals their function as sorting machineries toward exosomes. *The Journal of biological chemistry.* 2013;288(17):11649-11661.

151. Trajkovic K, Hsu C, Chiantia S, et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science.* 2008;319(5867):1244-1247.

152. Ghossoub R, Lembo F, Rubio A, et al. Syntenin-ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2. *Nature communications.* 2014;5:3477.

153. Stenmark H. Rab GTPases as coordinators of vesicle traffic. *Nature reviews Molecular cell biology.* 2009;10(8):513-525.

154. Chacon-Heszele MF, Choi SY, Zuo X, Baek JI, Ward C, Lipschutz JH. The exocyst and regulatory GTPases in urinary exosomes. *Physiological reports.*

2014;2(8).

155. Waters MG, Hughson FM. Membrane tethering and fusion in the secretory and endocytic pathways. *Traffic.* 2000;1(8):588-597.

156. Chen YA, Scheller RH. SNARE-mediated membrane fusion. *Nature reviews Molecular cell biology.* 2001;2(2):98-106.

157. Gao Y, Zorman S, Gundersen G, et al. Single reconstituted neuronal SNARE complexes zipper in three distinct stages. *Science.* 2012;337(6100):1340-1343.

158. Tian T, Zhu YL, Hu FH, Wang YY, Huang NP, Xiao ZD. Dynamics of exosome internalization and trafficking. *Journal of cellular physiology.* 2013;228(7):1487-1495.

159. Mulcahy LA, Pink RC, Carter DR. Routes and mechanisms of extracellular vesicle uptake. *Journal of extracellular vesicles.* 2014;3.

160. Escrevente C, Keller S, Altevogt P, Costa J. Interaction and uptake of exosomes by ovarian cancer cells. *BMC cancer.* 2011;11:108.

161. Rana S, Zoller M. Exosome target cell selection and the importance of exosomal tetraspanins: a hypothesis. *Biochemical Society transactions.* 2011;39(2):559-562.

162. Nazarenko I, Rana S, Baumann A, et al. Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation. *Cancer research.* 2010;70(4):1668-1678.

163. Rana S, Yue S, Stadel D, Zoller M. Toward tailored exosomes: the exosomal tetraspanin web contributes to target cell selection. *Int J Biochem Cell Biol.* 2012;44(9):1574-1584.

164. Rana S, Claas C, Kretz CC, Nazarenko I, Zoeller M. Activation-induced

internalization differs for the tetraspanins CD9 and Tspan8: Impact on tumor cell motility. *The international journal of biochemistry & cell biology.* 2011;43(1):106-119.

165. Christianson HC, Svensson KJ, van Kuppevelt TH, Li JP, Belting M. Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proceedings of the National Academy of Sciences of the United States of America.* 2013;110(43):17380-17385.

166. Morelli AE, Larregina AT, Shufesky WJ, et al. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood.* 2004;104(10):3257-3266.

167. Hwang I, Shen X, Sprent J. Direct stimulation of naive T cells by membrane vesicles from antigen-presenting cells: distinct roles for CD54 and B7 molecules. *Proceedings of the National Academy of Sciences of the United States of America.* 2003;100(11):6670-6675.

168. Conner SD, Schmid SL. Regulated portals of entry into the cell. *Nature.* 2003;422(6927):37-44.

169. Svensson KJ, Christianson HC, Wittrup A, et al. Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. *The Journal of biological chemistry.* 2013;288(24):17713-17724.

170. Tian T, Zhu YL, Zhou YY, et al. Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. *The Journal of biological chemistry.* 2014;289(32):22258-22267.

171. Feng D, Zhao WL, Ye YY, et al. Cellular internalization of exosomes occurs through phagocytosis. *Traffic.* 2010;11(5):675-687.

172. Fitzner D, Schnaars M, van Rossum D, et al. Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. *Journal of cell science.* 2011;124(Pt 3):447-458.

173. White IJ, Bailey LM, Aghakhani MR, Moss SE, Futter CE. EGF stimulates annexin 1-dependent inward vesiculation in a multivesicular endosome subpopulation. *EMBO J.* 2006;25(1):1-12.

174. Savina A, Furlan M, Vidal M, Colombo MI. Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J Biol Chem.* 2003;278(22):20083-20090.

175. Savina A, Fader CM, Damiani MT, Colombo MI. Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. *Traffic.* 2005;6(2):131-143.

176. Parolini I, Federici C, Raggi C, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem.* 2009;284(49):34211-34222.

177. Atienzar-Aroca S, Flores-Bellver M, Serrano-Heras G, et al. Oxidative stress in retinal pigment epithelium cells increases exosome secretion and promotes angiogenesis in endothelial cells. *Journal of cellular and molecular medicine.* 2016.

178. Clayton A, Turkes A, Navabi H, Mason MD, Tabi Z. Induction of heat shock proteins in B-cell exosomes. *J Cell Sci.* 2005;118(Pt 16):3631-3638.

179. Taylor DD, Lyons KS, Gercel-Taylor C. Shed membrane fragment-associated markers for endometrial and ovarian cancers. *Gynecologic oncology.* 2002;84(3):443-448.

180. Diamant M, Nieuwland R, Pablo RF, Sturk A, Smit JW, Radder JK. Elevated

numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type-2 diabetes mellitus. *Circulation.* 2002;106(19):2442-2447.

181. Deng ZB, Poliakov A, Hardy RW, et al. Adipose tissue exosome-like vesicles mediate activation of macrophage-induced insulin resistance. *Diabetes.* 2009;58(11):2498-2505.
182. Lehmann BD, Paine MS, Brooks AM, et al. Senescence-associated exosome release from human prostate cancer cells. *Cancer research.* 2008;68(19):7864-7871.
183. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nature reviews Immunology.* 2002;2(8):569-579.
184. Chernyshev VS, Rachamadugu R, Tseng YH, et al. Size and shape characterization of hydrated and desiccated exosomes. *Analytical and bioanalytical chemistry.* 2015;407(12):3285-3301.
185. Osteikoetxea X, Sodar B, Nemeth A, et al. Differential detergent sensitivity of extracellular vesicle subpopulations. *Organic & biomolecular chemistry.* 2015;13(38):9775-9782.
186. Vidal M, Sainte-Marie J, Philippot JR, Bienvenue A. Asymmetric distribution of phospholipids in the membrane of vesicles released during *in vitro* maturation of guinea pig reticulocytes: evidence precluding a role for "aminophospholipid translocase". *Journal of cellular physiology.* 1989;140(3):455-462.
187. Mobius W, Ohno-Iwashita Y, van Donselaar EG, et al. Immunoelectron microscopic localization of cholesterol using biotinylated and non-cytolytic perfringolysin O. *The journal of histochemistry and cytochemistry : official journal*

of the Histochemistry Society. 2002;50(1):43-55.

188. de Gassart A, Geminard C, Fevrier B, Raposo G, Vidal M. Lipid raft-associated protein sorting in exosomes. *Blood.* 2003;102(13):4336-4344.

189. Wubbolts R, Leckie RS, Veenhuizen PT, et al. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *The Journal of biological chemistry.* 2003;278(13):10963-10972.

190. Laulagnier K, Motta C, Hamdi S, et al. Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. *Biochem J.* 2004;380(Pt 1):161-171.

191. Fomina AF, Deerinck TJ, Ellisman MH, Cahalan MD. Regulation of membrane trafficking and subcellular organization of endocytic compartments revealed with FM1-43 in resting and activated human T cells. *Exp Cell Res.* 2003;291(1):150-166.

192. Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood.* 1999;94(11):3791-3799.

193. Altadill T, Campoy I, Lanau L, et al. Enabling Metabolomics Based Biomarker Discovery Studies Using Molecular Phenotyping of Exosome-Like Vesicles. *PLoS One.* 2016;11(3):e0151339.

194. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol.* 2006;Chapter 3:Unit 3 22.

195. van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R. Optical and non-optical methods for detection and characterization of microparticles and exosomes. *Journal of thrombosis and haemostasis : JTH.* 2010;8(12):2596-2607.

196. Lotvall J, Hill AF, Hochberg F, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles.* 2014;3:26913.

197. Lai RC, Arslan F, Lee MM, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res.* 2010;4(3):214-222.

198. Lai RC, Arslan F, Tan SS, et al. Derivation and characterization of human fetal MSCs: an alternative cell source for large-scale production of cardioprotective microparticles. *J Mol Cell Cardiol.* 2010;48(6):1215-1224.

199. Arslan F, Lai RC, Smeets MB, et al. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res.* 2013;10(3):301-312.

200. Giricz Z, Varga ZV, Baranyai T, et al. Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles. *J Mol Cell Cardiol.* 2014;68:75-78.

201. Laflamme MA, Murry CE. Heart regeneration. *Nature.* 2011;473(7347):326-335.

202. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature.* 2001;410(6829):701-705.

203. Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic

myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nature medicine.* 2001;7(4):430-436.

204. Wollert KC, Meyer GP, Lotz J, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet.* 2004;364(9429):141-148.

205. Meyer GP, Wollert KC, Lotz J, et al. Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. *Circulation.* 2006;113(10):1287-1294.

206. Schachinger V, Erbs S, Elsasser A, et al. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *The New England journal of medicine.* 2006;355(12):1210-1221.

207. Pak HN, Qayyum M, Kim DT, et al. Mesenchymal stem cell injection induces cardiac nerve sprouting and increased tenascin expression in a Swine model of myocardial infarction. *Journal of cardiovascular electrophysiology.* 2003;14(8):841-848.

208. Murry CE, Soonpaa MH, Reinecke H, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature.* 2004;428(6983):664-668.

209. Ziegelhoeffer T, Fernandez B, Kostin S, et al. Bone marrow-derived cells do not incorporate into the adult growing vasculature. *Circulation research.* 2004;94(2):230-238.

210. Dai W, Hale SL, Martin BJ, et al. Allogeneic mesenchymal stem cell

transplantation in postinfarcted rat myocardium: short- and long-term effects. *Circulation.* 2005;112(2):214-223.

211. Iso Y, Spees JL, Serrano C, et al. Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. *Biochemical and biophysical research communications.* 2007;354(3):700-706.

212. Erbs S, Linke A, Schachinger V, et al. Restoration of microvascular function in the infarct-related artery by intracoronary transplantation of bone marrow progenitor cells in patients with acute myocardial infarction: the Doppler Substudy of the Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial. *Circulation.* 2007;116(4):366-374.

213. Noiseux N, Gnecchi M, Lopez-Ilasaca M, et al. Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. *Molecular therapy : the journal of the American Society of Gene Therapy.* 2006;14(6):840-850.

214. Fazel S, Cimini M, Chen L, et al. Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. *The Journal of clinical investigation.* 2006;116(7):1865-1877.

215. Kinnaird T, Stabile E, Burnett MS, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms. *Circulation research.* 2004;94(5):678-685.

216. Mirotsou M, Zhang Z, Deb A, et al. Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proceedings of the National Academy of Sciences of the United States of America.* 2007;104(5):1643-1648.

217. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. *Journal of cellular biochemistry.* 2006;98(5):1076-1084.

218. Gnecchi M, He H, Liang OD, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nature medicine.* 2005;11(4):367-368.

219. Gnecchi M, He H, Noiseux N, et al. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2006;20(6):661-669.

220. Timmers L, Lim SK, Arslan F, et al. Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem cell research.* 2007;1(2):129-137.

221. Yu B, Kim HW, Gong M, et al. Exosomes secreted from GATA-4 overexpressing mesenchymal stem cells serve as a reservoir of anti-apoptotic microRNAs for cardioprotection. *Int J Cardiol.* 2015;182:349-360.

222. DeSantiago J, Bare DJ, Semenov I, et al. Excitation-contraction coupling in ventricular myocytes is enhanced by paracrine signaling from mesenchymal stem cells. *Journal of molecular and cellular cardiology.* 2012;52(6):1249-1256.

223. Ibrahim AG, Cheng K, Marban E. Exosomes as critical agents of cardiac regeneration triggered by cell therapy. *Stem Cell Reports.* 2014;2(5):606-619.

224. Gallet R, Dawkins J, Valle J, et al. Exosomes secreted by cardiosphere-derived cells reduce scarring, attenuate adverse remodelling, and improve function in acute and chronic porcine myocardial infarction. *Eur Heart J.* 2016.

225. de Couto G, Liu W, Tseliou E, et al. Macrophages mediate cardioprotective cellular postconditioning in acute myocardial infarction. *J Clin Invest.* 2015;125(8):3147-3162.

226. Guitart K, Loers G, Buck F, Bork U, Schachner M, Kleene R. Improvement of neuronal cell survival by astrocyte-derived exosomes under hypoxic and ischemic conditions depends on prion protein. *Glia.* 2016;64(6):896-910.

227. Song X, Ding Y, Liu G, et al. Cancer Cell-Derived Exosomes Induce Mitogen-Activated Protein Kinase-Dependent Monocyte Survival by Transport of Functional Receptor Tyrosine Kinases. *J Biol Chem.* 2016.

228. Bang C, Batkai S, Dangwal S, et al. Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. *J Clin Invest.* 2014;124(5):2136-2146.

229. Yang Y, Li Y, Chen X, Cheng X, Liao Y, Yu X. Exosomal transfer of miR-30a between cardiomyocytes regulates autophagy after hypoxia. *J Mol Med (Berl).* 2016.

230. Yu X, Deng L, Wang D, et al. Mechanism of TNF-alpha autocrine effects in hypoxic cardiomyocytes: initiated by hypoxia inducible factor 1alpha, presented by exosomes. *J Mol Cell Cardiol.* 2012;53(6):848-857.

231. Zhang X, Wang X, Zhu H, et al. Hsp20 functions as a novel cardiokine in promoting angiogenesis via activation of VEGFR2. *PLoS One.* 2012;7(3):e32765.

232. Gupta S, Knowlton AA. HSP60 trafficking in adult cardiac myocytes: role of the

exosomal pathway. *Am J Physiol Heart Circ Physiol.* 2007;292(6):H3052-3056.

233. Yamaguchi T, Izumi Y, Nakamura Y, et al. Repeated remote ischemic conditioning attenuates left ventricular remodeling via exosome-mediated intercellular communication on chronic heart failure after myocardial infarction. *Int J Cardiol.* 2015;178:239-246.

234. Eldh M, Ekstrom K, Valadi H, et al. Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. *PLoS One.* 2010;5(12):e15353.

235. Li J, Rohailla S, Gelber N, et al. MicroRNA-144 is a circulating effector of remote ischemic preconditioning. *Basic Res Cardiol.* 2014;109(5):423.

236. Vicencio JM, Yellon DM, Sivaraman V, et al. Plasma exosomes protect the myocardium from ischemia-reperfusion injury. *J Am Coll Cardiol.* 2015;65(15):1525-1536.

237. Clark JB, Palmer CJ, Shaw WN. The diabetic Zucker Fatty rat. *Proc Soc Exp Biol Med.* 1983;173(1):68-75.

238. Phillips MS, Liu Q, Hammond HA, et al. Leptin receptor missense mutation in the fatty Zucker rat. *Nat Genet.* 1996;13(1):18-19.

239. Picard F, Deshaies Y, Lalonde J, Samson P, Richard D. Topiramate reduces energy and fat gains in lean (Fa/?) and obese (fa/fa) Zucker rats. *Obes Res.* 2000;8(9):656-663.

240. Hempe J, Elvert R, Schmidts HL, Kramer W, Herling AW. Appropriateness of the Zucker Diabetic Fatty rat as a model for diabetic microvascular late complications. *Lab Anim.* 2012;46(1):32-39.

241. Whittaker P, Kloner RA, Przyklenk K. Intramyocardial injections and protection

against myocardial ischemia. An attempt to examine the cardioprotective actions of adenosine. *Circulation.* 1996;93(11):2043-2057.

242. Himori N, Matsuura A. A simple technique for occlusion and reperfusion of coronary artery in conscious rats. *Am J Physiol.* 1989;256(6 Pt 2):H1719-1725.

243. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol.* 2011;29(4):341-345.

244. Shen B, Wu N, Yang JM, Gould SJ. Protein targeting to exosomes/microvesicles by plasma membrane anchors. *J Biol Chem.* 2011;286(16):14383-14395.

245. Sun D, Zhuang X, Xiang X, et al. A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol Ther.* 2010;18(9):1606-1614.

246. Claycomb WC, Lanson NA, Jr., Stallworth BS, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A.* 1998;95(6):2979-2984.

247. Baranyai T, Herczeg K, Onodi Z, et al. Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods. *PLoS One.* 2015;10(12):e0145686.

248. Shelke GV, Lasser C, Gho YS, Lotvall J. Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum. *J Extracell Vesicles.* 2014;3.

249. Jella KK, Yu L, Yue Q, Friedman D, Duke BJ, Alli AA. Exosomal GAPDH from Proximal Tubule Cells Regulate ENaC Activity. *PLoS One.* 2016;11(11):e0165763.

250. del Valle HF, Lascano EC, Negroni JA. Ischemic preconditioning protection against stunning in conscious diabetic sheep: role of glucose, insulin, sarcolemmal and mitochondrial KATP channels. *Cardiovasc Res.* 2002;55(3):642-659.

251. del Valle HF, Lascano EC, Negroni JA, Crottogini AJ. Absence of ischemic preconditioning protection in diabetic sheep hearts: role of sarcolemmal KATP channel dysfunction. *Mol Cell Biochem.* 2003;249(1-2):21-30.

252. Hotta H, Miura T, Miki T, et al. Angiotensin II type 1 receptor-mediated upregulation of calcineurin activity underlies impairment of cardioprotective signaling in diabetic hearts. *Circ Res.* 2010;106(1):129-132.

253. Sasaki H, Ogawa K, Shimizu M, et al. The insulin sensitizer pioglitazone improves the deterioration of ischemic preconditioning in type-2 diabetes mellitus rats. *Int Heart J.* 2007;48(5):623-635.

254. Sivaraman V, Hausenloy DJ, Wynne AM, Yellon DM. Preconditioning the diabetic human myocardium. *J Cell Mol Med.* 2010;14(6B):1740-1746.

255. Fullmer TM, Pei S, Zhu Y, et al. Insulin suppresses ischemic preconditioning-mediated cardioprotection through Akt-dependent mechanisms. *J Mol Cell Cardiol.* 2013;64:20-29.

256. Najafi M, Farajnia S, Mohammadi M, et al. Inhibition of mitochondrial permeability transition pore restores the cardioprotection by postconditioning in diabetic hearts. *J Diabetes Metab Disord.* 2014;13(1):106.

257. Hepponstall M, Ignjatovic V, Binos S, et al. Remote ischemic preconditioning (RIPC) modifies plasma proteome in humans. *PLoS One.* 2012;7(11):e48284.

258. Pang T, Zhao Y, Zhang NR, Jin SQ, Pan SQ. Transient limb ischemia alters

serum protein expression in healthy volunteers: complement C3 and vitronectin may be involved in organ protection induced by remote ischemic preconditioning. *Oxid Med Cell Longev.* 2013;2013:859056.

259. Lang SC, Elsasser A, Scheler C, et al. Myocardial preconditioning and remote renal preconditioning--identifying a protective factor using proteomic methods? *Basic Res Cardiol.* 2006;101(2):149-158.

260. Hibert P, Prunier-Mirebeau D, Beseme O, et al. Apolipoprotein a-I is a potential mediator of remote ischemic preconditioning. *PLoS One.* 2013;8(10):e77211.

261. Hibert P, Prunier-Mirebeau D, Beseme O, et al. Modifications in rat plasma proteome after remote ischemic preconditioning (RIPC) stimulus: identification by a SELDI-TOF-MS approach. *PLoS One.* 2014;9(1):e85669.

262. Helgeland E, Breivik LE, Vaudel M, et al. Exploring the human plasma proteome for humoral mediators of remote ischemic preconditioning--a word of caution. *PLoS One.* 2014;9(10):e109279.

263. Hepponstall M, Ignjatovic V, Binos S, et al. Remote ischemic preconditioning (RIPC) modifies the plasma proteome in children undergoing repair of tetralogy of fallot: a randomized controlled trial. *PLoS One.* 2015;10(3):e0122778.

264. Ueno K, Samura M, Nakamura T, et al. Increased plasma VEGF levels following ischemic preconditioning are associated with downregulation of miRNA-762 and miR-3072-5p. *Sci Rep.* 2016;6:36758.

265. Dragovic RA, Gardiner C, Brooks AS, et al. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine.* 2011;7(6):780-788.

266. Lee JS, Wu Y, Schnepp P, et al. Proteomics analysis of rough endoplasmic

reticulum in pancreatic beta cells. *Proteomics.* 2015;15(9):1508-1511.

267. Agouni A, Lagrue-Lak-Hal AH, Ducluzeau PH, et al. Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome. *Am J Pathol.* 2008;173(4):1210-1219.

268. Kiss A, Tratsiakovich Y, Gonon AT, et al. The role of arginase and rho kinase in cardioprotection from remote ischemic perconditioning in non-diabetic and diabetic rat *in vivo*. *PLoS One.* 2014;9(8):e104731.

269. Kersten JR, Schmeling TJ, Orth KG, Pagel PS, Warltier DC. Acute hyperglycemia abolishes ischemic preconditioning *in vivo*. *Am J Physiol.* 1998;275(2 Pt 2):H721-725.

270. Zalesak M, Blazicek P, Pancza D, et al. Severity of lethal ischemia/reperfusion injury in rat hearts subjected to ischemic preconditioning is increased under conditions of simulated hyperglycemia. *Physiol Res.* 2014;63(5):577-585.

271. Yang Z, Tian Y, Liu Y, Hennessy S, Kron IL, French BA. Acute hyperglycemia abolishes ischemic preconditioning by inhibiting Akt phosphorylation: normalizing blood glucose before ischemia restores ischemic preconditioning. *Oxid Med Cell Longev.* 2013;2013:329183.

272. Ishihara M, Inoue I, Kawagoe T, et al. Effect of acute hyperglycemia on the ischemic preconditioning effect of prodromal angina pectoris in patients with a first anterior wall acute myocardial infarction. *Am J Cardiol.* 2003;92(3):288-291.

273. Baranyai T, Nagy CT, Koncsos G, et al. Acute hyperglycemia abolishes cardioprotection by remote ischemic perconditioning. *Cardiovasc Diabetol.* 2015;14:151.

274. Bellodi G, Manicardi V, Malavasi V, et al. Hyperglycemia and prognosis of acute

myocardial infarction in patients without diabetes mellitus. *Am J Cardiol.* 1989;64(14):885-888.

275. Malmberg K, Ryden L, Hamsten A, Herlitz J, Waldenstrom A, Wedel H. Mortality prediction in diabetic patients with myocardial infarction: experiences from the DIGAMI study. *Cardiovasc Res.* 1997;34(1):248-253.

276. Lonborg J, Vejlstrup N, Kelbaek H, et al. Impact of acute hyperglycemia on myocardial infarct size, area at risk, and salvage in patients with STEMI and the association with exenatide treatment: results from a randomized study. *Diabetes.* 2014;63(7):2474-2485.

277. Whittaker P, Przyklenk K. Reduction of infarct size *in vivo* with ischemic preconditioning: mathematical evidence for protection via non-ischemic tissue. *Basic Res Cardiol.* 1994;89(1):6-15.

278. Gu W, Kehl F, Krolikowski JG, Pagel PS, Warltier DC, Kersten JR. Simvastatin restores ischemic preconditioning in the presence of hyperglycemia through a nitric oxide-mediated mechanism. *Anesthesiology.* 2008;108(4):634-642.

279. Jeppesen DK, Hvam ML, Primdahl-Bengtson B, et al. Comparative analysis of discrete exosome fractions obtained by differential centrifugation. *J Extracell Vesicles.* 2014;3:25011.

280. Bobrie A, Colombo M, Krumeich S, Raposo G, Thery C. Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation. *J Extracell Vesicles.* 2012;1.

281. Ren X, Wang Y, Jones WK. TNF-alpha is required for late ischemic preconditioning but not for remote preconditioning of trauma. *J Surg Res.*

2004;121(1):120-129.

282. Jones WK, Fan GC, Liao S, et al. Peripheral nociception associated with surgical incision elicits remote nonischemic cardioprotection via neurogenic activation of protein kinase C signaling. *Circulation.* 2009;120(11 Suppl):S1-9.

283. Jensen RV, Zachara NE, Nielsen PH, Kimose HH, Kristiansen SB, Botker HE. Impact of O-GlcNAc on cardioprotection by remote ischaemic preconditioning in non-diabetic and diabetic patients. *Cardiovasc Res.* 2013;97(2):369-378.

284. Brussee V, Guo G, Dong Y, et al. Distal degenerative sensory neuropathy in a long-term type-2 diabetes rat model. *Diabetes.* 2008;57(6):1664-1673.

285. Liu Z, Zhao N, Zhu H, et al. Circulating interleukin-1beta promotes endoplasmic reticulum stress-induced myocytes apoptosis in diabetic cardiomyopathy via interleukin-1 receptor-associated kinase-2. *Cardiovasc Diabetol.* 2015;14:125.

286. Baxter GT, Kuo RC, Jupp OJ, Vandenabeele P, MacEwan DJ. Tumor necrosis factor-alpha mediates both apoptotic cell death and cell proliferation in a human hematopoietic cell line dependent on mitotic activity and receptor subtype expression. *J Biol Chem.* 1999;274(14):9539-9547.

287. Wang TD, Chen WJ, Mau TJ, Lin JW, Lin WW, Lee YT. Attenuation of increased myocardial ischaemia-reperfusion injury conferred by hypercholesterolaemia through pharmacological inhibition of the caspase-1 cascade. *Br J Pharmacol.* 2003;138(2):291-300.

288. Jung O, Jung W, Malinski T, Wiemer G, Schoelkens BA, Linz W. Ischemic preconditioning and infarct mass: the effect of hypercholesterolemia and endothelial dysfunction. *Clin Exp Hypertens.* 2000;22(2):165-179.

289. Kocic I, Konstanski Z, Kaminski M, Dworakowska D, Dworakowski R.

Experimental hyperlipidemia prevents the protective effect of ischemic preconditioning on the contractility and responsiveness to phenylephrine of rat-isolated stunned papillary muscle. *Gen Pharmacol.* 1999;33(3):213-219.

290. de Vries JE, Vork MM, Roemen TH, et al. Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes. *J Lipid Res.* 1997;38(7):1384-1394.

291. Marzetta CA, Foster DM, Brunzell JD. Conversion of plasma VLDL and IDL precursors into various LDL subpopulations using density gradient ultracentrifugation. *J Lipid Res.* 1990;31(6):975-984.

292. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol.* 2014;30:255-289.

293. Horsburgh K, Kelly S, McCulloch J, Higgins GA, Roses AD, Nicoll JA. Increased neuronal damage in apolipoprotein E-deficient mice following global ischaemia. *Neuroreport.* 1999;10(4):837-841.

294. Wang H, Anderson LG, Lascola CD, et al. ApolipoproteinE mimetic peptides improve outcome after focal ischemia. *Exp Neurol.* 2013;241:67-74.

295. Perman JC, Bostrom P, Lindbom M, et al. The VLDL receptor promotes lipotoxicity and increases mortality in mice following an acute myocardial infarction. *J Clin Invest.* 2011;121(7):2625-2640.

296. Castellano J, Farre J, Fernandes J, et al. Hypoxia exacerbates Ca(2+)-handling disturbances induced by very low density lipoproteins (VLDL) in neonatal rat cardiomyocytes. *J Mol Cell Cardiol.* 2011;50(5):894-902.

297. Adiels M, Boren J, Caslake MJ, et al. Overproduction of VLDL1 driven by

hyperglycemia is a dominant feature of diabetic dyslipidemia. *Arterioscler Thromb Vasc Biol.* 2005;25(8):1697-1703.

ABSTRACT

REMOTE PRECONDITIONING: EVALUATING THE EFFICACY OF CARDIOPROTECTION IN TYPE-2 DIABETES AND EXPLORING THE MECHANISTIC ROLE OF EXOSOMES

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Remote preconditioning is a promising and robust treatment for myocardial ischemia/reperfusion injury that evokes cardioprotection through endogenous neural and/or humoral signaling. A recent study has reported that protective signaling is mediated by exosomes through the circulation; however this concept is supported by limited and inconsistent evidence. Despite overwhelming success in preclinical studies, the efficacy of remote preconditioning in human studies is inconclusive. Importantly, the majority of remote preconditioning studies use healthy animal models despite growing evidence that comorbidities, such as type-2 diabetes, may negatively influence outcomes. Nonetheless, the efficacy of remote preconditioning in the setting of type-2 diabetes has not been investigated.

Using an established model of myocardial ischemia/reperfusion in the Zucker model of type-2 diabetes and a model of hypoxia/reoxygenation in cultured HL-1 cardiomyocytes we tested four hypotheses:

i. remote preconditioning is ineffective in early-stage type-2 diabetes *in vivo*;

ii. the traditional ultracentrifugation technique for exosomes isolating is inadequate

to isolate protective factor(s) from remote preconditioning;

iii. enhanced ultracentrifugation technique for exosome isolation sequesters a protective fraction of serum;

iv. the humoral component of remote preconditioning is defective in type-2 diabetes.

In support of Hypothesis I, we demonstrate that remote preconditioning failed to reduce infarct size caused by ischemia/reperfusion in the Zucker model of early-stage type-2 diabetes. Our results illustrate that the loss in efficacy is not the result of hyperglycemia per se nor sensitization of the myocardium to ischemia/reperfusion. Subsequently, we sought to isolate a subfraction of serum from remote preconditioned rats which contained exosomes that could communicate protection and render HL-1 cardiomyocytes resistant to hypoxia/reoxygenation-induced cell death. In agreement with Hypothesis II, we report that the traditional ultracentrifugation isolation technique (100,000 xg for 2 hr) did not isolate the protective component with the exosome-rich pellet from serum, suggesting that the protective component remained in the supernatant. In accordance with these observations, we enhanced the ultracentrifugation technique to improve exosome sedimentation and obtain a protective sub-fraction of serum. In agreement with Hypothesis III, the enhanced ultracentrifugation technique (300,000 xg for 12 hr) isolated a protective exosome-rich supernatant fraction from remote preconditioned serum. However, our enhanced ultracentrifugation technique also yielded an additional, exosome-rich pellet and an exosome-depleted fraction, neither of which evoked protection. Lastly, in support of Hypothesis IV, we demonstrate that unfractionated serum and the exosome-rich supernatant fraction obtained from remote preconditioned diabetic Zucker Fatty rats did not protect HL-1 cardiomyocytes from hypoxia/reoxygenation. In conclusion, our results

illustrate for the first time that the infarct-sparing efficacy of remote preconditioning is abolished in the setting of early-stage type-2 diabetes. We demonstrate that exosomes, although not sufficient for protection, may be requisite in the humoral component of remote preconditioning. Finally, we report that the humoral component of remote preconditioning is defective in the setting of type-2 diabetes – a defect that may contribute to the failure of remote conditioning to limit infarct size in this comorbid model.

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Publications

1. Kumar R, Bukowski MJ, **Wider JM**, Reynolds CA, Calo L, Lepore B, Tousignant R, Jones M, Przyklenk K, Sanderson TH. Mitochondrial dynamics following global cerebral ischemia. Mol Cell Neurosci. 2016 Oct;76:68-75. doi: 10.1016/j.mcn.2016.08.010. PMID: 27567688 Free Article
2. Gremmel T, Yanachkov IB, Yanachkova MI, Wright GE, **Wider J**, Undyala VV, Michelson AD, Frelinger AL 3rd, Przyklenk K. Synergistic Inhibition of Both P2Y1 and P2Y12 Adenosine Diphosphate Receptors As Novel Approach to Rapidly Attenuate Platelet-Mediated Thrombosis. Arterioscler Thromb Vasc Biol. 2016 Mar;36(3):501-9. doi: 10.1161/ATVBAHA.115.306885. PMID: 26743169
3. **Wider J**, Przyklenk K. Ischemic conditioning: the challenge of protecting the diabetic heart. Cardiovasc Diagn Ther. 2014 Oct;4(5):383-96. doi: 10.3978/j.issn.2223-3652.2014.10.05. Review. PMID: 25414825
4. Mentzer RM Jr, **Wider J**, Perry CN, Gottlieb RA. Reduction of infarct size by the therapeutic protein TAT-Ndi1 *in vivo*. J Cardiovasc Pharmacol Ther. 2014 May;19(3):315-20. doi: 10.1177/1074248413515750. PMID: 24367006 Free PMC
5. Sanderson TH, **Wider JM**. 2-vessel occlusion/hypotension: a rat model of global brain ischemia. J Vis Exp. 2013 Jun 22;(76). doi: 10.3791/50173. PMID: 23851591 Free PMC Article
6. Lagina AT 3rd, Calo L, Deogracias M, Sanderson T, Kumar R, **Wider J**, Sullivan JM. Combination therapy with insulin-like growth factor-1 and hypothermia synergistically improves outcome after transient global brain ischemia in the rat. Acad Emerg Med. 2013 Apr;20(4):344-51. doi: 10.1111/acem.12104. PMID: 23701341 Free Article
7. Przyklenk K, Undyala VV, **Wider J**, Sala-Mercado JA, Gottlieb RA, Mentzer RM Jr. Acute induction of autophagy as a novel strategy for cardioprotection: getting to the heart of the matter. Autophagy. 2011 Apr;7(4):432-3. PMID: 21187719
8. Sala-Mercado JA, **Wider J**, Undyala VV, Jahania S, Yoo W, Mentzer RM Jr, Gottlieb RA, Przyklenk K. Profound cardioprotection with chloramphenicol succinate in the swine model of myocardial ischemia-reperfusion injury. Circulation. 2010 Sep 14;122(11 Suppl):S179-84. doi: 10.1161/CIRCULATIONAHA.109.928242. PMID: 20837911

Abstracts

1. Przyklenk K, Whittaker P, **Wider J**, Undyala V, Gottlieb RA, Mentzer RM. Metabolic support of mitochondrial complex I with yeast Ndi1 evokes significant cardioprotection in the *in vivo* rat model of acute myocardial infarction. *CIRCULATION. 2011;* 124 (21).
2. Hüttemann M, Sanderson TH, Whittaker P, **Wider J**, Lee I, Przyklenk K. Non-pharmacologic modulation of mitochondrial function: a novel strategy to attenuate lethal reperfusion injury in brain and heart. *CIRCULATION.* 2009; 120 (118) S641-S641.
3. Hüttemann M, Lee I, **Wider J**, Grossman LI, Przyklenk K, Sanderson TH. Non-invasive modulation of cytochrome *C* oxidase activity with specific infrared light for the treatment of reperfusion injury. Biochimica et biophysica acta (BBA)-bioenergetics. 2012; 1817, S114.
4. Sanderson TH, **Wider J**, Reynolds CA, Lepore BD, Przyklenk K Hütteman M. Modulation of cytochrome c oxidase activity with specific infrared light wave lengths: noninvasive therapy for reperfusion injury. *CIRCULATION.* 2014; 130 (suppl2), A8-A8.
5. **Wider J**, Kulek A, Undyala V, Przyklenk K. Remote Ischemic Preconditioning: Do Exosomes Play a Requisite Role in Evoking Cardioprotection? *AHA Scientific Sessions.* 2015: AOS.04.03.