Can exercise mediate similar beneficial effects on endothelial function and endothelial reperfusion injury as ischemic preconditioning?

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Abstract

Introduction: The cardioprotective benefits of exercise are partly explained via long-term improvements in cardiovascular risk factors and/or physiological remodelling of (coronary) arteries. Nevertheless, work in animals revealed that a single bout of exercise offers immediate cardioprotection, similar to that of ischemic preconditioning (IPC). Whilst there is evidence that IPC (short repeated bouts of non-lethal ischemia followed by reperfusion) offers a biphasic pattern of protection and has both local (at site of injury) and remote effects (site distant to injury e.g. forearm), little is known about the cardioprotective properties of preconditioning from exercise in humans. To date, a small number of studies have investigated the immediate cardioprotective effects of exercise preconditioning in both animal and human models. In animals, 30 min of running exercise reduced infarct size immediately following exercise, with the protective effects lasting for up to 60 hours after the exercise (Yamashita et al., 1999) and the level of protection being more persistent when more than one bout was performed (Hoshida et al., 2002). Similarly, in the limited number of human studies performed, it has been demonstrated that a single bout of lower limb exercise is able to provide protection against endothelial ischemia reperfusion (IR)-injury (Seeger et al., 2015). However, more research is warranted to further explore the potential of (single bouts of) exercise as a form of preconditioning and to determine the most effective way to employ it. The overall aim of this study was to compare exercise preconditioning with IPC on the ability to provide immediate and clinically relevant protection against endothelial IR-injury in healthy individuals, and whether this protection was present in both the local and remote stimulus. Also, given that ageing negates the impact of IPC on endothelial IR injury (Loukogeorgakis et al., 2005) another aim was to examine whether exercise (squatting) preconditioning was

similarly attenuated with age. **Methods:** Fifteen healthy individuals (23.9 ± 3.1 yrs; BMI 25 ± 2 kg.m2) attended the laboratory on four separate occasions. Assessment of forearm maximal voluntary contraction was followed by three visits for each experimental condition: control, handgrip exercise and IPC. During each experimental visit, bilateral brachial artery flow-mediated dilation (FMD) was assessed at rest (baseline). Participants then either rested in the supine position for 40 min (Control), performed handgrip exercise (4 x 5 min of unilateral handgrip exercise, separated by 5 min rest) or were administered IPC (4 x 5 min upper-arm cuff inflation separated by 5 min reperfusion) followed by a 15-minute upper-arm occlusion (220 mm Hg) with 15-minute reperfusion to induce a temporary endothelial IR stimulus, followed by FMD assessment (post IR). A separate sub-study was conducted, to further explore whether the remote effects of preconditioning are also present when the preconditioning stimulus is applied to the lower limbs, but also whether preconditioning effects are present in older subjects. For this purpose, 12 young (23.5 ± 8.0 yrs; BMI 25 ± 3 kg.m2) and six elderly individuals (61.5 ± 5.2 yrs; BMI 25 ± 4 kg.m2) attended the laboratory for 3 experimental preconditioning visits (control, squats exercise and leg IPC). During each visit unilateral brachial FMD were assessed and IR stimulus was administered at the same time points as the handgrip study. Participants either rested in the supine position for 40 min (Control), performed squats exercise (4 x 5 min of body weight squats, separated by 5 min rest) or were administered leg IPC (4 x 5 min bilateral thigh cuff inflation separated by 5 min reperfusion). **Results:** In the handgrip study, in the local arm (i.e. the arm performing exercise and IPC) there was a trend for a condition*time interaction (P = 0.08). The reduction in FMD post IR-injury was less in handgrip exercise (0.5 % [1.7, 0.6]) and IPC (0.3 % [1.6, 1.0]) compared to control (1.8 % [2.4, 1.2]). In the remote arm (i.e.

the arm not performing exercise and IPC) there was a main effect of condition with FMD being 1.0 % (1.6, 0.4) lower in the control condition compared with handgrip exercise ($P < 0.01$) and 1.1 % (1.9, 0.3) lower in the control condition compared with IPC ($P = 0.01$). In the squats study, in the young group there was a main effect of condition with FMD being 0.9 % (1.9, 0.0) lower in the control condition compared with leg IPC ($P = 0.05$). FMD was also lower in the squats condition by 1.1 % (1.9, 0.3) compared with leg IPC ($P = 0.02$). In the elderly group, there was a trend for a condition*time interaction ($P = 0.09$), the reduction in FMD was less following both exercise (0.6 % [2.1, 0.9]) and ischemic preconditioning (1.7 % [4.2, 0.7]) compared to control (3.0 % [4.6, 1.4]). **Conclusion:** This study suggests exercise preconditioning may offer both local and remote protection against endothelial IR-injury in young, healthy individuals in a similar capacity to traditional IPC and possibly to a greater extent in older individuals.

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Declaration

I declare that the work contained within this thesis is entirely my own.

Poster Communications

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List of Abbreviations

ATP	Adenosine triphosphate
BMI	Body mass index
CVD	Cardiovascular disease
CV	Cardiovascular
eNOS	Endothelial nitric oxide synthase
FMD	Flow-mediated dilation
IPC	Ischemic preconditioning
IR	Ischemia reperfusion
MI	Myocardial infarction
MVC	Maximal voluntary contraction
NADPH	Nicotinamide adenine dinucleotide phosphate
NIRS	Near infrared spectroscopy
ROS	Reactive oxygen species

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1 Chapter 1
Literature Review

1.1 Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide and explains four million deaths per year in Europe (Nichols et al., 2012). Physical inactivity is a significant and preventable risk factor that leads to development and progression of CVD (Thijssen et al., 2018). Leading a physically active lifestyle can provide primary protection and reduce development of CVD by up to 40% (Manson et al., 1999). Furthermore, previous studies have shown that the positive effects of a physically active lifestyle are greater than those connected with drug interventions (Naci and Ioannidis, 2013). The positive direct effects of physical activity on CV events was shown in a seminal bus conductor study comparing drivers, who spend a greater amount of time sitting, compared to conductors, who stand for most of their job. The drivers had an increased myocardial infarction (MI) occurrence compared with conductors, and more drivers died as a result of MI (Morris and Crawford, 1958).

Exercise training has direct effects on the vasculature that can in turn enhance vascular function which can cause positive remodelling of the artery (i.e. increase in lumen size and decreased thickness of walls) (Tinken et al., 2008). Furthermore, exercise training can have a positive effect in reducing CVD risk factors, which mostly occurs after several months of regular exercise. Although it has been suggested that the above factors combined only partly explain the cardioprotective benefits of exercise (Green et al., 2017). The question arises as to whether a single bout of exercise can provide an immediate and clinically relevant benefit in humans that may contribute to explaining the cardioprotective effects of exercise. The focus of this literature review is two-fold; firstly to introduce ischemia reperfusion (IR)-injury and the detrimental effects it causes, and to explore the role of endothelial function and how this can be

assessed. Secondly, to examine the concept of preconditioning to attenuate these detrimental effects, and how exercise can lead to these preconditioning effects.

1.2 Ischemia Reperfusion Injury

Cardiovascular events, diseases and interventions (e.g. MI, peripheral artery disease, surgery, respectively) can expose cardiovascular tissue to prolonged periods of ischemia that can lead to tissue necrosis (Downey, 1990). Following such ischemic periods, reperfusion is mandatory, which in itself can have further negative effects and contributes to exaggerate the injury, known as an ischemia reperfusion (IR)-injury (Murry et al., 1986, Przyklenk et al., 1993, Hausenloy and Yellon, 2008). The extent of the damage caused during a cardiovascular event is dependent on the IR-injury, e.g. the length of time of the ischemia before reperfusion and the amount of tissue occluded can alter the extent of the subsequent damage. IR-injury can occur both planned and unplanned in prominent clinical scenarios, for instance IR-injury occurs in response to planned cardiac ischemia, such as cardiac angioplasty or cardiac bypass surgery. Conversely, IR-injury will also occur during unplanned ischemic events such as myocardial infarction. Furthermore, IR-injury of tissue is a common occurrence during various surgical procedures, not necessarily related to the heart only, and is especially present in the case of organ transplants (Serracino-Inglott et al., 2001). Moreover, myocardial injury is even frequently reported in non-cardiac surgeries, with a previous study reporting a prevalence of myocardial injury in 8% of individuals aged over 45 years, but it often remains clinically silent (Abbott et al., 2016). Therefore, it is extremely important to find an effective method to negate the effect of IR-injury in both planned and unplanned instances. Regardless of the significant damage caused

by IR-injury there is still relatively little understanding of the pathophysiology behind it, leading to the poor availability of therapeutic strategies in order to combat it.

IR-injury is known to cause vascular endothelial dysfunction, which is a large contributor to CVD due to the impairment of the ability of vessels to dilate due to decreased production of nitric oxide (Moens et al., 2005). IR-injury occurs due to a period of ischemia to a vascular bed followed by rapid reperfusion to the same area. The restoration of blood flow to the ischemic organ or tissues leads to further damage, thus heightening the impairment of its functional integrity (Galaris et al., 2006). This has previously been referred to as the 'oxygen paradox', due to the importance of returning oxygenation to the vessel in a timely manner to salvage tissue, whilst trying to avoid activating mechanisms (e.g. IR-injury) that will cause greater detriment to the tissue. An assortment of molecular pathways have been suggested in order to explain the phenomenon, however, there is still yet to be a definitive (single) mechanism. It has been proposed that ischemia reduces cellular oxidative phosphorylation, which in turn means phosphates such as adenosine triphosphate (ATP) cannot be resynthesized. This alters the membrane ATP-dependent ionic pump function, thus allowing a greater amount of calcium into the cell (Collard and Gelman, 2001). The entry of a large amount of calcium can then cause a loss of regulation of cell volume during reperfusion, which can further lead to increases in production of free radicals, affect function of mitochondria and cause damage to the sarcolemma – all of which can lead to cell swelling and cause tissue necrosis (Braunwald and Kloner, 1985).

It has been proposed that IR-injury diminishes vasodilation via the production of reactive oxygen species (ROS) and the disruption in endothelial nitric oxide synthase (eNOS) occurring within the opening minutes of reperfusion (Loukogeorgakis et al., 2010). This arises due to increased generation of superoxide anions (Eltzschig and

Eckle, 2011, Collard and Gelman, 2001) caused by mitochondrial and enzymatic sources such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Loukogeorgakis et al., 2010). During ischemia, ATP is degraded to form hypoxanthine. This is normally oxidised to xanthine by xanthine dehydrogenase, but due to lack of oxygen during ischemia xanthine dehydrogenase is converted to xanthine oxidase. Therefore, hypoxanthine isn't converted during ischemia and instead once oxygen is reintroduced (reperfusion), excess hypoxanthine is converted by xanthine oxidase which leads to the formation of ROS (Collard and Gelman, 2001). Superoxide, a type of ROS, is thought to decrease the bioavailability of nitric oxide (NO), as it reacts with NO to form peroxynitrate, a highly reactive and cytotoxic oxidant (Xia et al., 1996). Therefore, if production of superoxide is reduced, less endothelial dysfunction will be present after IR.

1.3 Endothelial Function

Previous studies have used an endothelial IR-injury model in order to examine whether cardioprotection can be achieved. This model has been used previously to produce a temporary ischemic injury in order to assess the efficacy of applying IPC prior to the injury (Kharbanda et al., 2001). A flow mediated dilation (FMD) assessment is performed in order to assess endothelial function prior to and after IR-injury, with an intervention usually taking place in-between (e.g. preconditioning). Brachial artery endothelial function is assessed as it has been shown to have a strong correlation with coronary endothelial function (Anderson et al., 1995) and thus can give an indication of the cardioprotection induced. The endothelium is a monolayer of endothelial cells that lines the lumen of the vascular beds, as well as extending into the vascular wall

and adventitial vasa vasorum. The endothelium is located strategically in order to carry out both mechanical and metabolic functions (Bonetti et al., 2003, Lerman and Zeiher, 2005). Early research suggested that the main function of the endothelium was to act as a barrier, but it is also an important signal transducer that allows for the modification of the vessel wall phenotype (Vita and Keaney, 2002). The endothelium plays a key role via its effect on vascular tone by releasing vasoactive molecules that can contract and relax the vessel (Deanfield et al., 2007). The most prominent of these vasoactive molecules is NO, which is generated via the oxidation of L-arginine by the action of eNOS. NO diffuses to the vascular smooth muscle cells where it activates guanylate cyclase, which ultimately leads to cGMP-mediated vasodilation. The most significant activator of eNOS is shear stress (i.e. the force of the flowing blood against the endothelial surface of a blood vessel) which allows for adaptation of organ perfusion to changes in cardiac output (Corson et al., 1996). In order to assess NO-dependent vasodilation many studies seek to use an increased shear stress stimulus which can help to assess NO bioavailability via FMD measurement (Pyke and Tschakovsky, 2005).

1.4 Flow mediated dilation

Peripheral vascular endothelial function can be assessed using the flow mediated dilation (FMD) technique. FMD will be the main outcome measure that will allow the effect of IR-injury on endothelial function to be assessed. FMD was first introduced in 1992 by Celermajer *et al* and is a frequently used measurement technique (Celermajer et al., 1992). Several observations led to this approach including evidence that suggested the endothelium played a key role in vasodilation and shear stress

having a strong mechanistic input. Celermajer et al. (1992) worked with the assumption that the observed dilation was NO-mediated without direct evidence. Subsequent research has suggested that the physiological stimulus to induce an endothelium-mediated vasodilation response in vivo is flow-associated shear (Thijssen et al., 2011). The majority of studies have shown that using NO blockades can significantly attenuate FMD (Joannides et al., 1995, Mullen et al., 2001, Kooijman et al., 2008). The evidence collated above highlights the validity of FMD as a technique implored to assess endothelium dependent and NO mediated endothelial function (Thijssen et al., 2011).

FMD uses high resolution B-mode ultrasound to obtain images of an artery. FMD is assessed by imaging the changes in artery diameter in response to a period of ischemia (usually 5 minutes) which is induced using a blood pressure cuff, inflated to supra-systolic level, distal from the imagining location around the forearm. The correct cuff placement during the FMD technique is vital in order to guarantee NO mediated vasodilation. This was shown by Doshi et al. (2001) who established that the dilatory response to FMD was abolished when the cuff was placed distal to imaged artery and an NO blockade was used. Although the NO blockade had a significantly less effect when the cuff was placed in a proximal position. Similarly, the duration of the ischemic period during FMD is vitally important. Following 5 minutes of ischemia NO blockades resulted in attenuated FMD, whereas after 15 minutes of ischemia they had a negligible impact (Mullen et al., 2001). In light of this and in order to accurately produce an NO mediated response, expert consensus guidelines have been developed to standardise FMD assessment (Thijssen et al., 2019b)

The effectiveness of FMD can also depend on various factors including the technical ability of the sonographer, subject preparation and environmental influences. Firstly,

proper FMD training is highly important in order to produce reliable data. Although, there is no specific protocol for FMD training, making it hard to quantify an exact duration of training, some research has indicated that expert training produces lower variability in results (van Mil et al., 2016). Subject preparation is also of great importance in order to collect reliable FMD data. As outlined by Thijssen et al. (2019b), there are various factors that can affect FMD which include: smoking, alcohol, food consumption, supplements/drugs, physical activity and mental stress. These factors can impact both NO release and also baseline vasomotor tone. Therefore, it is important to control for these factors prior to conducting all FMD tests.

FMD is an extremely useful non-invasive technique and, when performed in line with the standardised guidelines, can give an accurate outlook on the NO mediated dilation of conduit arteries in response to increased blood flow and shear stress. Importantly, FMD can be used to assess both the acute and longer-term impacts of physiological and pharamlogical interventions in humans (Thijssen et al., 2019b).

1.5 Ischemic Preconditioning

The aforementioned FMD technique can be used to assess the efficacy of preconditioning interventions on attenuating endothelial IR-injury. Given the detrimental effects caused by IR-injury, it is vital that interventions that target preventing the extent of IR-injury, or reducing its occurrence, are explored. Many studies have examined the impact of ischemic preconditioning (IPC) in order to produce positive cardioprotective effects and protect against IR-injury, with the idea that small bouts of non-lethal ischemia may offer protection. IPC is defined as repeated exposure to short periods of ischemia followed by tissue reperfusion. In healthy

humans, IPC is administered by inflating a blood pressure cuff on a limb followed by deflation, this offers strong cardioprotective effects including reducing infarct size (Bøtker et al., 2010). The potential cardioprotective effects of IPC were introduced by Murry et al. (1986) and involved occlusion of the left anterior descending artery in dogs for 5 minutes, alternated with 5 minutes of reperfusion and repeated 4 times. Following this a 40 minute period of ischemia was applied to the same artery. When IPC preceded the 40 minute bout of ischemia subsequent infarct size was reduced by 75% in comparison to control animals that experienced a sham-intervention. This led to clinical observations that suggested post myocardial infarction patients with a prior history of angina (i.e. myocardial ischemia) demonstrated greater ejection fraction (Matsuda et al., 1984) and smaller infarct size (Ottani et al., 1995). Therefore, this contributed to the idea that exposure to non-lethal bouts of cardiac ischemia before coronary ischemia may offer protection against the impact of IR-injury and subsequent myocardial damage.

Subsequent research has shown that IPC can attenuate infarct size when IPC is performed locally to the coronary artery that was occluded to cause the MI (Murry et al., 1986) but also when IPC was performed on a coronary artery distant to the site of the MI (Przyklenk et al., 1993), thus suggesting that IPC can offer both local and remote effects. It was demonstrated that cyclical ischemia and reperfusion of the circumflex coronary artery was associated with the protection of cardiac territory supplied by the left anterior descending artery, an area distant from the circumflex coronary artery in dogs (Przyklenk et al., 1993). This led to further research examining the potential effects of remote IPC in humans. For instance, Bøtker *et al.,* (2010) conducted a study that demonstrated that the remote effects of IPC allowed it to be performed on an artery away from the coronaries. In this study, IPC was performed

on the brachial artery using a blood pressure cuff to cause cyclical bouts of temporary ischemia in an ambulance on route to hospital during an acute MI. The group that received IPC during transportation had a reduction in final infarct size in comparison to the group who received no IPC (Bøtker *et al.*, 2010). Similarly, Kharbanda et al. (2002) found that a brief period of limb ischemia to the forearm via cuff inflation induced preconditioning of human arterial vessels not only locally but also remotely. This suggests that IPC may be able to protect distant organs that may undergo an IR-injury, and that short-term temporary ischemia in order to precondition the arteries prior to/or during MI can attenuate infarct size and the amount of damage caused.

Moreover, it has been suggested that IPC protection occurs in a biphasic manner with early protection lasting 1-2 hours which then reappears 18-24 hours later and can remain present for up to 72 hours (Hausenloy and Yellon, 2008). Thus suggesting that IPC can offer both immediate and sustained cardioprotection against ischemia reperfusion injuries. There have been a large amount of proposed mechanisms relating to IPC. It has been suggested that both neuronal and humoral factors are likely to play a role in the signal transduction of IPC but no single, definitive mechanism has been identified. The proposed neuronal mechanism relates to the activation of nociceptive fibres, which release an unidentified molecule into the blood or signal through the spinal cord which lead to the release of cardioprotective substances (Heusch et al., 2015). The humoral mechanism was identified in the study by Michelsen et al. (2012) which proposed that a blood-borne factor may be crucial in providing protection in both IPC. The study showed that protection was evident when they used preconditioned blood from human participants as a dialysate for naïve rabbit hearts which underwent an IR-injury protocol, thus clearly showing a humoral pathway. It is

important to note that specifics of these mechanisms have not been identified, but it has been heavily suggested that humoral and neuronal factors play a key role.

Conversely, translation of IPC from preclinical to clinical research is not always successful (Hausenloy et al., 2010). For instance, a recent review concluded that remote IPC did not significantly reduce incidence of acute MI and mortality in adult cardiac surgery patients (Xie et al., 2018). There is also some debate as to how IPC should be administered, as well as concern over cuff inflation time periods, although it has been deemed as both feasible and safe whilst being well tolerated by awake patients (Koch et al., 2011). Studies have also questioned whether the traditional IPC stimulus is sufficiently strong for patient groups. Given these limitations, other techniques that may induce cardioprotection should be considered.

Table 1.1 *Overview of studies investigating ischemic preconditioning interventions.*

Author (Year)	Population	Local or Remote	IPC Protocol	Occlusion/Injury Protocol	Findings
Murry *et al.*, (1986)	Dogs	Local	4 x 5 min occlusion/reperfusion	40 min occlusion (4 days reperfusion)	↓ Infarct size ~ 75% compared to control group.
Przyklenk *et al.*, (1993)	Dogs	Remote	4 x 5 min occlusion/reperfusion	1 hour occlusion (4.5 hours reperfusion)	↓ Infarct size ~ 65% compared to control group.
Kharbanda *et al.*, (2002)	Healthy, middle aged	Remote	3 x 5 min occlusion/reperfusion	Endothelial IR-injury (20 min occlusion/ 15 min reperfusion)	↑ Vascular function (strain-gauge plethysmography)
Loukogeorgakis *et al.*, (2005)	Healthy, middle aged	Remote	3 x 5 min occlusion/reperfusion	Endothelial IR-injury (20 min occlusion/ 20 min reperfusion)	↑ Vascular function (FMD)
Bøtker *et al.*, (2010)	Suspected first acute MI	Remote	4 x 5 min occlusion/reperfusion	First acute MI	↑ Myocardial salvage
Van den Munckhof *et al.*, (2013)	Healthy young and elderly	Remote	3 x 5 min occlusion/reperfusion	Endothelial IR-injury (20 min occlusion/ 15 min reperfusion)	↑ Vascular function in the young (FMD) No protective effect in elderly

1.6 Exercise Preconditioning

The idea of exercise being able to offer cardioprotection is not new. Previous studies have commented on a phenomenon termed ‘warm up angina’. This phenomenon relates to the clinical observation that the time to clinical angina complaints and/or ST-depression during exercise is extended during the second bout of exercise. This developed into ‘walk through angina’, which is a similar phenomenon, but without a period of rest. This paradox of patients recovering from an angina episode and being less susceptible to further effort induced angina bares resemblance to the ‘oxygen paradox’. These clinical observations have indirectly lead to the discovery of IPC (Marber et al., 1994). Furthermore, warm-up angina has been show to produce a similar biphasic response to that which is seen in IPC (Lambiase et al., 2003). Overall, this suggests that exercise is able to attenuate cardiac ischemia in patients with angina during a second bout of exercise. This phenomenon supports the concept that exercise may possess preconditioning effects in humans.

To further explore this interesting hypothesis, studies in animals have shown that a single bout of exercise is associated with smaller infarct size. Yamashita et al. (1999) conducted a study with rats that performed 25-30 minutes of treadmill running, subsequently followed by an IR-injury of the heart at different time-points (i.e. immediately after exercise up to 60 hours after the cessation of exercise). This study revealed that infarct size was reduced when IR-injury occurred immediately, but this cardioprotection was lost at 3 hours post-exercise. Although, it returned at 36 hours post exercise and was still evident up to 60 hours post-exercise. This suggests that exercise induced protection follows the characteristic biphasic pattern as with IPC (i.e. early protection occurring in the first hour, which later re-appears after 24 hours and can remain present up to 72 hours). Interestingly, other animal research has shown that

repeated bouts of exercise may elicit greater cardioprotection. A study conducted by Hoshida et al. (2002) investigated the time course of cardioprotection after repeated physiological stresses (i.e. exercise) in rats. They discovered that when two bouts of exercise (30 minute treadmill exercise separated by 48 hours) were performed, the beneficial effects of ameliorating IR-injury were observed in a monophasic manner with reduced infarct size being present from 30 minutes persistently for up to 60 hours. This therefore suggests that repeated exercise bouts may offer more persistent cardioprotection than a single bout of exercise.

The findings from animal studies led to further research in humans as to whether exercise can offer cardioprotection. For instance, Seeger et al. (2015) established that a single bout of lower limb interval exercise effectively prevented brachial artery endothelial IR-injury, although moderate-intensity endurance exercise did not prevent IR-injury. During the interval exercise session, participants performed a 10 min warmup at 30% of maximal workload, then ten 1 min cycle exercise bouts at 100% of maximum workload separated by 2 min recovery periods cycling at 25% of maximal workload, followed by a 5 min cooldown at 30% maximal workload. The continuous exercise session consisted of the same warmup and cooldown either side of a 28 min continuous cycle at 50% of maximal workload. This was compared to a control group who lay rested in the supine position for 43 min. The finding that interval leg exercise provided protection in the brachial artery suggests it can offer remote preconditioning effects, resulting in attenuating endothelial IR-injury.

To date only one study, by Michelsen et al. (2012), has directly compared the acute impact of exercise with IPC. Healthy participants underwent exercise (4 intermittent running bouts of 2 minutes) and IPC (4 x 5 minutes of arterial occlusion). Blood was taken from the human participants after preconditioning and was used as a dialysate

to perfuse a rabbit heart using the Langendorff model of myocardial infarction. The rabbit heart was then exposed to 40 min occlusion of a coronary artery, subsequent infarct size was assessed relative to risk area. The results showed that exercise and IPC reduced infarct size equally by ~50%, thus suggesting that an acute bout of exercise may offer cardioprotection. There are clear comparisons between IPC and exercise, which could be vital in determining the most effective way to employ exercise in order to have a beneficial cardioprotective effect. For example, it has been shown that when exercise is performed in an interval mode (similar to the occlusion/reperfusion cycle of IPC) it is able to provide a level of protection. Whereas, it was also demonstrated that continuous exercise was found to offer no preconditioning effect when compared to control, even though it offered similar total deoxygenation to interval exercise but not in the same pattern (Seeger et al., 2015). Moreover, Seeger et al. (2015) also established that lower limb exercise (cycling) was able to provide protection and negate IR-injury in the brachial artery, this suggest that exercise may be able to offer remote protection. Thus, examining the local and remote effects of exercise will be a focus of the study contained within this thesis.

There have been a large array of proposed mechanisms behind both IPC and exercise preconditioning, with studies suggesting that similar mechanisms may be in play (Michelsen et al., 2012). It has been suggested that both neuronal and humoral factors are likely to play a role in the signal transduction of IPC but no single, definitive mechanism has been identified (Heusch et al., 2015). The first study to directly compare IPC and exercise preconditioning, by Michelsen et al. (2012), proposes that a blood-borne factor may be crucial in providing protection in both IPC and exercise. The study showed that protection was evident when they used preconditioned blood from human participants as a dialysate for naïve rabbit hearts which underwent an IR-

injury protocol, thus clearly showing a blood-borne factor is evident. Although, specific circulating effector(s) of remote IPC are yet to be discovered, thus meaning it cannot be confirmed that the humoral mechanism behind exercise preconditioning is the same as IPC.

1.7 Influence of Ageing

It is important to note that some previous studies have suggested that the aptitude of ischemic preconditioning is reduced in the presence of CVD risk factors and thus there may be some moderating factors of exercise preconditioning (Thijssen et al., 2018). In particular, ageing is a risk factor that is associated with decreased efficacy of ischemic preconditioning to protect against IR-injury (Munckhof et al., 2013). Whether decreased efficacy of preconditioning stimuli in the older population also relates to exercise is currently unknown. In light of this, the impact of ageing on exercise negating IR-injury will be examined in the study contained within this thesis. This is highly relevant as, ultimately, the older population represents a logical target population for clinical translation of (exercise) preconditioning.

Interestingly, the detrimental effects of ageing can be partially reversed by exercise training. Abete et al. (2000) found that post-ischemic myocardial injury was reduced in older, trained rats in comparison to a sedentary group. A later cross-sectional study that looked at the effect of lifelong exercise in humans found that older athletes had maintained the ability of preconditioning to attenuate IR-injury (Maessen et al., 2017). Overall, these findings suggest that age can have a negative effect on the efficacy of preconditioning, but this can be overcome by exercise training. Perhaps exercise represents a stronger preconditioning stimulus than ischemia alone, making it possible

that exercise (but not ischemic) preconditioning is still effective to prevent or reduce endothelial IR-injury in older populations.

1.8 Summary

This literature review has sought to provide an overview around the concept of exercise preconditioning from animal models to more recent human models. It has also aimed to highlight the efficacy of exercise to offer cardioprotection as a form of preconditioning to protect against IR-injury. Therefore, the aim of the study contained within this thesis was to (i) compare exercise preconditioning with IPC on the ability to provide immediate and clinically relevant protection against endothelial IR-injury in healthy individuals, and (ii) whether this protection was present in both the local and remote stimulus. Also, given that ageing negates the impact of IPC on endothelial IR-injury (Loukogeorgakis et al., 2005), exercise may represent a more powerful preconditioning stimulus. This is supported by the fact that exercise training can partly restore the age related attenuated efficacy of IPC, and thus another aim was to (iii) examine whether exercise preconditioning (squatting) attenuated endothelial IR-injury in an older population. The hypothesis of the thesis is that exercise will offer similar protective benefits to IPC and these benefits will be evident both locally and remotely. Moreover, exercise may produce a more powerful preconditioning stimulus in the elderly than IPC, and thus offer an increased level of protection.

2 Chapter 2

Can exercise, similar to ischemic preconditioning, protect against endothelial reperfusion injury in humans?

2.1 Introduction

Regular exercise improves the risk against cardiovascular (CV) disease (Green et al., 2017) and can have a greater effect than some drug interventions (Naci and Ioannidis, 2013). The cardioprotective benefits of exercise are partly explained via gradual, but modest, improvements in CV risk factors and/or physiological remodelling of (coronary) arteries (Green et al., 2010). Whilst these effects are well established, it takes several weeks or months for them to develop. Nevertheless, a single bout of acute exercise may also offer cardioprotection, which is hypothesised to be present for up to 72 hours following exercise. To date, a small number of studies have investigated the immediate cardioprotective effects of exercise preconditioning in both animal and human models. In animals, 30 min of running exercise reduced infarct size immediately following exercise, the protective effects lasted for up to 60 hours after the exercise (Yamashita et al., 1999) and were more persistent when more than one bout was performed (Hoshida et al., 2002). In human studies, participants performed intermittent running exercise, following which a blood sample was collected; the preconditioned blood was then used as a dialysate to perfuse a rabbit heart using the Langendorff model of myocardial infarction. Infarct size was reduced by ~50% in the animals that received the preconditioned blood (Michelsen et al. (2012). In a subsequent study, Seeger et al. (2015) found that a single bout of interval exercise offered protection, against endothelial IR-injury. Therefore, there is accumulating evidence that exercise has immediate protective effects.

To understand these effects, previous work compared the protection against injury mediated by a bout of exercise with other preconditioning stimuli, most commonly IPC. In humans, IPC is applied by short repeated bouts of non-lethal ischemia followed by reperfusion using a blood pressure cuff on a limb (4 x 5 min of arterial occlusion

followed by reperfusion). The comparison of exercise preconditioning with IPC was employed in the study by Michelsen et al. (2012) using human blood dialysate to perfuse a rabbit heart together with the Langendorff model of myocardial infarction, the infarct size reduction was comparable when exercise or IPC was performed by the human before the blood sample. Importantly, exercise may offer a more practical alternative to IPC given that patients and clinicians (i) might find exercise is a more acceptable with less discomfort; (ii) are more familiar with exercise and; (iii) exercise also offers additional benefits by improving CVD risk factors, although this may take a longer time period to develop (Thijssen et al., 2018). However, several questions pertaining to the specifics of the exercise preconditioning stimulus are currently unanswered.

The observations from a large number of research studies have shown the cardioprotective effects of IPC are evident from 1-72 hrs, in a biphasic pattern, and importantly are evident both locally (i.e. limb that IPC is performed on) and remotely (i.e. contralateral limb) (Przyklenk et al., 1993, Kharbanda et al., 2002). Given the evidence from research studies that preconditioning with exercise mediates immediate protective effects that can be transferred through a blood-borne substance from an exercised human to a naïve animal, it may be suggested that exercise preconditioning displays remote effects that are similar to IPC.

Therefore, the aim of this study was to (i) compare exercise preconditioning with IPC on the ability to provide immediate and clinically relevant protection against endothelial IR-injury in healthy individuals, and (ii) whether this protection was present in both the local and remote stimulus. In this study, the impact of remote exercise preconditioning was examined in two ways. Firstly, by employing unilateral handgrip exercise (4 x 5 min exercise bouts followed by reperfusion) and examining

vascular protection on both the exercised (local) forearm and the contralateral (remote) forearm. Secondly, by employing double leg squatting exercise (4 x 5 min exercise bouts followed by reperfusion), e.g. exercise using a larger muscle mass, and examining vascular protection on the forearm (remote). This will help to better understand whether the mode of exercise (small muscle mass and large muscle mass) impact protection against IR-injury. Finally, given that ageing negates the impact of IPC on endothelial IR-injury (Loukogeorgakis et al., 2005), exercise may represent a more powerful preconditioning stimulus. This is supported by the fact that exercise training can partly restore the age related attenuated efficacy of IPC, and thus another aim was to (iii) examine whether exercise preconditioning (squatting) attenuated endothelial IR-injury in an older population.

2.2 Methods

2.2.1 Participants

For the *handgrip study*, 15 healthy male individuals (age 23.9 ± 3.1 years, BMI 25 ± 2 kg.m2) were recruited (Table 1). For the *squat study*, 12 young (age 23.5 ± 8.0 years, BMI 25 ± 3 kg.m2) and six elderly, healthy male individuals (age 61.5 ± 5.2 years, BMI 25 ± 4 kg.m2) were recruited. Individuals were free from cardiovascular and metabolic diseases, did not have any arm injury, non-smokers and were not taking any regular medication. Participants were informed of the study protocol verbally and in writing before providing fully informed verbal and written institutionally approved informed consent. The study was approved by the University ethics committee and adhered to the standards set out in the Declaration of Helsinki (World Medical Association, 2013). All data collection took place at Liverpool John Moores University.

2.2.2 Research Design

For the *handgrip study*, participants attended the laboratory on four separate occasions. One for an initial visit to assess forearm maximal voluntary contraction. Then three experimental visits, performed at the same time of day, separated by at least 3 days, having fasted overnight (12hrs), refraining from alcohol and exercise for 24 hrs and caffeine for 12 hrs before each visit. During each visit, bilateral brachial artery endothelial function [using flow-mediated dilation (FMD)] was examined at resting baseline prior to any intervention and following an endothelial ischemia reperfusion (IR)-injury (15 min of arm ischemia and 15 min of reperfusion). Following

the resting baseline FMD, Participants then either rested in the supine position for 40 min (Control), performed handgrip exercise (4 x 5 min of unilateral handgrip exercise, separated by 5 min rest) or were administered IPC (4 x 5 min forearm cuff inflation separated by 5 min reperfusion) [Figure 1 A]. The order of the intervention (control, exercise and IPC) was administered in a randomised counterbalanced order on the dominant arm. For the *squat study*, during each experimental visit, unilateral brachial artery FMD was also examined at resting baseline prior to any intervention and following an endothelial IR-injury. Following the resting baseline FMD, participants either rested in the supine position for 40 min (Control), performed squats exercise (4 x 5 min of body weight squats, separated by 5 min rest) or were administered leg IPC (4 x 5 min bilateral thigh cuff inflation separated by 5 min reperfusion) [Figure 1 B]. The order of the intervention (control, exercise and IPC) was administered in a randomised counterbalanced order.

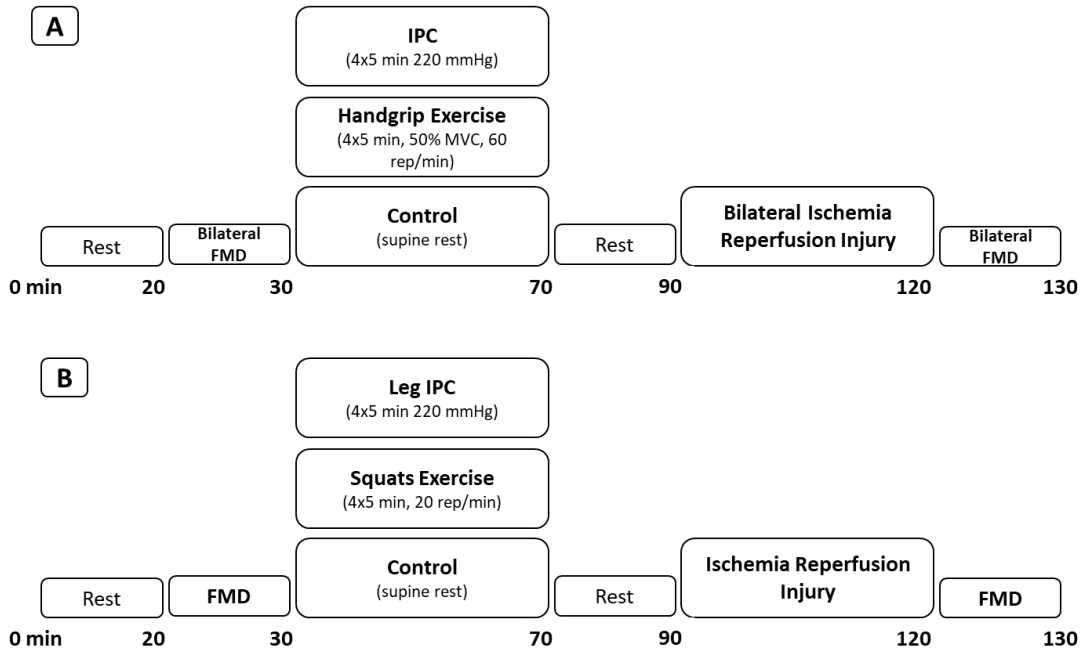

Figure 2.1 *A schematic of the research design for the handgrip (A) and the squat (B) studies.*

2.2.3 Measurements

Brachial artery endothelial function. This was assessed using the FMD. Following 20 min of supine rest, the arm was extended and positioned 80° from the torso. A rapid inflation/deflation pneumatic cuff (D.E. Hokanson, Bellevue, WA) was placed around the forearm (immediately distal to the olecranon) to produce the stimulus of forearm ischemia. A 15-MHz multifrequency linear array probe, attached to a high-resolution ultrasound machine (T3300; Terason, Burlington, MA), was then used to image the brachial artery in the distal third of the upper arm. When an optimal image was obtained, the probe was held stable and the ultrasound parameters were set to optimize the longitudinal, B-mode image of the lumen–arterial wall interface. The ultrasound was also used to attain simultaneous continuous Doppler velocity using the lowest possible insonation angle (60°). A recording of resting diameter and velocity was taken for 1 min, then the forearm cuff was inflated (>200 mm Hg) for 5 min. Both diameter and velocity recordings resumed 30 seconds before cuff deflation and continued for 3 min post deflation. Analysis of brachial artery diameter was performed using custom designed edge-detection and wall-tracking software, which is largely independent of investigator bias. Previous articles contain detailed descriptions of our analytical approach (Black et al., 2008, Woodman et al., 2001). From synchronized diameter and velocity data, blood flow (the product of lumen cross sectional area and Doppler velocity) were calculated at 30 Hz. Shear rate (an estimate of shear stress without viscosity) was calculated as four times mean blood velocity/vessel diameter. Reproducibility of diameter measurements using this semi-automated software is significantly better than manual methods, significantly reduces observer error, and possesses within-day coefficient of variation of 6.7% (Woodman et al., 2001). All FMD measurements were performed by two sonographers. Sonographer 1 had a

coefficient of variation in FMD% of 19% and a coefficient of variation of 2% for baseline artery diameter. Sonographer 2 had a coefficient of variation in FMD% of 18% and a coefficient of variation of 3% for baseline artery diameter.

Ischemia Reperfusion. A temporary, endothelial IR-injury was induced by inflating a cuff around the upper arm to 220 mmHg for 15 min using a rapid inflation pneumatic device. This was followed by a 15 min reperfusion period before the FMD protocol was repeated.

2.2.4 Interventions

Exercise: For the *handgrip study* exercise intensity was calculated at 50% of MVC. Participants were in a seated position, with the dominant arm placed at an angle on a table. Participants performed 5-minutes of rhythmic (using a metronome) handgrip contractions on a dynamometric handheld force transducer, completing 60 reps/min, followed by 5 min rest. For the *squat study*, participants performed 5-minutes of rhythmic (using a metronome) squatting, at 20 reps/min, followed by 5 min rest. For both exercise protocols, this was repeated 4 times in total. Ratings of perceived exertion was taken in the final 30 sec of each bout of handgrip exercise using the Borg scale.

Ischemic Preconditioning. For the *handgrip study* IPC was performed in the supine position and cuff inflation pressure set at a standardized pressure (220 mm Hg) with the use of a rapid inflator (E20) and air source (AG101) (Hokanson, Washington, USA). In the *handgrip study* forearm cuff was placed on the upper arm and inflated for 5 minutes followed by deflation for 5 minutes, allowing for reperfusion. In the *squat study*, the cuff was placed on the upper leg and was inflated for 5 minutes

followed by deflation for 5 minutes, allowing for reperfusion, this was repeated four times.

Control. Participants lay rested in a supine position for 40 min for both handgrip and squat studies.

Defining exercise intensity: Forearm Maximum Voluntary Contraction. Participants attended the laboratory on their initial visit and performed short (three second) maximal voluntary handgrip isometric contractions (MVC); with each effort separated by 90 seconds rest. Each participant produced three efforts in total. A dynamometric handheld force transducer was used to determine force generation. The maximum-recorded value (kg) from these three efforts was used to determine MVC.

2.2.5 Pilot Study; matching ischemia between preconditioning protocols

A pilot study was conducted in order to match exercise to IPC to produce similar amounts of ischemia. For the *handgrip study* (n = 4 males, (26 ± 8 yrs; BMI 25 ± 1 kg.m2), near-infrared spectroscopy (NIRS) was used on the extensor carpi radialis longus in order to measure muscle oxygenation during a range of different handgrip exercise protocols. A range of protocols were tested with intensity between 30-75% MVC and 30-60 reps/min).This was then compared to NIRS data from traditional IPC on the same muscle to match as closely as possible to the level of ischemia produced for both preconditioning modes. The most feasible, well matched protocol was 60 reps/min at 50% MVC. Similarly, for the *squats study* (n = 4 males, (26 ± 8 yrs; BMI 25 ± 1 kg.m2), NIRS was used on the vastus lateralis muscle during a range of different squat exercise protocols. A range of protocols were tested with differing squat hold

durations and squats per/min. This was then compared to NIRS data from leg IPC and matched as closely as possible to the level of ischemia produced. The most feasible, well matched protocol was 20 reps/min, with squats being held for 1.5 sec.

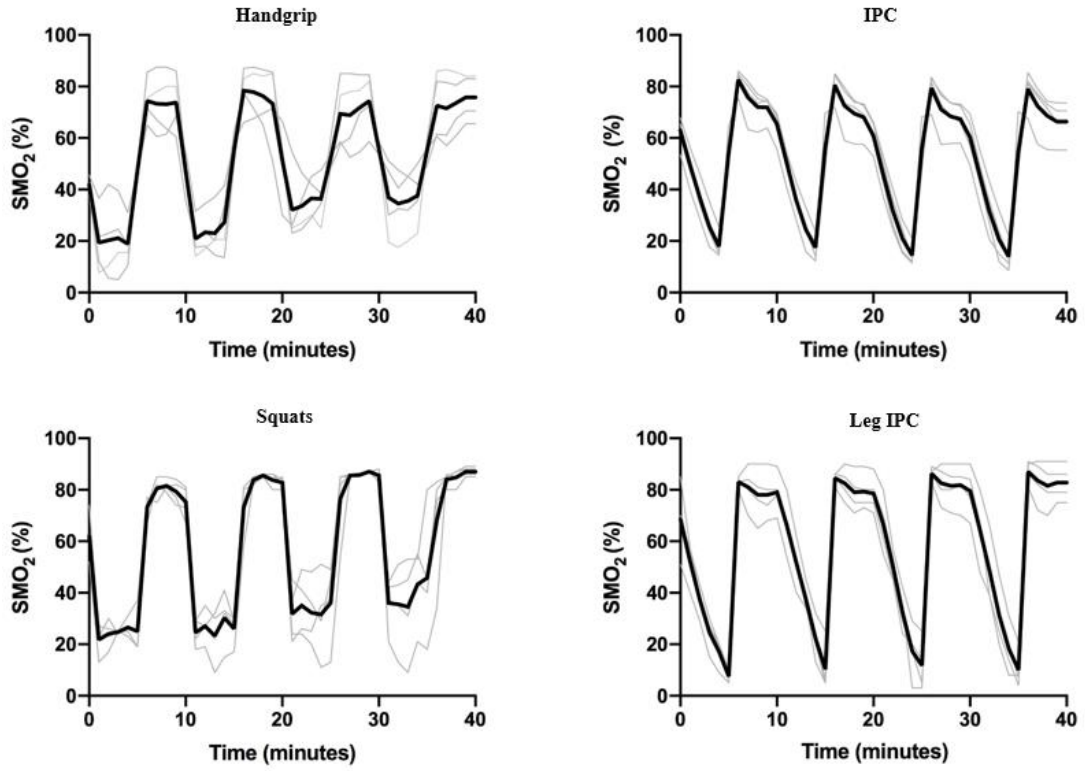

Figure 2.2 *Muscle oxygen saturation (%) for the matched study intervention protocols. Handgrip and IPC shows SMO_2 % of the carpi radius longus and Squats and Leg IPC shows SMO2 % of the vastus lateralis during each protocol respectively.*

2.2.6 Statistical Analysis

For the *handgrip study*, two factor general linear models was employed with condition (3 levels: control, handgrip and IPC) and time (2 levels: resting baseline and post IR injury) for FMD data obtained on both the local (intervention arm) and the remote (contralateral arm) to analyse FMD variables during each condition. For the *squat study* the same statistical test was employed with condition (3 levels: control, squats

and leg IPC) and time (2 levels: resting baseline and post IR injury) for all FMD variables. Statistically significant interactions were followed up with the least significant difference (LSD) approach to multiple comparisons. Analysis was conducted using Statistical Package for Social Sciences (Version 26: SPSSS Inc., Chicago, IL). Statistical significance was delimited at $P < 0.05$ and exact P values are cited (P values of '0.00' provided by the statistics package are reported as '< 0.01'). Data are presented in the text as mean and 95% confidence intervals (95%CI).

2.3 Results

Table 2.1 *Descriptive characteristics of participants*

	Handgrip (***n*= 15**)	**Squats Young** (***n*= 12**)	**Squats Elderly** (***n*= 6**)
Age (*years*)	23.9 ± 3.1	23.5 ± 8.0	61.5 ± 5.2
Weight (*kg*)	79.1 ± 11.4	76.8 ± 11.7	76.8 ± 16.4
BMI (kg/m^2)	25 ± 2	25 ± 3	25 ± 4
MAP (mmHg)	78 ± 6	77 ± 6	91 ± 8
SBP (*mmHg*)	115 ± 8	112 ± 9	128 ± 11
DBP (*mmHg*)	60 ± 5	59 ± 5	73 ± 10

Values are means ± SD. Abbreviations; BMI, Body Mass Index; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

2.3.1 Handgrip study

Local arm: FMD was reduced by 0.9 % (1.5, 0.3) from resting baseline to post IR-injury (main effect of time: P = 0.01). There was a trend for a condition*time interaction but this did not reach statistical significance (P = 0.08). In the control condition, FMD was reduced by 1.8 % (2.4, 1.2) following IR-injury. In both handgrip exercise and IPC the reduction in FMD was less, 0.5 % (1.7, 0.6) and 0.3 % (1.6, 1.0), respectively. There was no main effect of condition (P = 0.33). A post hoc power calculation using G*Power (3.1.5) indicated that a sample size of n=15 provided 71% power to detect a difference in IR injury between local IPC and handgrip exercise.

Remote arm: FMD was reduced by 2.0 % (2.8, 1.2) from resting baseline to post IR-injury (main effect of time: P < 0.01). There was no condition*time interaction (P = 0.14). There was a main effect of condition with FMD being 1.0 % (1.6, 0.4) lower in the control condition compared with handgrip exercise (P < 0.01). Similarly, FMD

was 1.1 % (1.9, 0.3) lower in the control condition compared with IPC (P = 0.01). A post hoc power calculation using G*Power (3.1.5) indicated that a sample size of n=15 provided 82% power to detect a difference in IR injury between remote IPC and handgrip exercise. Resting diameter was increased by 0.01 cm (0.0, 0.0) from resting baseline to post IR-injury (main effect of time: P = 0.02). There was a trend for a condition*time interaction but this did not reach statistical significance (P = 0.06). In the control condition, resting diameter increased by 0.02 cm (0.0, 0.0) following IR-injury. In both handgrip exercise and IPC the increase in resting diameter was less > 0.01 cm (0.0, 0.0) and 0.01 cm (0.0, 0.0) respectively. There was also a main effect for condition in resting baseline diameter (P = 0.03). Resting baseline in the control condition was 0.02 cm (0.0, 0.0) and 0.01 cm (0.0, 0.0) higher than in the handgrip and IPC conditions, respectively.

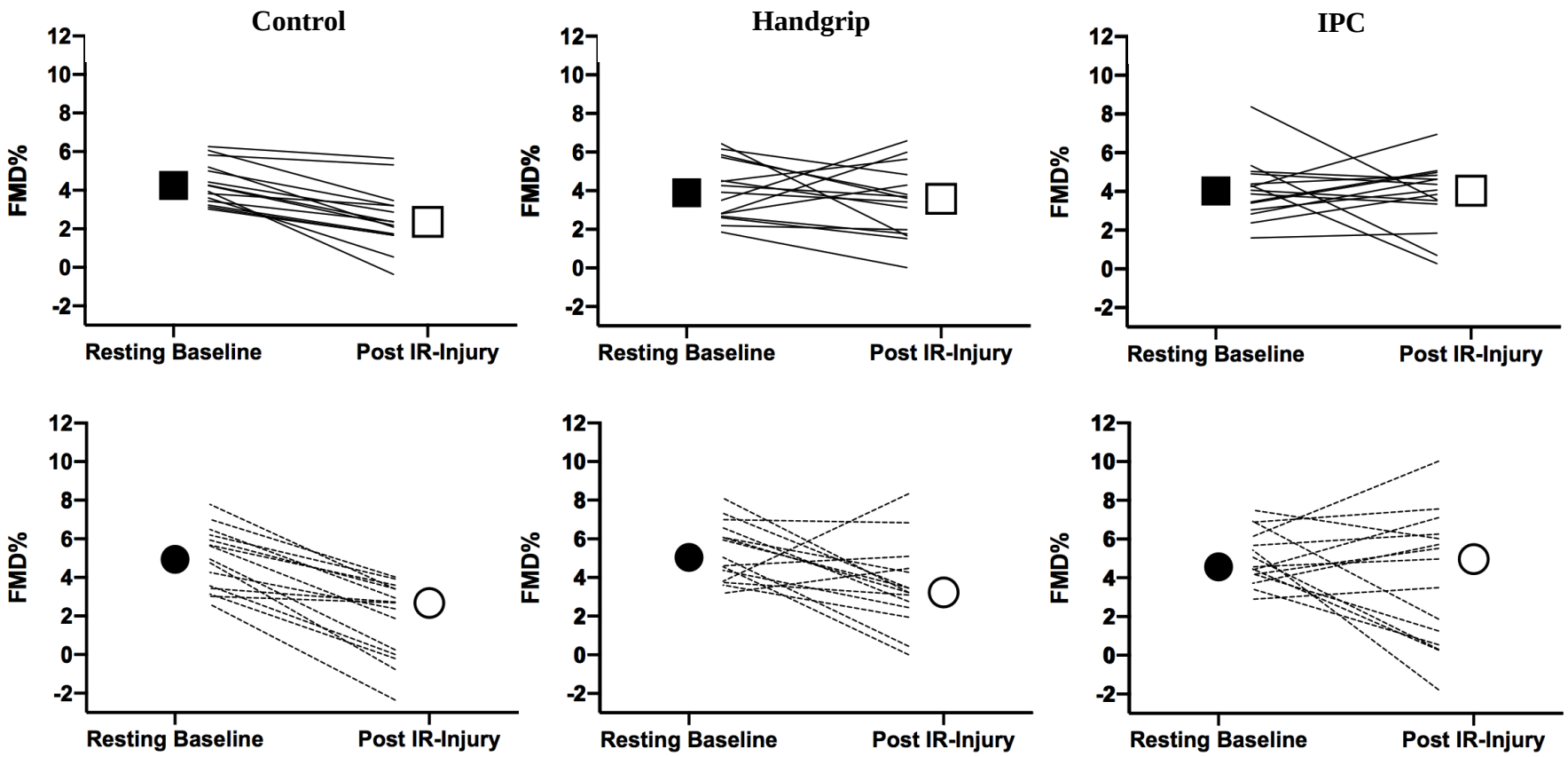

Figure 2.3 Brachial artery FMD at resting baseline (solid markers) and post IR-injury (open markers) in both local (squares) and remote (circles) arms. Symbols denote the mean values.

Table 2.2 *Brachial artery flow mediated dilation at resting baseline and post ischemia-reperfusion injury after either control, handgrip exercise or IPC in the local and remote arm.*

Local	**Baseline**			**Post IR-injury**			**P-value**		
	Control	**Handgrip**	**IPC**	**Control**	**Handgrip**	**IPC**	**Condition**	**Time**	**Condition* Time**
Resting Diameter (cm)	0.41 ± 0.06	0.41 ± 0.06	0.41 ± 0.06	0.42 ± 0.07	0.43 ± 0.07	0.42 ± 0.07	0.33	0.03*	0.63
Peak Diameter (cm)	0.43 ± 0.06	0.43 ± 0.06	0.43 ± 0.06	0.43 ± 0.06	0.44 ± 0.07	0.43 ± 0.07	0.38	0.10	0.28
FMD %	4.4 ± 1.1	4.0 ± 1.5	4.1 ± 1.6	2.5 ± 1.6	3.4 ± 1.8	3.8 ± 1.7	0.33	0.01*	0.08
Time to peak (sec)	51 ± 21	41 ± 12	43 ± 11	40 ± 19	39 ± 19	42 ± 20	0.31	0.30	0.39
Shear AUC (10^3)	16.6 ± 8.5	13.9 ± 6.6	15.1 ± 3.9	11.7 ± 3.5	11.7 ± 5.1	13.6 ± 6.7	0.29	< 0.01*	0.36
Remote	**Baseline**			**Post IR-injury**			**P-value**		
	Control	**Handgrip**	**IPC**	**Control**	**Handgrip**	**IPC**	**Condition**	**Time**	**Condition* Time**
Resting Diameter (cm)	0.41 ± 0.06	0.40 ± 0.6	0.40 ± 0.06	0.42 ± 0.07	0.40 ± 0.07	0.41 ± 0.06	0.03*	0.02*	0.06
Peak Diameter (cm)	0.42 ± 0.06	0.42 ± 0.06	0.42 ± 0.07	0.43 ± 0.06	0.41 ± 0.07	0.42 ± 0.07	0.13	0.90	0.21
FMD %	5.0 ± 1.6	5.3 ± 1.5	5.0 ± 1.4	1.9 ± 2.0	3.5 ± 2.1	3.9 ± 3.4	0.02*	< 0.01*	0.14
Time to peak (sec)	44 ± 15	45 ± 14	38 ± 7	53 ± 38	53 ± 46	48 ± 35	0.58	0.34	0.99
Shear AUC (10^3)	15.8 ± 6.1	19.4 ± 8.2	16.1 ± 5.9	12.9 ± 6.6	15. 7± 7.5	13.8 ± 5.2	0.07	0.03*	0.55

2.3.2 Squats study

In the young group, FMD was reduced by 1.9% (2.9, 0.9) from resting baseline to post IR-injury (main effect for time: $P < 0.01$). There was no condition*time interaction ($P = 0.81$). There was a main effect of condition with FMD being 0.9 % (1.9, 0.0) lower in the control condition compared with leg IPC ($P = 0.05$). FMD was also lower in the squat exercise by 1.1 % (1.9, 0.3) compared with leg IPC ($P = 0.02$)

In the elderly group, FMD was reduced by 1.8 % (3.1, 0.4) from resting baseline to post IR-injury (main effect for time: $P = 0.02$). There was a trend for a condition*time interaction but this did not reach statistical significance ($P = 0.09$). In the control condition, FMD was reduced by 3.0 % (4.6, 1.4) following IR-injury. In both squats and leg IPC the reduction in FMD was less 0.6 % (2.1, 0.9) and 1.7 % (4.2, 0.7), respectively. There was no significant main effect of condition ($P = 0.49$). In the elderly group, SR_{AUC} (10^3) was reduced by 5.2 (2.5, 8.0) from resting baseline to post IR-injury (main effect for time: $P < 0.01$). There was a significant interaction for a condition*time ($P = 0.05$). In the control condition, SR_{AUC} (10^3) was reduced by 8.5 (5.6, 11.3) following IR-injury. In both squats and leg IPC the reduction in SR_{AUC} (10^3) was less 4.0 (0.8, 8.8) and 3.3 (0.8, 7.5), respectively. There was no significant main effect of condition ($P = 1.0$).

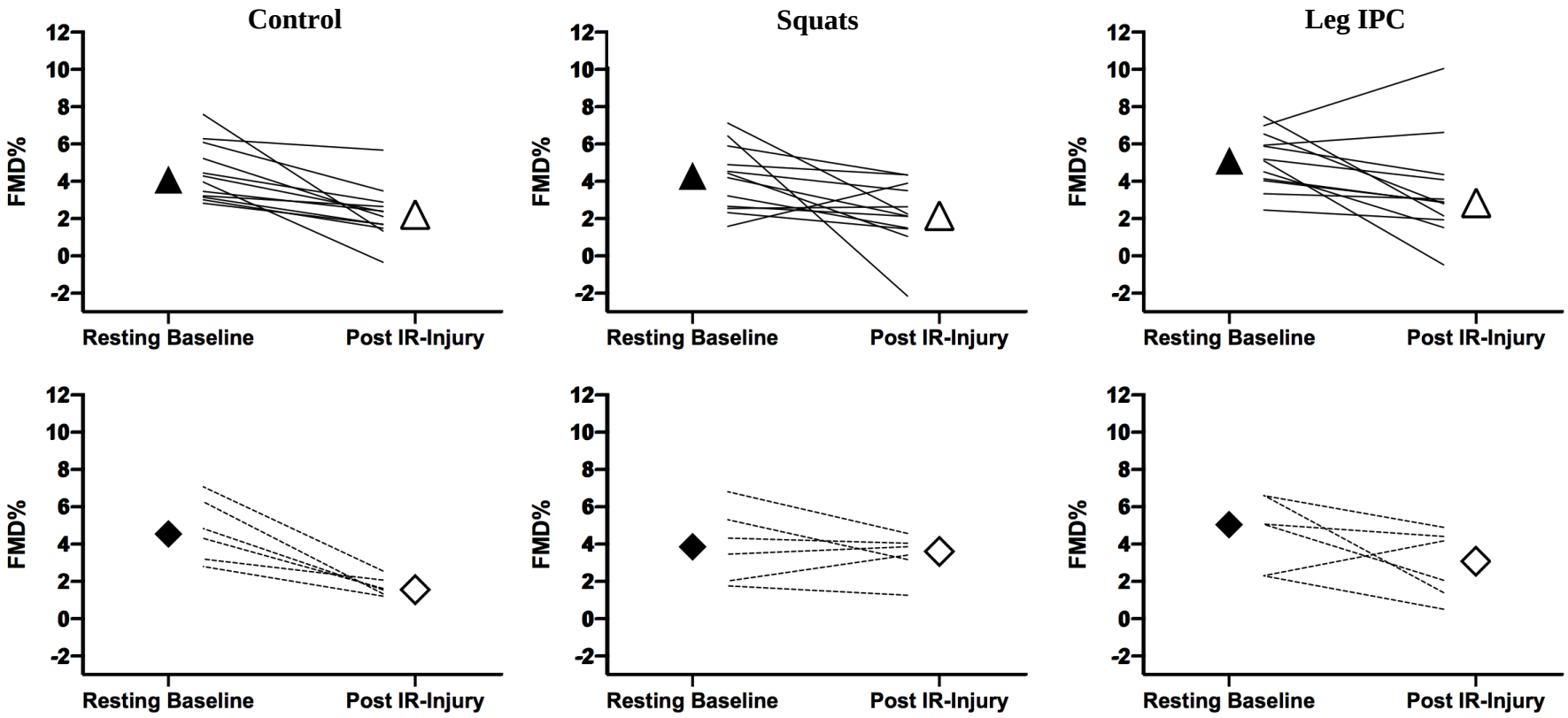

Figure 2.4 Brachial artery FMD at resting baseline (solid markers) and post IR-injury (open markers) in both young (triangles) and elderly (diamonds) groups. Symbols denote the mean values.

Table 2.3 *Brachial artery flow mediated dilation at resting baseline and post ischemia-reperfusion injury after either control, squats or Leg IPC in the young and elderly group.*

Young	**Baseline**			**Post IR-injury**			**P-value**		
	Control	**Squats**	**Leg IPC**	**Control**	**Squats**	**Leg IPC**	**Condition**	**Time**	**Condition* Time**
Resting Diameter (cm)	0.43 ± 0.06	0.42 ± 0.06	0.42 ± 0.06	0.44 ± 0.06	0.43 ± 0.07	0.44 ± 0.07	0.56	0.04*	0.11
Peak Diameter (cm)	0.45 ± 0.06	0.44 ± 0.06	0.44 ± 0.06	0.45 ± 0.06	0.44 ± 0.06	0.45 ± 0.07	0.64	0.40	0.24
FMD %	4.4 ± 1.5	4.1 ± 1.7	5.1 ± 1.5	2.3 ± 1.4	2.2 ± 1.8	3.5 ± 2.7	0.02*	< 0.01*	0.81
Time to peak (sec)	47 ± 24	73 ± 36	59 ± 21	49 ± 22	65 ± 39	72 ± 36	0.08	0.66	0.42
Shear AUC (10^3)	13.9 ± 10.6	20.2 ± 7.2	18.6 ± 7.6	11.7 ± 3.9	12.3 ± 4.0	12.1 ± 5.1	0.10	< 0.01*	0.18
Elderly	**Baseline**			**Post IR-injury**			**P-value**		
	Control	**Squats**	**Leg IPC**	**Control**	**Squats**	**Leg IPC**	**Condition**	**Time**	**Condition* Time**
Resting Diameter (cm)	0.44 ± 0.04	0.44 ± 0.03	0.43 ± 0.03	0.44 ± 0.03	0.44 ± 0.03	0.43 ± 0.03	0.67	< 0.01*	0.78
Peak Diameter (cm)	0.46 ± 0.03	0.46 ± 0.04	0.45 ± 0.03	0.48 ± 0.04	0.49 ± 0.05	0.47 ± 0.05	0.73	0.03*	0.47
FMD %	4.7 ± 1.8	3.9 ± 1.9	4.6 ± 1.9	1.7 ± 0.5	3.4 ± 1.2	2.9 ± 1.8	0.49	0.02*	0.09
Time to peak (sec)	72 ± 23	70 ± 29	65 ± 26	52 ± 28	93 ± 44	70 ± 37	0.41	0.83	0.23
Shear AUC (10^3)	16.3 ± 3.6	14.1 ± 3.9	13.9 ± 5.6	7.9 ± 2.7	10.2 ± 3.9	10.6 ± 4.9	1.00	< 0.01*	0.05*

2.4 Discussion

The aim of this study was to compare exercise preconditioning with IPC on the ability to provide immediate and clinically relevant protection against endothelial IR-injury in healthy individuals, and whether this protection was present in both the local and remote stimulus. Also, given that ageing negates the impact of IPC on endothelial IR-injury (Loukogeorgakis et al., 2005), exercise may represent a more powerful preconditioning stimulus. This is supported by the fact that exercise training can partly restore the age related attenuated efficacy of IPC, and thus another aim was to examine whether exercise preconditioning (squatting) attenuated endothelial IR-injury in an older population. This study provides evidence that exercise preconditioning (i) can attenuate endothelial IR-injury; (ii) can offer remote vascular protection and; (iii) has greater protective effects in an older population. This is the first study to demonstrate these similar attributes of exercise preconditioning to IPC in a human model. Taken together, the data indicate acute exercise has preconditioning effects that could be a potentially useful in a clinical environment to those at increased CVD risk or prior to planned interventions that cause IR injuries.

The current study has provided evidence that exercise attenuates the decline in FMD following IR-injury and is at least as effective as IPC. These findings are in agreement with previous work in humans suggesting interval exercise (exercise which includes periods of rest) can negate brachial artery endothelial IR-injury (Seeger et al., 2015). The current study is comparable to previous studies who have implemented this temporary ischemia-reperfusion injury model. In the handgrip study IR-injury reduced brachial artery FMD by ~1.8 and ~3.1 % in the local and remote arms respectively, in the control condition. This is a similar reduction as seen in other studies (2.0 to 3.8 %) (Munckhof et al., 2013, Loukogeorgakis et al., 2010, Loukogeorgakis et al., 2005). In

the current study, for the first time, IPC and exercise were matched for ischemia time and the level of ischemia and were at least similar in negating endothelial IR-injury in the local (handgrip) and the remote (squats) limbs. Thus, exercise preconditioning could be a useful intervention (i.e. within a clinical environment) to protect against IR-injury.

A novel finding of the current study is the evidence that exercise has local and remote effects. The handgrip preconditioning mediated vascular protective effects locally that were, at least, similar to local IPC. Nevertheless, the remote effect on the contralateral arm (in the handgrip study) or in the leg (squat study) were not as large as the local effect. However, there was some evidence that both exercise and ischemic preconditioning had some impact on negating the endothelial IR-injury compared to the control condition. This contrasts the previous research which has demonstrated an attenuation in brachial FMD in response to endothelial IR-injury can be obtain from lower limb interval exercise (Seeger et al., 2015). The present study provides evidence of a condition effect, in both the remote upper limb protocols and the lower limb protocols in the young, but no significant interaction and therefore should be taken with caution as this includes differences in baseline FMD. In the study by Seeger et al. (2015) they demonstrated that lower limb exercise was able to remotely prevent brachial artery endothelial IR-injury. Although, it is important to note that the interval exercise performed in that study may have produced a larger stimulus in young healthy individuals and could account for the difference in findings. Moreover, the similarity in responses between remote squats exercise and remote handgrip exercise suggest that even with a large muscle mass (at the same relative intensity) the same effect is evident. Thus, suggesting that exercise preconditioning is equally applicable when performed in a larger muscle mass compared to a smaller muscle mass. This is an

important finding as it allows for a better understanding as to which dose, exercise modes and protocols work to provide cardioprotection.

The squats exercise was able to attenuate the decline in FMD after IR-injury in the elderly population, to a larger extent than that seen in the young population. In the squats exercise post IR-injury FMD was reduced by ~0.6 and ~1.9 % in the elderly and young group respectively. There is however no previous research that looks at the effect of an acute bout of exercise preconditioning in a healthy, older population. Moreover, the squats exercise had a greater impact on reducing IR-injury in comparison to leg IPC in the elderly population. The majority of previous research into the efficacy of IPC has been conducted in healthy animals or healthy young humans, whereas ischemia related diseases more often occur in older populations. A recent study by Munckhof et al. (2013) has suggested that the efficacy of IPC to protect against IR-injury is attenuated with age, although, it is unclear as to whether age is underlying factor behind the reduced efficacy of IPC or whether lifestyle changes that occur with increased age (i.e. less physically active) play a greater role (Abete et al., 2000). Therefore, the finding that exercise produced a greater impact on attenuating IR-injury is important as it shows that exercise preconditioning may be a more effective intervention than IPC in an elderly population.

2.4.1 Methodological Considerations

In this study, a model of ischemia/reperfusion was used to mimic IR-injury in the upper limb, this model has been used in previous studies to produce a temporary ischemic injury (Kharbanda et al., 2001, Loukogeorgakis et al., 2005). However, there is an obvious inability to assess whether the remote effects of preconditioning are

transferable to the cardiac tissue. Although, the endothelial IR-injury model is often used and has been shown to have a strong correlation to coronary endothelial function (Anderson et al., 1995). It is important to note that the latest expert consensus guidelines (Thijssen et al., 2019b) have been followed in order to assess vascular function. There are some limitations to this research, a relatively small number of participants completed the research protocols, although this is a similar number, in terms of young participants, to other studies looking at preconditioning (Seeger et al., 2015). Although, the sample size of the elderly group is very small, meaning that further research on the effect of exercise preconditioning in this population is warranted.

2.4.2 Clinical Perspectives

Exercise is emerging as a potential form of preconditioning which may be able to offer clinically relevant cardioprotection (Thijssen et al., 2018). Observations from the current study suggest that exercise may be a more beneficial alternative to traditional IPC. This study has shown that exercise has some remote preconditioning effects, as well as being more effective in an elderly population. This in turn could be clinically relevant as these types of exercise are easy to perform and can be done frequently, whilst also offering other benefits outside of preconditioning when compared with IPC. Inaba et al. (2010) suggested that a 1% decrease in FMD is associated with a 13% increase in risk of future cardiovascular events. Therefore, the attenuation of the decline in FMD after preconditioning may be clinically relevant and exercise preconditioning may potentially be a potent producer of cardioprotection and offer an alternative to traditional IPC.

Acute MI is one of the leading causes of death worldwide, in 2007 close to 8 million individuals were affected by AMI (Yang et al., 2010). Re-establishing blood flow to an ischemic tissue area is hugely important to attenuate damage, nevertheless the reperfusion that follows can itself cause further damage. Thus, IR-injury is of great clinical relevance and techniques to combat it should be extensively explored. This study is in agreement with previous work (Seeger et al., 2015, Yamashita et al., 1999, Hoshida et al., 2002, Thijssen et al., 2019a, Michelsen et al., 2012) that certain forms of exercise may be a useful tool in providing cardioprotection against IR-injury.

2.4.3 Future Directions

Future studies should consider the mode and intensity of exercise performed and whether it is more useful to employ, whole body exercise or specific limb exercise. This will allow further examination of whether different preconditioning stimuli offer varying levels of cardioprotection against IR-injury. Given that ischemic events and therefore IR-injury is more likely to occur in older, clinical populations, further research is warranted in order to assess the efficacy of the exercise to offer cardioprotection within this population. Future studies should consider comparing the efficacy of both whole body exercise and specific limb exercise in a large elderly population in order to more comprehensively assess the impact of exercise preconditioning.

2.4.4 Conclusion

In summary, this study suggests exercise preconditioning may offer both local and remote protection against endothelial IR-injury in young, healthy individuals in a similar capacity to traditional IPC and possibly to a greater extent in older individuals.

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