Ischaemic preconditioning in exercise and disease: One size fits all?

Joost Seeger

A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy.

This research programme was carried out in collaboration with the Radboud University Medical Centre.

November 2015

Table of Contents

ABSTRACT

Ischaemia reperfusion injury (IR-injury) occurs when blood supply to a certain area of the body is blocked, and is subsequently followed by reperfusion. During the period of ischaemia, tissue is damaged as a result of lack of oxygen. Rapid reperfusion is mandatory, but unfortunately causes damage in addition to the damage induced by ischaemia alone. While a prolonged period of ischaemia is harmful to the bodily tissue, short periods of ischaemia interspersed with short bouts of reperfusion have protective effects. This mechanism is called ischaemic preconditioning (IPC). In this thesis, the impact of co-morbidity and age on IR-injury and IPC are explored. Moreover, the possible role of IPC to enhance exercise performance is investigated. Finally an attempt is made to understand the interchangeable effects of IPC and exercise performance in the prevention of IR-injury.

Using the brachial artery endothelial function as a surrogate marker, first the consequences of IR-injury in both young and older individuals on endothelial function were studied. It was also assessed whether IPC could prevent endothelial IR-injury. It was found that endothelial function in both groups declined, when IR-injury was not preceded with IPC. However, when IPC was applied prior to IR-injury, a protective effect was detected in young subjects, but not in older participants. In chapter 5, this study was repeated in patients with heart failure, as they are at an increased risk for IR-injury. While in both groups a significant decline in endothelial function was observed, a much larger decline was established in the heart failure group. Moreover, IPC failed to protect against endothelial dysfunction in heart failure patients after IR-injury.

The third study presented in this thesis, focused on the question whether exercise performance enhancement during a 5-km time trial was comparable when IPC on the upper legs was applied immediately before the time trial *versus* 24 hours (24-IPC) prior to exercise. Interestingly, a significant and strong correlation was found in finish time between acute IPC and 24-IPC, suggesting comparable effects of IPC and 24-IPC on exercise performance. In a follow-up study**,** it was determined whether local IPC applied on the upper arm, or remote IPC applied on the legs, would lead to an improved maximum incremental arm crank exercise test in individuals with a complete spinal cord lesion. The main finding was that upper arm IPC led to an increased performance enhancement, whilst remote IPC (stimulus below the lesion) did not lead to any significant differences. These studies help to inform the best or most practical application of IPC in daily life situations.

Some previous work has suggested that exercise may resemble some of the effects of IPC. More specifically, acute exercise might possess the same protective effects against ischaemia-reperfusion injury as IPC. Therefore, in young healthy individuals it was studied, whether an acute bout of endurance or interval exercise is able to protect against brachial endothelial IR-injury. It was established that interval exercise prevented endothelial dysfunction after an IR stimulus, while no protective effect of endurance exercise was found. It was concluded that interval exercise, but not endurance exercise, prevented endothelial dysfunction after an ischaemic period.

In conclusion, this thesis provides further evidence for the protective effects of (remote) IPC, both on the prevention of endothelial IR-injury as well as improvement in exercise performance. However, effects may depend on the protocol and population studied.

1

General introduction

Ischaemic heart disease remains the world's leading cause of death [1]. When an artery is blocked, immediate reperfusion of this artery is mandatory, as prolonged periods of lack of oxygen supply will lead to severe cellular damage. However, reperfusion itself also leads to a series of adverse events, which are potentially even more lethal to the affected tissue then ischaemia alone [2]. The negative consequences of a prolonged period of ischaemia followed with reperfusion are referred to as IR-injury, and first described by Jennings et al. [3].

While a prolonged period of ischaemia is potentially lethal, short and repeated bouts of ischaemia can exert protective effects when applied prior to IR-injury [4]. This intervention is termed IPC, and has been subject of several investigations [5]. The effects of IPC are not simply restricted to the tissue that was directly exposed to the repeated bouts of ischaemia, which means that organs remote to the ischaemic challenge are protected when IPC is applied [6]. In addition, IPC is known to exert its effects acutely up to three hours, where after it reappears after 24 hours for approximately 48-72 hours [7, 8]. The ability of IPC to exert effects in remote organs or tissues, as well as the ability to benefit of IPC well after the application, makes IPC an attractive and potentially relevant intervention [9].

The general aims of this thesis were twofold. **Part 1** relates to the uncertainty regarding the efficacy of IPC in specific groups. First, it was studied whether and how cardiovascular risk factors and/or disease affect IR-injury and the protective effect of IPC. For this purpose, older humans and patients with heart failure *in vivo* were included (**Chapters 4 and 5**). We hypothesised that both, older age and heart failure, are associated with greater IR-injury and diminished protectiveness from IPC, as preclinical studies have shown attenuated responses of the ageing heart or in patients with different types of co-morbidities after application of IPC [10, 11]. To examine the impact of IR-injury the endothelial function of the brachial

artery was assessed before and after IR-injury of the forearm, and presented as a surrogate marker of IR-injury.

A translation of IPC-like strategies to the clinical arena is often disappointing [11], to date there remains a lot of uncertainty regarding the potent effects of IPC and pharmacological preconditioning in preclinical research in specific groups [12]. As most studies are performed in (young) animals, one may question whether a typical patient also benefits from IPC. The majority of patients confronted with cardiovascular disease are of older age, as ageing is a major risk factor for developing cardiovascular disease [13]. Therefore, in **Chapter 4,** the impact of older age was examined, on the protective effect of IPC on endothelial IR-injury in humans *in vivo*.

Patients with heart failure have a poor prognosis and have an increased risk for adverse cardiovascular events and are therefore a key-target for IPC-application [14]. Whilst IPC may be an effective approach, the effectiveness of IPC in heart failure is also debated. Little is known whether these patients can benefit from this intervention as research in this typical group is lacking [15]. In **chapter 5,** an *in vivo* study is presented, in compared the protective effects of IPC between heart failure patients and their age-matched controls were compared. In both groups, the magnitude of endothelial IR-injury and the ability of IPC to reduce these possible adverse effects were examined.

Part 2 relates to the interchangeable effects of IPC and exercise. First, a number of studies suggest that IPC is an effective intervention to enhance exercise performance, as first

described by de Groot et al. [16]. Moreover, another recent study has found some preliminary evidence for the effectiveness of acute exercise to prevent the myocardium against IR-injury in animals [17]. It was concluded that exercise might has similar preconditioning effects as IPC to prevent the negative consequences of IR-injury. Therefore, our second general aim is to study the relationship between IPC and exercise in more detail (**chapters 6, 7 and 8**). It was hypothesised that exercise has potential preconditioning effects but that the effect may differ between different modes of exercise. More specifically, it was hypothesised that interval exercise (given the remarkable similarities with the repeated periods of ischaemia in IPC) has stronger preconditioning effects than endurance exercise. In addition, potential of IPC to enhance exercise performance was further explored and an attempt was being made to study the related mechanisms.

Studies exploring the potential of IPC to enhance exercise have typically applied IPC in the 1-2 hours preceding the exercise event, which is in line with most (pre)clinical work on IPC that have demonstrated that the cardioprotective effects are present up to 1-3 h after application of IPC. Interestingly, the cardioprotective effects of IPC reappear after 24-h, which is referred to as the second window of protection (SWOP) [5]. Whether a SWOP is also present in relation to the benefits for exercise performance is currently unknown. Such an effect could improve practical application of IPC before an exercise event. Therefore, in **chapter 6** it was examined whether the effects of IPC on performance enhancement during a 5-km running trial are similar to the effects when IPC is applied 24-h before the exercise test.

Studies have shown the ability of IPC to enhance exercise performance in healthy humans, predominantly exercise involving the lower limbs. It is for example described that IPC can enhance both cycling and running exercise [16, 18]. Subjects with a spinal cord injury are

restricted to upper limb exercise. Therefore, in **chapter 7** the impact of IPC on upper limb arm crank exercise performance in spinal cord injured individuals was examined. In addition, it was studied whether IPC has remote effects on upper limb arm crank exercise performance by application of IPC on the legs preceding exercise, as this has never been studied in relation to exercise before.

Some recent studies suggested that exercise may have preconditioning effects, which are suggested to be similar as observed after the application of IPC [17, 19]. These observations raise the question whether some types of exercise might have similar effects as IPC, especially since some types of exercise (such as interval exercise) share the characteristic repeated exposure to short bouts of ischaemia like in IPC. Therefore, in **chapter 8** a study was conducted, in which we explored whether exercise can prevent endothelial damage after prolonged ischaemia and reperfusion injury. As different types of exercise might lead to different outcomes, it was also examined whether different types of exercise possess similar preconditioning effects. The protective effects of moderate endurance exercise and high intensity interval in young, healthy subjects were compared on the ability to prevent endothelial IR-injury

2

Literature review

Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death in Europe, and still responsible for over 4 million deaths per year, i.e. close to half of all deaths in Europe and 40% in the European Union [20]. The annual cost of CVD is estimated to be €169 billion a year in the European Union [21].

Although health care has been improved remarkably in the last few decades and death rates have consistently fallen since the early 1960s, there are still a large number of people that suffer from coronary heart disease and its consequences. It is for example estimated that in the Netherlands alone, 730.000 persons are diagnosed with coronary heart disease [22]. Heart failure is commonly diagnosed, in a further 120.000 individuals. Due to the ageing population in the Netherlands, it has been estimated that these numbers will rise to 200.000 in the coming decade [23].

Determinants of cardiovascular disease

In understanding the development of cardiovascular disease, several studies have correlated the presence of behavioural factors and subject characteristics (such as biomarkers) to the future presence of cardiovascular disease. Classic behavioural risk factors associated with the development of CVD are tobacco smoking, physical inactivity, unhealthy diets and harmful use of alcohol. Indeed, it is estimated that for example in the UK, more than 20,000 deaths each year from cardiovascular disease can be attributed to smoking [24]. Non-modifiable risk factors include sex, ethnic origin and family history, whilst ageing is the most important non-modifiable risk factor. Prevalence of CVD increases dramatically with age, which is demonstrated to be independent of other cardiovascular risk factors. Not only the

prevalence of CVD, but also the morbidity and mortality caused by CVD increases exponentially with advancing age [25, 26], with the incidence of myocardial infarction doubling between the ages of 45 to 65 [27].

Already In the 1950s, Morris et al. [28] reported that active bus workers and postmen demonstrated a 50% lower event rate from coronary artery disease in comparison to colleagues who were physically less active. [28]. Ever since, much research has shown the benefits of a physical active lifestyle, while leaving the question on adopting the optimal strategy still open for debate. In general, it is now widely accepted that higher fitness level and physical activity patterns are beneficial for the prevention of CVD [29, 30]. Although these traditional modifiable and non-modifiable risk factors explain the development of CVD to a large extent, other unknown factors act as important mediators in the development of CVD. For example, approximately 41% of the risk reduction associated with exercise cannot be explained by established traditional risk factors [31]. To explain this 'risk factor gap', Green et al. proposed an important role for the vascular endothelium [32], which plays a crucial role in the regulation of vascular tone and in the adaptations of blood vessel structure. The endothelium produces numerous paracrine hormones, including nitric oxide (NO), which are anti-atherogenic. Endothelial dysfunction precedes and predicts the development of atherosclerotic disease and interventions of known cardiovascular benefit improve endothelial function. Indeed, Halcox et al. [33] demonstrated that systemic endothelial function was associated with progression of preclinical carotid arterial disease over a 6-year period and was more closely related to changes in carotid intima-media thickness than conventional risk factors [33]. Accordingly, coronary and peripheral endothelial dysfunction are independent predictors for cardiovascular events, and improvement in endothelial function improves prognosis [34]. Therefore endothelial

dysfunction can be considered as an early and integral manifestation of vascular disease and improvement in endothelial function should have a significant impact on CV risk [32].

Ischaemic reperfusion injury

When an artery is blocked, immediate reperfusion of this artery is mandatory, as prolonged periods of lack of oxygen supply will lead to severe cellular damage. Consequences of depriving an organ of its blood supply have long been recognised as the critical factor in the clinical outcome. However, reperfusion itself also leads to a series of adverse events, which are potentially even more lethal to the affected tissue then ischaemia alone [35]. For example, the histological changes of injury after 3 hour of liver or intestinal ischaemia followed by 1 hour of reperfusion are far worse than the changes observed after 4 hour of ischaemia alone [36, 37]. The injury of body tissue after restoration of blood flow following an ischaemic period is called ischaemic reperfusion injury (IR-injury) and can occur in a variety of clinical settings, including reperfusion after thrombolytic therapy, coronary angioplasty, organ transplantation, aortic cross-clamping or cardiopulmonary bypass [35]. It is believed that tissue damage caused by reperfusion injury has important clinical implications, because IR-injury provides clinicians with an opportunity to intervene with cardioprotective drugs or other measures that eventually (partly) prevent tissue damage due to IR-injury [38].

The combined lethal effect of ischaemia and reperfusion were first described for the myocardium in the early 1960s by Jennings and his co-workers [3]. They reported cell-swelling, contracture of myofibrils, disruption of the sarcolemma, and the appearance of

intramitochondrial calcium phosphate particles [3, 39]. After this landmark study, many others have performed comparable experiments to confirm and further understand the mechanisms of the detrimental impact of reperfusion after a period of ischaemia in humans and animals [39].

Pathophysiology of ischaemic reperfusion injury

Human body cells tightly regulate cell volume and composition of intracellular and extracellular compartments. Of special importance is the balance of different ions between the intracellular and extracellular environment. This balance is complex, in that both active and passive transport take place. Passive transport of ions across the cell membrane is regulated by processes like osmosis and is contingent on the balance between cations and anions. Active transport is of special importance, because this allows ions to move against their gradient across the cell membrane. Intracellular solute composition differs from the extracellular fluid in both volume as well as chemical and electrical potential. These differences are essential for a cell to maintain adequate function (Table 1).

Table 1. Chemical compositions of extracellular and intracellular fluids (adopted from Guyton and Hall. textbook of medical physiology 12th edition).

	Extracellular fluid	Intracellular fluid
Na^+	142 mEq/L	10 mEq/L
K^+	4 mEq/L	140 mEq/L
Ca^{2+}	2.4 mEq/L	0.0001 mEq/L
MG^{2+}	1.2 mEq/L	58 mEq/L
CL^-	103 mEq/L	4 mEq/L
$HCO3^-$	28 mEq/L	10 mEq/L
Phosphates	4 mEq/L	75 mEq/L
$SO4^{2-}$	1 mEq/L	2 mEq/L
Glucose	90 mg/dl	0 to 20 mg/dl
Amino Acids	30 mg/dl	200 mg/dl
Cholesterol/Phospholipids/ Neutral fat	0.5 g/dl	2 to 95 g/dl
Po_2	35 mm Hg	20 mm Hg
PCO_2	46 mm Hg	50 mm Hg
pH	7.4	7.0
proteins	2 g/dl	16 g/dl

Ischaemia

Intracellular use of both ATP and high-energy phosphates is critical to maintain cellular homeostasis [40]. ATP is needed to facilitate active transport, with Na-K pump (or Na,K-ATPase) being the most important and demanding transport mechanism. During ischaemia,

ATP is progressively depleted. The lack of oxygen prevents mitochondrial respiration and oxidative phosphorylation, leading to anaerobic glycolysis, thereby causing a gradual increase in acidity of the intracellular environment. To buffer the resulting cellular decrease in pH, excessive H^+ is excreted by accelerated Na^+/H^+ exchange, which in turn causes substantial Na^+ inflow into the intracellular space [41].

Normally, the sodium pump would excrete redundant Na^+. However, with the sodium pump being ATP-dependent and ATP being depleted during periods of ischaemia, intracellular Na^+ cannot be removed efficiently during ischaemia. Meanwhile, intracellular depletion of ATP also gradually inactivates ATPases, such as the Na-K pump, impacting ATP-dependent Ca^{2+} reuptake, and active Ca^{2+} excretion. This ultimately results in Ca^{2+} overload and a subsequent reduction in membrane potential [40, 42]. Calcium loading will additionally lead to cellular osmotic swelling as a consequence of disturbed homeostatic intra-extracellular balance, and any possibility of damage repair during the ischaemic period is prevented by the gradual depletion of ATP [43]. Ultimately, the energy compromise and final breakdown of cellular components leads to necrotic cell death with rupture of the plasma membrane and subsequent inflammatory responses as the tissue is invaded by neutrophils [39].

Reperfusion

When reperfusion occurs after a prolonged period of ischaemia, oxygen levels are rapidly restored and the extracellular pH returns to normal. Despite the restoration of oxygen levels and extracellular pH, the intracellular pH is still acidic and this pH gradient facilitates extrusion of H^+ from the cell in exchange for Na^+ [44]. The increased cytosolic Na^+ is at least

partly extruded in exchange for Ca^{2+} (reversed function of the Na^{+}-Ca^{2+} exchanger), thereby resulting in a further increase in Ca^{2+} intracellular levels.

During reperfusion, mitochondria are re-energised and the rapid delivery of oxygen leads to a massive production of reactive oxygen species (ROS), particularly during the initial phase of reperfusion [39]. The increase in ROS during ischaemia and reoxygenation is thought to be due to damage to electron transport chain components resulting in inefficient transfer of electrons, generating superoxide [44]. Consequently, the marked increased production of ROS causes further oxidative damage to cellular structures, including the smooth endoplasmic reticulum, leading to even more Ca^{2+} release [45].

Another important feature during reperfusion is the opening of the mitochondrial permeability transition pore (mPTP), a non-specific, multimeric pore structure spanning the mitochondrial inner and outer membranes [43]. mPTP serves as a high-conductance, nonselective, voltage-dependent channel and is closed under normal physiologic conditions. However, under conditions of cell stress, the formation of the open pore in the inner membrane results in the loss of membrane impermeability and rapid collapse of the mitochondrial membrane potential [45]. During ischaemia, opening of the mPTP is inhibited by the acid cellular environment. However, when pH returns to normal during reperfusion, the mPTP is no longer inhibited and therefore reperfusion favours mPTP opening. Opening of the mPTP, causes the mitochondria to break down ATP and if unrestrained, will ultimately lead to necrotic cell death [43].

Taken together, although reperfusion after ischaemia is obligatory in order to prevent further tissue damage, both ischaemia and reperfusion lead to unfavourable cellular processes that provoke greater cellular damage and lead to possible cellular death.

Ischaemic reperfusion injury of the endothelium

The endothelium is the inner layer of a blood vessel and has the ability to secrete different vasoactive substances and plays a pivotal role into regulating vascular tone and thereby blood flow [46]. Endothelial function is often determined with a non-invasive ultrasound technique called 'flow-mediated dilation' (FMD), which will be described in the methods section into more detail. In healthy blood vessels, the endothelium responds to shear stress, with the release of substances (mainly nitric oxide), leading to vasodilation [34]. In general, healthy vessels show a larger response, measured as the amount of dilation, as opposed to vessels characterised with endothelial dysfunction. Ischaemia-reperfusion refers to a prolonged period of ischaemia (when applied as a model in humans typically 15-20 minutes), followed by reperfusion of 15-20 minutes.

After exposure to such IR-injury, a decline in blood vessel dilation is found [47, 48]. Endothelial cells are particularly sensitive to IR, with reperfusion leading to both structural and functional damage of the endothelium. Subsequent endothelial injury and swelling due to IR can contribute to further ischaemia by impeding blood flow upon reperfusion, which has been termed the 'no-reflow phenomenon' and is present in the myocardium [49] as well as the brain [50].

This model of 20-minutes of ischaemia leading to impaired endothelial function (but not necrosis) is clearly different to the clinical situation of a prolonged (~60 minutes) ischaemia of the heart, leading to impaired endothelial function and necrosis. Although much shorter in duration of ischaemia than in a myocardial infarction, this model of 20-minutes of forearm ischaemia induces reduction in endothelial function. Furthermore, the duration of ischaemia may be inferior to the presence of reperfusion. It was for example found in cats, that a 90

minutes period of ischaemia alone, did not lead to detectable endothelium damage, while a short period of reperfusion after 90 minutes of ischaemia led to attenuated endothelial functioning [51]. The negative impact of reperfusion was greater after 20 minutes, than after only 2.5 minutes, which is suggestive a pivotal role for (the time of) reperfusion in relation to structural and functional damage to the endothelium. Despite the obvious dissimilarities with a myocardial infarction, our model is relevant in that it induces endothelial damage due to ischaemia-reperfusion injury.

Ischaemic preconditioning

Efficacy of treatment of acute ischaemic heart disease has improved the in the last decades. Procedures that allow the rapid return of blood flow to the ischaemic zone of the myocardium, have led to a 50% reduction in mortality rate [45]. However, as indicated in the paragraph above, reperfusion itself also leads to further complications like stunning, cardiac arrhythmia and even irreversible cell injury. As described earlier, these complications relate for example to the endothelium and the heart, but complications of reperfusion occur in every organ when exposed to a prolonged period of ischaemia, such as the brain, the limb after knee surgery or the kidney duration transplants [52]. As reperfusion after a period of ischaemia is both unavoidable but lethal, scientists have sought for efficient strategies to prevent secondary complications after reperfusion therapy.

A possible protective effect of short bouts of ischaemia preceding actual arterial occlusion was first described by cardiologists, who observed that patients reporting angina in the 24 hours preceding a myocardial infarction show a significantly better outcome in terms of development of congestive heart failure, reinfarction, and ultimately, mortality [53]. These 'protective' effects of preceding angina were present despite a paradoxical increase in total

time of exposure to ischaemia, and have been referred to as the cardiac warm-up phenomenon [54].

Murry et al. [4] were the first to actually describe and demonstrate such an effective strategy in an experimental animal model. In a dog, the left anterior descending artery was occluded (4 times 5 minutes) interspersed with 5 minutes of reperfusion periods where after the same artery was occluded for 40 minutes. After the intervention, they found a significant reduction in infarction size compared to the control animals, suggesting a protective effect on the heart muscle cells. This intervention has been infinitely repeated in similar experiments afterwards, and is termed IPC [44]. In general, IPC is defined as an increased tolerance to ischaemia and reperfusion induced by a previous (series of) sub-lethal periods of ischaemia. Indeed, such procedures can reduce infarct size by up to 75%, which indicates the importance and potency of prevention of IR-injury in the clinical management of ischaemic insults [39]. After the first experiments performed in cardiac tissue, many others have investigated the impact of IPC in IR-injury on different organ sites. These studies have shown IPC to be effective in different organ sites, like the liver, brain, vascular endothelium and skeletal muscle [55-58].

Most studies use occlusion periods of 5 minutes, interspersed with 5 minutes of reperfusion, which is repeated 3-4 times. Although much more variations are possible within this protocol, only a few studies have tried to determine whether different protocols lead to differences (in the magnitude) of the infarct-sparing effect [59, 60]. In addition, most studies are performed in animals, leading to significant inter-species bias, as animals tend to show differences between species in response after application of IPC [5]. For example, studies using rats of pigs found that the strength of the stimulus was related to the infarction size of

the heart [61, 62], while no differences were found in rabbits and dogs [5]. Also in the human cardiac tissue, no differences were found in relation to the strength of the stimulus [63]. As to date, there is still uncertainty about the optimal protocol to induce maximal protection since almost all studies use a protocol in which 3-4 cycles of 5 minutes of occlusion are used.

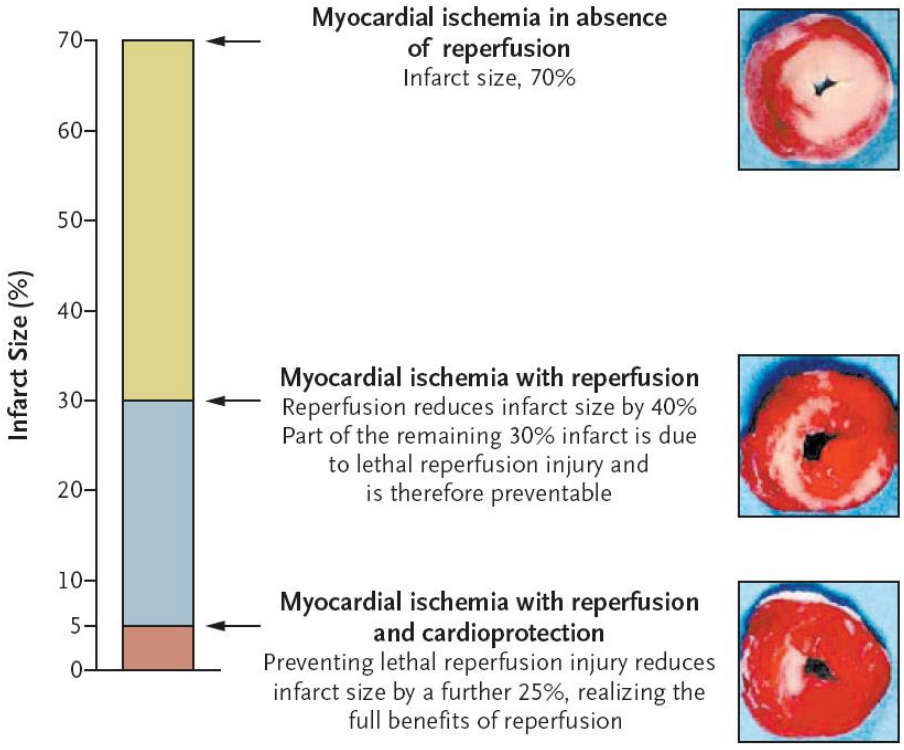

Figure 1. *Adopted from Yellon et al. [39]. This figure shows the hypothetical effect of myocardial damage after ischaemia with and without reperfusion and with and without cardioprotection which may be realised with ischaemic preconditioning. At the top bar in yellow, lethal myocardial injury is shown after ischaemia without subsequent reperfusion. The importance of reperfusion after an ischaemic period is shown in the middle (blue), leading to a 40% reduction in cardiac damage. However, cardioprotection can reduce the harmful effects of Ischaemic reperfusion injury to a large extent as is shown at the*

bottom in red with another reduction of 25% . Protection against the negative consequences of ischaemic reperfusion injury is therefore mandatory, as it can significantly reduce tissue damage to a large extend and thus improve clinical outcome

Physiology of ischaemic preconditioning

The mechanisms of IPC are not fully understood. However, currently there are some well-accepted theories used to explain the protective effect of IPC. When considering preconditioning, it is useful to think in terms of triggers, mediators, memory and end-effectors.

Triggers

Triggers are released during the short periods of ischaemia and carry the signal for ischaemia protection. They are the first step in this complicated signal cascade. Some of the best described 'key triggers', which are important in starting the IPC signal are adenosine, bradykinin and opioids [5, 64] (Figure 2). As can be depicted from figure 2, other important triggers are the reactive oxygen species (ROS). While large amounts of ROS are harmful for the cellular components, smaller numbers of ROS can induce IPC, commonly referred to as the oxygen paradox. Also norepinephrine is a well-known IPC trigger, while the role of NO is still debated regarding classical IPC. It is interesting to note that blockade of a single receptor type only raises the ischaemic threshold instead of completely blocking it [5]. This implies that different triggers act together, or the presence of a redundancy in systems, in order to bring the cell in a protective phenotype.

Mediators and end-effectors

Mediators are the interconnection between the triggers that start the preconditioning cascade, and the end-effectors which are responsible for getting a cell in the preconditioning state. Extracellular signals, surpass the cellular plasma membrane and multiple signal transductions pathways converge finally on the mitochondria, which have an important lethal function during IR-injury. These pathways either preserve ATP synthesis or prevent the onset of mPTP formation [45]. The exact signal transduction pathways have been studied extensively. These studies established that the protein kinase C (PKC) exerts an important role as a mediator during IPC. PKC can be either directly activated by adenosine and indirectly via bradykinin and opioid peptides and therefore serves as an intermediate in the signal cascade.

An important end-effector leading to early protection after preconditioning is the ATP-sensitive potassium channel (K_{atp} channel). While the exact mechanism is still debated, opening of this channel very likely works as both a trigger and an end-effector of preconditioning. While early evidence favoured the role of sarcolemma K_{atp} channels [65], latter studies have revealed a possible and far more important role for the mitochondrial inner membrane K_{atp} channels [66].

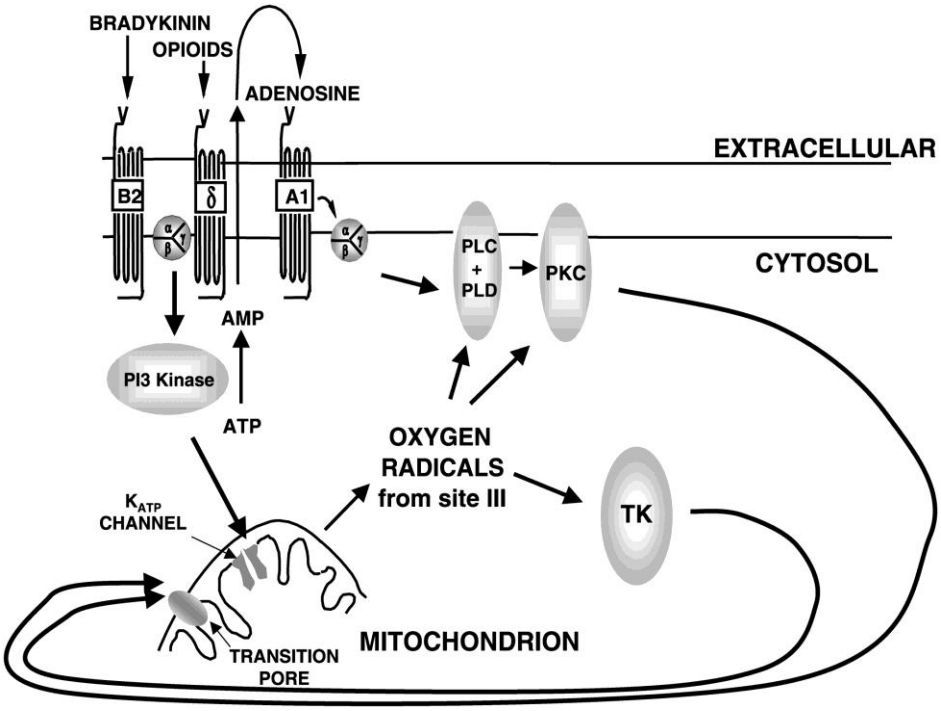

Figure 2. *Adopted from Yellon et al [5].* A proposed scheme for classical ischaemic preconditioning, in which bradykinin, opioids, adenosine and free radicals are able to trigger protection via distinct pathways which finally converge on the mitochondrion.

Remote preconditioning

In addition to the effects of IPC that prevent local cellular injury in the area that was directly exposed to the IPC stimulus, studies have also revealed a beneficial effect of similar magnitude of IPC on remote regions. This indicates that tissues that were not directly exposed to ischaemia are as equally protected against prolonged ischaemia as the tissue directly exposed to the IPC stimulus. This phenomenon is commonly referred to as remote preconditioning (RIPC) and was first described first by Przyklenk and her group [6]. They demonstrated that brief preconditioning occlusions of the circumflex artery also limit infarct size from subsequent sustained occlusion of the left anterior descending artery in the dog heart [6, 64]. Moreover, the remote protective effects are also present when IPC was

applied to a different organ, rather than a different area of the target organ, as shown in figure 3. For example, McClanahan, reported renal ischaemia to be protective in preconditioning the heart in a rabbit model [67]. This study was followed by numerous (animal) studies providing additional evidence that IPC of an organ results in protective effects of other organs not directly exposed to the IPC stimulus [68].

The favourable effects of RIPC are of great value in clinical practice, as it allows for the application of IPC to an extremity (i.e. arm or leg) to reduce IR-injury of an affected organ site that is not directly accessible, like for example the heart or brain tissue. Indeed, Kharbanda et al. [48] examined endothelial IR-injury of the forearm before and after IPC applied to the contra-lateral arm. They demonstrated that IPC completely abolished the negative effects of IR-injury when IPC was applied to the uni- as well as the contra-lateral limb compared to IR-injury without IPC. In addition, they demonstrated in a pig animal model that IPC performed in a lower limb significantly reduced myocardial infarction size after 40 minutes of coronary occlusion compared to a control condition. Subsequently, others have shown that RIPC is effective in different mammals and on different organ sites, with several studies observing that ischaemic events in the brain adapt cardiac tissue to ischaemia [69], or that renal artery occlusion is as effective as direct myocardial IPC in reducing infarct size [67, 69, 70].

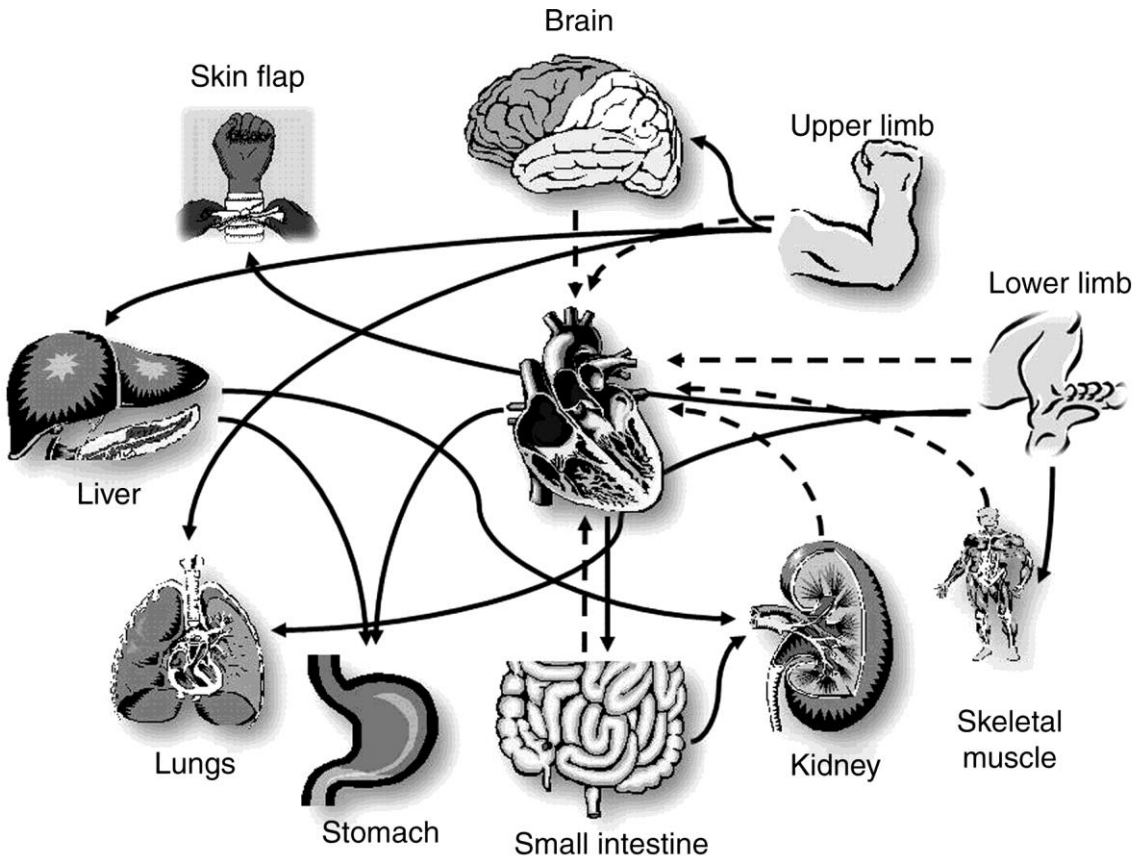

Figure 3. *Adopted from Hausenloy et al. As depicted in the figure, ischemic preconditioning functions remotely, with potential beneficial effects on many organs [71]*

Delayed ischaemic preconditioning

In 1993, two independent groups, reported that the protective effect of ischaemic preconditioning reappears after 24 hours and can last up to 72 hours [7, 72]. This SWOP, may be somewhat less powerful than the early protection, but can still significantly reduce infarct size by 50% [7]. Although both classical and delayed protection largely share common signalling pathways, several essential differences are present, with the synthesis of new proteins being the most striking one [64]. The autocoid factors that trigger classical IPC, are also responsible for the protection seen during the SWOP. Again, adenosine is one of the

important initiators of protection, since pharmacological blockade of adenosine receptors completely abolished the SWOP [8]. Also Bradykinin and ROS are known to have important trigger functions [45]. One major difference between the early and late protection relates to the role of NO as an essential trigger during the late phase of protection, while its role during the early phase is still a matter of debate. Via eNOS-derived NO and superoxide, peroxynitrite anion is formed, which in turn activates PKC. DNA microarray studies have revealed that preconditioning changes the gene expression pattern in rat hearts, which suggest that complex cellular mechanisms are involved in the evolution of late preconditioning [45, 73].

Ageing and Ischaemic preconditioning

A strong negative predictor of recovery from myocardial infarction in humans is older age [74]. Although IR-induced myocardial injury can occur in people of all ages, the risk of experiencing an IR insult increases with age, with the greatest risk of IR-injury occurring in both men and women over the age of 60 [75]. Despite the potent effects of RIPC and pharmacological preconditioning in preclinical research, translation of these strategies to the clinical area is often disappointing [76]. This may relate to the inclusion of young and healthy animals in preclinical studies, whereas application of IPC in the clinical setting typically relates to the older patients with co-morbidities. A potential explanation for the detrimental effect of advanced age for the development (and consequences) of cardiovascular disease may relate to changes in the magnitude of IR-injury and the potential of IPC to prevent this effect. For example, ageing affects cardiomyocytes, which undergo complex changes that

finally result in loss of contractile function and loss of endogenous protection against irreversible injury [25].

Studies in older animals, suggest that the protective effects of IPC are attenuated in ageing hearts [77-79] or brain [80], although data is conflicting [81, 82]. Another study using human biomaterial performed with isolated arterial trabeculae from elderly patients, found that IPC has no beneficial effect on the functional recovery after stimulated IR [83]. It must be emphasised that no previous study in humans has examined the direct impact of ageing of the efficacy of IPC *in vivo*. One possible explanation for the reduced efficacy of IPC might relate to the mitochondrion, where an age-dependent unfavourable increase in ROS occurs [84]. Whereas low amounts of ROS function as signalling molecules and are essential for cardioprotective signalling cascades, high amounts of ROS are detrimental as described earlier [25]. The higher amounts of ROS are both caused by enhanced ROS formation and a decreased age-dependent antioxidant capacity which collectively leads to oxidative stress induces mitochondrial dysfunction [85, 86].

Decreased bioavailability of NO, measured either with FMD or as plasma nitrite concentration, is another important hallmark of ageing [87]. NO functions as a potent vasodilator, but also as an important cellular signalling molecule during the SWOP. Other suggested responsible mechanisms for the age-related decline in protectiveness of IPC are the decrease of norepinephrine to IPC stimulus [88], impaired PKC translocation in response to IPC [89] and an enhanced dephosphorylation by protein phosphates [90, 91].

As ageing highly influences the impact of IR-injury and the protection afforded by IPC, the use of animals as an appropriate model for ageing is debated. Although some studies have used older animals, most have used young animals, which raises the question of whether

these young animals reflect the same pathophysiological changes during their relatively short lifespan as seen with the ageing process in humans. Abete [92] stated that, while pathophysiological modifications occurring with age are not dichotomous but progressive, the effect of IPC may gradually reduce with increasing age. It is therefore almost impossible to study a progressive ageing model in animals and therefore *in vivo* studies performed in humans are necessary.

DeVan et al. [47] conducted a study in humans in which they measured endothelial function before and after IR-injury in young and middle-aged subjects to gain insight into the primary effects of ageing. They found a greater magnitude and delayed recovery from endothelial IR-injury indicating the unfavourable effects of ageing. Interestingly, habitual exercise in the elderly provided partial protection against the endothelium IR-injury, possibly as a result of an increased NO-bioavailability related to better physical fitness. The efficacy of IPC to prevent or attenuate IR-injury was not explored (in this study) and is topic of chapter 4 of this thesis.

Efficacy of IPC in clinical groups

Although IPC has been proven to be effective in reducing IR-injury to a large extent, in clinical practice some practical considerations arise when IPC is applied [93]. As described earlier, most events happen in elderly and IPC related interventions are still not efficient in reducing IR-injury induced tissue damage. Another important factor that may contribute to impaired efficacy of IPC may relate to chronic diseases, like heart failure and diabetes [40]. Patients with heart failure are at increased risk of both fatal and nonfatal major adverse

cardiovascular events [94]. In heart failure, an increased rate of cardiovascular death in post-infarcted failing heart suggests that endogenous protective mechanisms against IR-injury might be lost or attenuated by the ongoing disease [45]. Indeed, heart failure is characterised by a variety of morphological alterations in G-proteins and protein kinase activation which might impact on the signal transduction cascade of preconditioning [45]. These changes suggest that patients with chronic (cardiovascular) disease are especially at risk but with limited effectiveness of IPC.

Interestingly, animal studies show some conflicting data, stressing again the need for human *in* vivo research. A previous study in animals found that HF is associated with an increased injury of cardiomyocytes after IR-injury [95], which may relate to the larger risk for cardiac events in HF. In comparison, another animal study found an attenuated protective effect of IPC against IR-injury in the failing rabbit heart muscle [96]. However, to date, no previous studies have examined the impact of HF on IR-injury or whether HF alters the cardioprotective effects of IPC seen in healthy humans. This will be topic of research in chapter 5 of this thesis.

Ischaemic preconditioning and exercise

Green et al. postulated that the traditional risk factors can only explain up to 60% of the exercise-induced improvement in CHD risk reduction and that therefore other unknown mechanism must be responsible for these favourable adaptations [32]. One possible explanation for the benefits of exercise (training) might relate to a positive effect of exercise (training) on IR-injury and/or IPC, especially since both exercise and IPC can be characterised

by repeated exposure to short periods of (local) ischaemia. Recent studies have found preliminary evidence from cross-sectional comparisons between highly trained subjects and their sedentary peers that exercise training is associated with a reduced endothelial IR-injury [47, 97]. These effects may partly contribute to the protective effects of exercise training. More importantly, this observation raises the question as to whether exercise training has preconditioning effects on the vasculature that eventually contribute to the lower endothelial IR injury in trained subjects. Another important hallmark of exercise relates to different types of exercise training causing different effects, with high-intensity interval training leading to superior effects on physical fitness and the vasculature compared to 'traditional' moderate-intensity exercise [98, 99].

Interestingly, a recent study from Michelsen and co-workers [17] provided some first preliminary evidence that (high-intensity) exercise has IPC-like effects on the protection of tissue against prolonged ischaemia [100]. They found that high-intensity interval running exercise (4 X 2-min) and ischaemic preconditioning (4x5-min) in humans, followed by blood withdrawal and perfusion through isolated rabbit hearts, were similarly able to reduce infarct size induced by ligation [100]. These authors concluded that high-intensity exercise preconditioning elicits cardioprotection through a humoral mediated mechanism, similar to that of ischaemic preconditioning. Whether acute bouts of exercise possess the ability to prevent endothelial IR injury in humans *in vivo* is currently unknown. This will be subject of research in chapter 8 of this thesis.

Ischaemic preconditioning and exercise performance

The potential benefits of IPC may be present beyond the 'traditional' area of cardiology, especially given the apparent similarities that exercise and IPC share. In exercise performance, the ultimate goal for all elite and professional athletes is to improve performance. De Groot et al. [16] were the first to test the hypothesis that application of IPC might be an effective, non-harmful and legal technique to increase performance [16]. They postulated that elevated levels of adenosine and K_{atp} channels related improvement of oxygen and substrate delivery after IPC might positively influence exercise performance. This study showed, for the first time, that ischaemic preconditioning improves maximal performance in humans. In fifteen healthy subjects, a significant increase in maximal oxygen consumption and maximal power output was established, which suggested that ischaemic preconditioning was capable of augmenting performance. Others have also shown the beneficial impact of IPC on maximal performance [101, 102]. More recently, Bailey et al. found that repeated IPC of the lower limbs prior to a 5 km trial preserved endothelial function of the brachial artery, whereas in the control (no IPC) running group endothelial function significantly decreased after exercise [103]. IPC may therefore operate through improvements in vascular function, which regulate blood flow to remove and transport lactate for uptake and utilization. It is indeed known that IPC improves muscular blood flow through intramuscular ATP-sensitive potassium channels and adenosine levels [64]. Shortly after this first study published by Bailey et al., the same group showed that IPC improves performance during a 5 km trial [104]. In addition, they also showed that overall increase in blood lactate during submaximal exercise was lower when exercise was preceded by IPC [104]. These results suggest that IPC may act as an effective tool to improve exercise

performance in elite athletes, although the exact underlying mechanisms of this effect remain unclear.

In conclusion, recent studies have revealed positive effects of IPC on exercise performance, but underlying mechanisms are poorly understood. It is for example, currently unknown whether IPC applied to the non-exercising muscles (i.e. remote IPC) is equally effective as IPC of the active muscles. Another issue relates to the timing of the IPC stimulus (first vs. second window), which is of special importance for an athlete when applying IPC. This thesis will address the question of whether acute and delayed IPC have potential benefit for exercise performance in chapter 6 and chapter 7.

3

General methods

Methods applied in this thesis:

Brachial artery endothelial ischaemia-reperfusion injury

Brachial artery endothelial function. To assess endothelial ischaemia-reperfusion injury (and the ability of IPC to attenuate this effect), we make use of assessment of the brachial artery endothelial function. For this purpose, brachial artery diameter and blood flow velocity were determined by using non-invasive echo-Doppler equipment. Subjects rested in a supine position with the right arm extended. Brachial endothelial (dys)function was measured with flow-mediated dilation (FMD). For the assessment of FMD, a rapid inflation/deflation pneumatic cuff (E10 rapid cuff inflator, Hokanson, USA) was placed around the right arm distal to the olecranon process to provide an ischaemic stimulus distal from the brachial artery to provoke vasodilation and subsequent shear stress. The right arm was extended to the side and positioned at heart level and was supported with towels to provide a stable position for recordings. A 10-MHz multifrequency linear array probe attached to a high-resolution ultrasound machine was used to perform imaging (T3000, Terason, Aloka, UK). The brachial artery was imaged in the distal third of the upper arm. Ultrasound parameters were set to optimize longitudinal B-mode images of the luminal-arterial wall interface. A continuous Doppler velocity assessment was obtained simultaneously and data were collected using the lowest possible insonation angle (always <60°), which did not vary during each study. After a resting period of at least 15 minutes, 1 minute of baseline recording of the arterial diameter and blood flow velocity was performed. Subsequently, the occlusion cuff was inflated to 220 mmHg for 5 minutes. Arterial diameter and blood flow velocity recordings were restarted at least 30 seconds before cuff deflation and continued for at least 3 minutes after deflation [34].

In order the minimise variance between measurements, assessments were performed by an experienced researcher (Dick Thijssen), who has performed >75 studies related to these measurements with the FMD technique.

Brachial artery ischaemia reperfusion (IR). IR-injury was either induced by a 5-minute ischaemic handgrip exercise stimulus followed by 15 minutes of reperfusion, or by a 20-minute ischaemic period caused by cuff occlusion of the upper arm. The ischaemic handgrip was performed at 30% of the maximal voluntary contraction. This protocol leads to a (near) maximal ischaemic stimulus and peak reactive hyperaemia [105]. This protocol of prolonged ischaemia, followed by reperfusion, is repeatedly found to result in an immediate decrease in brachial artery FMD [106-108]. The transient decrease in FMD is believed to reflect IR-induced endothelial dysfunction, a finding supported by studies that successfully prevented this decline in FMD by well-established pharmacological (i.e. statins and physical (i.e. ischaemic preconditioning [109, 110]) interventions that protect against IR-injury. This method is safe and frequently used. Previous studies from various groups found this protocol to result in an immediate decrease in brachial artery FMD [106-108], which is believed to reflect IR-induced endothelial dysfunction. As such, studies have typically adopted this model to examine IR-injury in conduit arteries. It is worthy of note that brachial artery FMD correlates well with coronary artery endothelial function in humans [111], and predicts the extent and severity of coronary atherosclerosis.

Ischaemic preconditioning

IPC was applied proximal on the upper arms, or proximal on the legs using a rapid inflation/deflation pneumatic cuff, with either 3 or 4 cycles of 5-minute of cuff inflation to 220 mmHg. This IPC-protocol is based on previous studies that have reported a protective effect of this stimulus in the heart or peripheral tissues [4, 110, 112-114], with putative mechanisms for protection related to the sympathetic nervous system and the production of reactive oxygen species [107, 109, 110].

Although IPC has been studied extensively, the most effective protocol is still a matter of debate [5, 115]. In our studies, a protocol with 3 of 4 cycles of 5 minutes occlusion was adopted, as this has been used by many others and has been proven to be effective in studying the brachial artery endothelium [48]. While other protocols with more cycles, or a longer duration of temporary ischaemia or reperfusion could be potentially more effective, this was not one of our aims, and we therefore adhered to well-known protocol which has been by others.

Brachial artery diameter and blood flow analysis

Analysis of the brachial artery diameter was performed using custom-designed edge-detection and wall-tracking software, which is independent of investigator bias [116]. Analyses were performed by the author of this thesis and supervised by an experienced researcher. Baseline data were calculated across the 1-minute preceding cuff inflation. Following cuff deflation, peak diameter was automatically detected according to an

algorithm as described in detail elsewhere [117]. Within-subject reproducibility of the FMD using this semi-automated software has a coefficient of variation of 6.7-10.5% [118]. Post-deflation shear rate data, derived from velocity and diameter measures, were used to calculate the shear rate area-under-the-curve (SR_{AUC}).

A recent study described that inadequate scaling for FMD would be present if the upper confidence limit of the regression slope of the relationship between logarithmically transformed base diameter and peak diameter is less than one [119]. In such an event, FMD% may not be an appropriate measure to estimate endothelial function. Therefore, we repeated the analysis for FMD in chapter 4 and chapter 5, using the allometric modelling solution [119].

Near-infrared spectroscopy

Near-infrared spectroscopy (NIRS) was used to measure local oxygenation in the lower limb tissue during exercise by assessing regional concentration changes in oxyhaemoglobin (O_2Hb) and deoxyhaemoglobin (HHb) using a continuous-wave near-infrared spectrophotometer (OXYMON, Artinis Medical Systems, the Netherlands). The NIRS optodes were positioned on the right thigh, twelve centimetres proximal to the fibular head, on the vastus lateralis muscle parallel to the long axis of the muscle. NIRS measurements were performed continuously. An inter-optode distance of 35 mm was used, resulting in a penetration depth of approximately 15-20 mm [120]. Briefly, this technique is based on the relative transparency of tissue for light in the near infrared region and on the changes in O_2-

dependent absorption of haemoglobin and myoglobin. As one cannot distinguish between myoglobin and haemoglobin, the combined effect of these two substances are studied.

The changes in absorption measured by NIRS are converted into estimates of concentration changes of O_2Hb and HHb, while no absolute values of flow can be obtained. The sum of O_2Hb and HHb reflects changes in blood volume, represented by the total haemoglobin signal (tHb). In addition, it is difficult to differentiate with NIRS between skin blood flow and muscle blood flow [121]. Therefore, we use NIRS mostly as an observational measurement in order to detect patters during acute exercise.

Incremental cycling test

Subjects completed an incremental exercise test on a stationary ergometer (Lode Excalibur, Groningen, The Netherlands) to determine VO_2max in $ml/O_2/kg/min$; maximum workload in wattage (W) and maximum heart rate (HR). Exercise was performed in a temperature controlled room (19°C). Oxygen uptake was measured continuously by analysing the expired ventilation with a continuous gas analyser (COSMED Quark CPET, Pavona di Albano, Italy), calibrated to known reference standards. Dependent on sex and expected fitness level, workload was increased every minute with 20 or 25 W. Workload increased every minute until volitional exhaustion, typically demonstrated by an inability to cycle at 60-70 revolutions per minutes. HR was measured continuously with a 12-lead ECG. BP was obtained every three minutes to monitor subjects' health (data not reported). Blood lactate level was measured with a finger stick ((Lactate Pro LT-1710, Arkray) before and within 2

minutes after the test was finished. All exercise tests were performed under supervision of a laboratory technician and a physician. All tests included in our study met at least 3 out of 4 most often used quality assessment points: ±10 beats/min of the predicted maximum HR (208-age*0.7), levelling off VO_2 (<150 mlO_2 during last minute), post-exercise RER >1.1, and post-exercise lactate of >8.0 mmol/L [122].

Incremental arm crank test

Incremental maximal test. An incremental exercise test was performed on an arm crank ergometer (Lode, Groningen, The Netherlands). The arm crank axis was positioned at shoulder height of the subject, while straps and wheel blockers were used to keep the wheelchair in position. The positioning of the wheelchair in relation to the arm crank was recorded and kept the same among the 3 testing days. The arm crank was configured to a 10 W protocol, starting at a power output of 10 W which increased by 10 W per minute until voluntary exhaustion. Subjects were instructed to keep cadence between 60 and 80 rpm, and exercise was terminated if subjects were unable to maintain the cadence >60 rpm. Exercise was performed in a temperature-controlled room (19°C). Oxygen uptake was measured continuously by analysing the expired ventilation with a continuous gas analyser (COSMED Quark CPET, Pavona di Albano, Italy), while HR was measured continuously with a Polar chest band (Polar RS800, Polar Electro Oy, Finland).

5 km running trial

In chapter 6, we used a 5 km running trial in order to assess performance after application of IPC or SHAM. A potential improved performance was measured as a decrease in total time needed for the 5 km trial. In order to keep environmental circumstances identical, the 5 km running trial was performed on an indoor treadmill, while keeping climate conditions similar between the tests. During the trial, subjects could chose to increase or decrease speed, but were blinded for speed, time and HR and were only informed about total distance covered. We chose to perform 3 familiarization sessions for each individual, since a minimum of at least one is described by Laursen et al. [123]

Ethics

All individuals, studied in chapter 4, 5, 6, 7 and 8, provided informed consent to participate and all these study were approved by the ethics committee of the Radboud University Nijmegen Medical Centre.

4

Ageing attenuates the protective effect of ischaemic preconditioning against endothelial ischaemia-reperfusion injury in humans

INGE VAN DEN MUNCKHOF, NIELS RIKSEN, JOOST P.H. SEEGER, TIM H. SCHREUDER, GEORGE F. BORM,

THIJS M.H. EIJSVOGELS, MARIA T.E. HOPMAN, GERARD A. RONGEN, DICK H.J. THIJSSEN

AMERICAN JOURNAL OF PHYSIOLOGY - HEART AND CIRCULATORY PHYSIOLOGY 2013 APRIL 19. 304: H1727–H1732

INTRODUCTION

Despite major advances in prevention and treatment, ischaemic cardiovascular disease remain the leading cause of morbidity and mortality worldwide. Current treatment for acute ischaemic events in heart, brain or kidney is timely reperfusion of the occluded artery. However, as explained earlier in chapter 2, reperfusion of the ischaemic tissue itself will also induce injury, commonly referred to as ischaemia–reperfusion (IR)-injury [39].

Endothelial cells are particularly sensitive to IR, with reperfusion leading to both structural and functional damage of the endothelium, as described in chapter 2. Subsequent endothelial injury and swelling due to IR can contribute to further ischaemia by impeding blood flow upon reperfusion, which has been termed the 'no-reflow phenomenon' and is present in the myocardium [49] as well as the brain [50]. Recent evidence support a central role for the nitric oxide (NO)-pathway in IR-induced endothelial dysfunction, as supplementation of tetrahydrobiopterin (BH-4) or L-arginine protects against IR-induced endothelial dysfunction in humans [124]. The no-reflow phenomenon is associated with worse clinical outcome and increased mortality in patients undergoing percutaneous coronary intervention (PCI) [49]. Therefore, novel therapies to limit IR-injury are urgently needed. Ischaemic preconditioning (IPC), i.e. repeated preceding short periods of ischaemia,[4] is the most powerful strategy to limit IR-injury *in vivo* [48, 110, 113]. A comparable protective effect can be established with remote IPC and pharmacological preconditioning [125].

Despite the potent effects of (remote) IPC and pharmacological preconditioning in preclinical research, translation of these strategies to the clinical arena is often disappointing [126].

This may relate to the inclusion of young and healthy animals in preclinical studies, whereas application of IPC in the clinical setting typically relates to older patients with co-morbidities. Evaluation of the impact of ageing on cardioprotection is therefore prioritised by a recent position paper [126]. Animal studies suggest that the protective effects of IPC are attenuated or even abolished in ageing hearts [78] or brain [127], although data is conflicting [81]. Also in isolated atrial trabeculae from elderly patients, IPC has no beneficial effect on the functional recovery after simulated IR [83]. To date, no previous study examined the impact of ageing on the protective effects of IPC in humans *in vivo*.

Therefore, in this study, the impact of advanced age on the protective effect of IPC on endothelial IR-injury in humans *in vivo* was examined. In line with previous animal data, as described in chapter 2, it was hypothesised that advanced age is associated with an abolished protective effect of IPC. Since ischaemia and reperfusion cannot be studied safely in heart or brain, brachial artery flow-mediated dilation (FMD; i.e. a measure of peripheral artery endothelial function) was used before and after ischaemia (20-min) and reperfusion (15-min) as described in chapter 3.

METHODS

Participants

Fifteen young (20-25 years) and 15 older healthy men (68-79 years) were included in this study. All subjects were free of any cardiovascular disease, diabetes mellitus, hypertension (diastolic ≥90 and/or systolic blood pressure (BP) ≥140 mmHg) and hypercholesterolaemia (total cholesterol ≥6.5 mmol/L). Moreover, (elite) athletes (performing >10 h/week),

smokers, obese subjects (BMI ≥30 kg/m^2) and those who were taking medication that interferes with our primary outcome parameters were also excluded. The study was registered at ClinicalTrials.gov (NCT01606410).

Experimental Design

Subjects attended to our laboratory twice (separated by at least 7 days, with a maximum of 28 days). Brachial artery endothelial function was measured with flow-mediated dilation (FMD) in the right arm. Brachial artery FMD was measured before and after 20 minutes of arm ischaemia and 15 minutes of reperfusion as described in more detail in chapter 3. FMD was assessed before and after IR-injury is performed, with or without a preceding ischaemic preconditioning stimulus [114]. IPC was performed as 3 cycles of 5-minute occlusion of the right upper arm followed by 5-minutes of deflation.

Measurements

Before each experiment, participants refrained from food ingestion ≥6 hours, caffeine and products with high levels of vitamin C intake ≥18 hours, and from strenuous physical activity ≥24 hours. Subjects were tested at the same time of day to prevent diurnal variation in FMD response. All measurements were performed in a temperature-controlled room (22.5°C) and using recent guidelines of FMD [34], as described in more detail in chapter 3.

IR-injury. The rapid inflation/deflation pneumatic cuff was positioned proximally around the upper arm to provide an occlusion for 20 minutes, so that the brachial artery was within the

ischaemic zone and was exposed to IR. The cuff was inflated for 20 minutes to 220 mmHg, which was followed by 15 minutes of reperfusion.

Brachial artery diameter and blood flow analysis

Analysis of the brachial artery diameter was performed using custom-designed edge-detection and wall-tracking software as described in chapter 3.

Statistical analysis

All data were analysed using the Statistical Package for the Social Sciences (SPSS, version 16). Data are presented as mean±SD unless stated otherwise. Baseline parameters were compared by paired *t*-tests, whilst baseline characteristics between groups were compared using an unpaired Student's *t*-test. In order to evaluate the effect of IPC on the impact of IR on FMD, the differences between the FMD before and after IR were calculated. These differences were then analysed with a linear mixed model analysis with random factor subject and fixed factors age group, IPC and the interaction age group*IPC. In an additional analysis, baseline characteristics were added as covariates (i.e. BMI, BP, cholesterol, and glucose). In a similar way, we also evaluated the impact of preconditioning within the age groups, using a mixed model analysis with random factor subject and fixed factor IPC.

To assess potential confounding by shear rate, which differed between our samples of young and elderly volunteers, we also examined the relation between the SR_{AUC} and the change in

FMD after IR using a Pearson's correlation coefficient. The level of statistical significance was set at 0.05.

RESULTS

Baseline characteristics are presented in Table 1. Compared to young men, older men demonstrated a higher BMI, systolic BP, total cholesterol, and glucose levels, whilst no differences between young and older men were found for body mass, height, diastolic BP, and HDL-cholesterol. All values were within the normal range.

Table 1: Subject characteristics in healthy young (n=15) and older men (n=15)(mean±SD).

Variable	Young men	Older men	P-value
Age(yrs)	22±1	72±4	<0.001
Body weight(kg)	76±10	81±9	0.20
Heigth(cm)	180±7	177±6	0.29
BMI(kg/m2)	23.4±2.5	25.5±2.5	0.03
Systolic blood pressure(mmHg)	119±8	128±9	0.007
Diastolic blood pressure(mmHg)	77±7	76±5	0.54
Cholesterol(mmol/L)	4.4±0.9	5.2±0.8	0.018
Triglycerides(mmol/L)	0.9±0.5	1.2±0.3	0.052
LDL-cholesterol(mmol/L)	2.7±0.7	3.4±0.6	0.005
HDL-cholesterol(mmol/L)	1.3±0.3	1.2±0.3	0.48
Glucose(mmol/L)	4.5±0.3	4.9±0.4	0.006

BMI; body mass index, LDL; low-density lipoprotein, HDL; high-density lipoprotein

IR and IPC: young men

There were no significant differences in baseline brachial artery diameter, brachial artery FMD (absolute (FMDmm) or relative change (FMD%) from baseline), time-to-peak or SR_{AUC} between measurement days (all P>0.05). In young men, FMD% decreased significantly after IR (Figure 1). When IR was preceded by IPC, this decrease in FMD was abolished (change from baseline, IPC vs. control: P=0.01). Also when FMD was presented as the absolute

change in mm, we found that the decrease in FMDmm after IR was abolished when preceded by IPC (change from baseline, IPC vs. control: P=0.03, Table 2).

We found no impact of IR (neither with or without IPC) on the time-to-peak diameter, whilst IR was associated with an increase in baseline diameter and decrease in SR_{AUC} (Table 2). The change in SR_{AUC} after IR-injury was similar between the control- and IPC-condition, whilst a significant interaction effect was found for the change in diameter (change from baseline, IPC vs. control: P=0.04). We found no significant relation between SR_{AUC} and the change in FMD after IR (r=0.16, P=0.57).

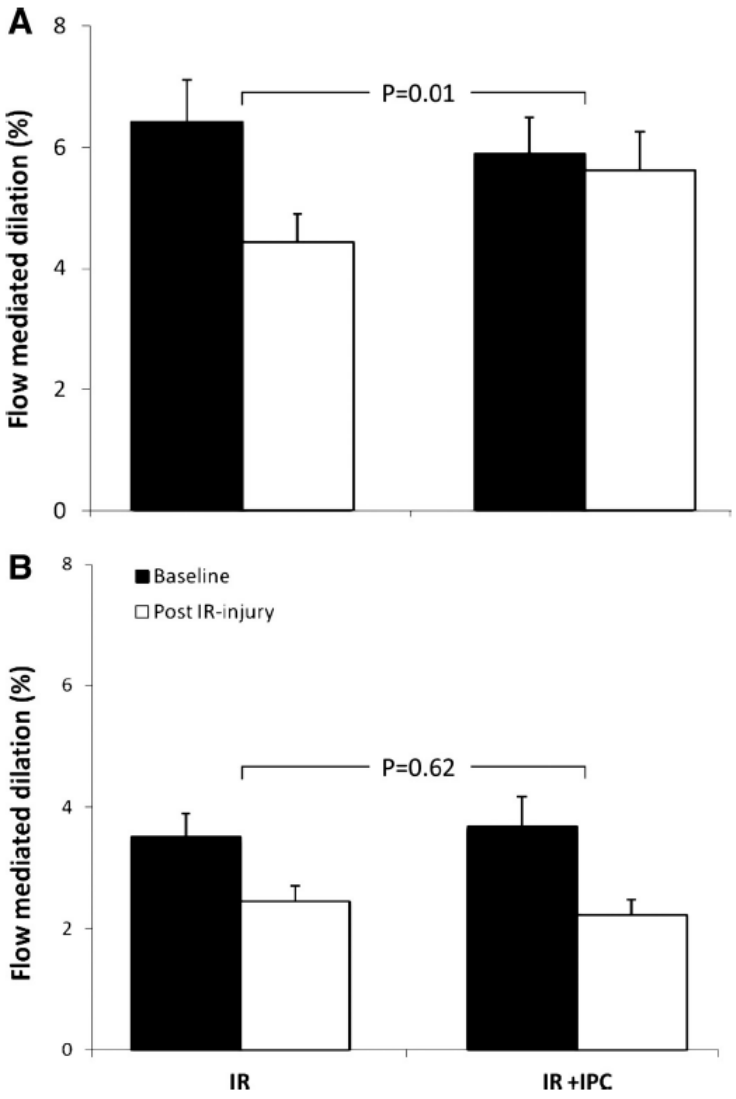

FIGURE 1. Brachial artery flow-mediated dilation before (baseline, black bars) and after ischaemia-reperfusion (Post IR, white bars) under control conditions (IR) and when preceded by ischaemic preconditioning (IR+IPC) in healthy young (A, n=15) and older men (B, n=15). A mixed model analysis revealed a statistically significant difference between the age groups (P=0.04). Error bars represent SE.

IR and IPC: older men

There were no significant differences in baseline brachial artery diameter, brachial artery FMD (in mm or in %), time-to-peak or SR_{AUC} between measurement days (all P>0.05). At baseline, older men demonstrated a significantly lower FMD% and FMDmm compared to young men (both P=0.001). Older men also demonstrated a significantly larger brachial artery resting diameter (P=0.02), and a longer time-to-peak (P=0.04) and a lower SR_{AUC} (P=0.003).

IR resulted in a significant decrease in brachial artery FMD in older men (Figure 1). When IR was preceded by IPC, however, a similar decrease in FMD was observed (IR*IPC interaction: P=0.62). Also when FMD was presented as the absolute change in mm, we found a comparable decrease in FMDmm after IR between both conditions in older men (change from baseline, IPC vs. control: P=0.52, Table 2). The primary endpoint of the study was the difference in IPC-induced protection between the young and elderly subjects. IPC-induced protection appeared to be significantly reduced in the elderly patients (P=0.04). After correction for the differences in baseline characteristics (BMI, BP, cholesterol, and glucose), the size of the difference in protection did not change, but the P-value increased to 0.07.

In addition, IPC did not alter the time-to-peak diameter, whilst IR was associated with an increase in resting diameter and decrease in SR_{AUC} in older men (Table 2). The changes in resting diameter and SR_{AUC} were similar between both conditions (Table 2). We found no significant relation between baseline SR_{AUC} and the change in FMD after IR in the older subjects or in the pooled data set (r=0.09 and P=0.75, r=0.24 and P=0.21, respectively), excluding relevant bias by differences in shear rate (SR_{AUC}).

Table 2: Brachial artery characteristics before and after ischaemia-reperfusion (IR) when preceded by a control intervention or ischaemic preconditioning (IPC) in healthy young and older men (n=15). Data is presented as mean±SD.

	Control		IPC		P-values	
Young men	*Baseline*	*IR*	*Baseline*	*IR*	*IPC vs control*[1]	*Older vs young*[2]
FDM (%)	6.4±2.7	4.4±1.8	5.9±2.3	5.6±2.5	0.01	
FMD (mm)	0.25±0.09	0.19±0.07	0.23±0.08	0.22±0.08	0.03	
Diameter (mm)	3.9±0. 5	4.3±0.6	4.0±0.6	4.2±0.7	0.04	
Time-to-peak diameter (s)	43±10	38±14	41±10	34±9	0.77	
SR_{AUC} (s, 10^3)	29.5±9.4	22.8±8.0	28.6±5.3	23.2±8.9	0.59	
Older men						
FDM (%)	3.5±1.7	2.5±1.0	3.7±2.1	2.2±1.1	0.62	0.04
FMD (mm)	0.15±0.05	0.11±0.04	0.16±0.08	0.10±0.05	0.52	0.06
Diameter (mm)	4.4±0.5	4.8±0.5	4.4±0.5	4.8±0.7	0.83	0.36
Time to peak diameter (s)	55±19	51±21	58±26	54±30	0.97	0.87
SR_{AUC} (s, 10^3)	20.5±5.3	14.9±7.4	20.7±8.3	16.0±9.2	0.67	0.85

[1]P-value for difference in change from baseline (IPC *vs* control), [2]P-value for difference in effect of IPC on change from baseline (young *vs* old)

DISCUSSION

The purpose of this study was to examine the impact of advanced age on the protective effect of IPC on endothelial IR-injury in humans *in vivo*. First, it was found that IR impairs brachial artery endothelial function, which is in agreement with various other studies as described in more detail in chapter 2. Second, IR-induced endothelial dysfunction in young men can be prevented when preceded by 3 cycles of 5-minute ischaemia of the upper-arm prior to the ischaemic event. More importantly, it was demonstrated for the first time in humans that older age is associated with an abolished effect of IPC to protect against IR-induced endothelial dysfunction in the brachial artery.

IR-induced endothelial dysfunction is of particular importance as this contributes to impeded blood flow upon reperfusion, commonly referred to as the 'no-reflow phenomenon [49]. The presence of the 'no-reflow phenomenon' in the coronary arteries is frequently reported in patients with an acute myocardial infarction. In this study, we adopted a model of IR-injury using prolonged ischaemia of the forearm. As demonstrated in various, independent laboratories [110, 128], the model of forearm IR-stress applied in the current study results in a significant decrease in brachial artery endothelial function in young as well as in older men. This indicates that our model is valid to detect IR-induced endothelial dysfunction. Moreover, in this similar model, IPC prevents against endothelial dysfunction after IR in healthy, young to middle-aged volunteers [110, 113]. In this study, we confirm these observations in a group of young, healthy men.

The main finding of our study is that the protective effect of IPC on brachial artery endothelial IR-injury was abolished in older men. This observation is in agreement with some previous suggestions in animals [78, 129] and a human *ex vivo* study on isolated atrial

trabeculae [83]. However, this latter study failed to correct for potential confounders, such as medication and cardiovascular risk factors. Although some report an age-related decline in efficacy of IPC [130, 131], these studies are limited by their use of endpoints which are not valid to assess effects of preconditioning (i.e. ST-segment elevation) [125] and the inclusion of subjects with cardiovascular disease and risk factors [130, 131]. In our study we included only healthy, older subjects without cardiovascular risk factors. Even statistically correcting for differences between young and older men in BMI, BP, cholesterol, and glucose did not change our major outcomes. More evidence for successfully including a representative healthy older population relates to the lower brachial artery FMD, but also a longer time-to-peak diameter and larger baseline diameter, compared to young subjects. These findings confirm previous observations in healthy older men [132]. Taken together, we provide the first evidence in a group of healthy older humans that the effect of IPC to prevent IR-induced endothelial dysfunction in the forearm is abolished.

Although it was not the purpose, our '*proof of concept*'-study raises the question about the potential underlying mechanism. First, the attenuated effect of IPC may relate to an elevated threshold for triggering IPC protection rather than a complete loss of IPC to be protective. Longer ischaemia and/or increased episodes of ischaemia/reperfusion in the IPC-protocol may be necessary in the older population for IPC to be protective, such as previously described for diabetes mellitus type 2 [133]. It is demonstrated, primarily based on evidence derived from cardiac tissue, that IPC cardioprotection is regulated via different pathways, such as the nitric oxide(NO)-pathway, reperfusion injury salvage kinase(RISK)-pathway and AMP-activated protein kinase. These pathways converge on mitochondria and prevent

opening of the mitochondrial permeability transition pore (mPTP) upon reperfusion and subsequent cell death [134]. A recent paper comprehensively discusses the impact of ageing on the signalling cascades that seem to contribute to the loss of cardioprotection by IPC in the aged heart [25]. Although the mechanisms of IPC-related protection may substantially differ between cardiac and vascular sites, ageing seems to result in a decreased protein expression and blunted responses of signalling molecules, such as heat shock protein 70 content, extracellular ligand (e.g. IGF-1 and IL-6), decreased levels of connexin 43 or lower expression level of sarcolemmal receptors (e.g. IGF-1 receptor, bradykinin receptors) [25]. Also, blunted activation of protein kinases (e.g. ERK1/2, Akt, GSK3beta, or p38) and age-related mitochondrial changes have been hypothesised to contribute to the age-related loss of cardioprotection via IPC [135]. Generally, these suggestions are typically made based on: 1. the importance of these signalling cascades for cardioprotection by IPC and 2. the impact of ageing on these cascades. Taken together, future studies are necessary to elucidate the underlying pathways and the potential interacting effects between pathways in humans, but also distinguish between cardiac and vascular tissue as the IPC-driven effects may differ between vessel sites.

Limitations. Strengths of this study include the controlled design, inclusion of homogenous healthy groups, within-subject comparison regarding the impact of IPC and the use of observer-independent analysis. The model that has been used has several limitations. First, the impact of IR-injury and IPC in the forearm vasculature was studied rather than directly in the cardiac tissue. The brachial artery was chosen because of earlier described (chapter 3) similarities in function with the coronary arteries. Nonetheless, one should take caution when extrapolating our findings to other vascular beds, especially since due to ethical reasons, we had to choose a relative short period of ischemia in order to attain the IR-injury.

Secondly, we did not use an endothelium independent vasodilator, so we cannot exclude that the detrimental effect of IR on FMD reflects a reduced sensitivity of the vascular smooth muscle cells to nitric oxide. Thirdly, the diameter of the brachial artery did not return to baseline after IR. Based on the inverse relation between baseline diameter and FMD [34], the increase in diameter may relate to the decrease in FMD. However, the magnitude of increase in diameter was consistent across young and older subjects and additional analyses corrected for these changes. Therefore, changes in diameter unlikely explain our principle findings. Another limitation is the lower SR_{AUC} in the older cohort compared to younger men. However, young and older men showed no correlation between SR_{AUC} and the change in FMD after IR. Moreover, the changes in SR_{AUC} after IR (with or without IPC), were similar in both groups. Therefore, it is unlikely that a difference in SR_{AUC} *per se* explains our findings. Finally, cardiovascular risk factors (e.g. hyperlipidaemia, diabetes, hypertension) are likely to interfere with the endogenous cardioprotective effects of IPC in humans [45]. In our study, BMI, BP, cholesterol and glucose levels were all within the normal range and none used medication known to interfere with these variables. The somewhat higher values in the older population may relate to physiological human ageing, which is associated with small, but gradual changes in these parameters. Finally, when statistically correcting for these parameters, we found a similar effect size of IPC in young and older subjects. Therefore, the conclusions drawn based on our observations in this study are valid for a typical, healthy cohort of older men.

Clinical Relevance. Our observation of an abolished effect of IPC raises questions about the impact of ageing on ischaemic post- or peri-conditioning; i.e. alternative strategies to prevent IR-injury. It is believed that the various methods of ischaemic conditioning share a final common pathway that prevents opening of the mPTP [136].

Although speculative, advanced age may therefore also impact upon the efficacy of ischaemic peri- or postconditioning as described in chapter 2. However, this requires further research in humans and is speculative at this stage. In contrast to *traditional* IPC, the application of *remote* IPC may be clinically more relevant as the latter procedure can be applied to a remote vascular bed. Whether ageing also affects remote IPC is unknown and represents a logical extension from our current findings.

The results from this study demonstrates that brachial artery endothelial ischaemia-reperfusion injury can be prevented by IPC in young healthy men but not in elderly subjects. This finding may have important implications for studies that examine the efficacy of IPC as an intervention to prevent endothelial IR-injury and limit cardiac damage in a clinical setting. IPC and alternative interventions based on IPC are currently being applied in a large number of randomised controlled trials to prevent cardiac IR injury in patients. Compared to preclinical studies, application of (remote) IPC in the clinical setting is often disappointing [126]. This may relate to the inclusion of healthy, young animals in preclinical studies, whilst clinical trials mostly involve elderly (discussed in more detail in the synthesis, chapter 9). Our observations, therefore, highlight the importance of first examining the effects of novel cardioprotective strategies in older animals before translation is made to humans. Also, the impact of age should be considered when examining the effect of interventions that prevent or attenuate endothelial dysfunction after IR by including an age-matched control group (preferably a one-by-one matching) and/or including age as a potential co-factor in statistical analyses.

The results from this study indicate that IR-injury is more pronounced in the elderly population, and that IPC does not protect against IR-injury in the elderly. As discussed in

more detail in the synthesis (chapter 9), most work in IPC has been performed in relatively young and healthy animals. Therefore, more research in the diseased populations is necessary to truly understand the impact of IPC in a relevant population. As the protocol used in this chapter has proven to be effective in measuring the consequences of IR-injury and IPC *in vivo* (in a clinically relevant population), this study set-up was used in a follow-up study using patients with heart failure and presented in the following chapter.

5

Heart failure is associated with exaggerated endothelial ischaemia-reperfusion injury and attenuated effect of ischaemic preconditioning

JOOST P.H. SEEGER, NATHALIE M.M. BENDA, NIELS P. RIKSEN, ARIE P.J. VAN DIJK, LOUISE BELLERSEN, MARIA T.E. HOPMAN, N. TIMOTHY CABLE, DICK H.J. THIJSSEN

EUROPEAN JOURNAL OF PREVENTIVE CARDIOLOGY 2014 NOV 11. PII: 2047487314558377.

INTRODUCTION

Heart failure (HF) is a major cause of death in developed countries and represents a growing public health problem, partly due to the ageing population, and is responsible for an increasing proportion of hospital admissions [137]. A potential explanation for the poor prognosis of HF patients may relate to an exaggerated ischaemia-reperfusion (IR)-injury in HF as demonstrated in rats [95]. Such an increased vulnerability to IR-injury is clinically relevant, as this may contribute to worsening of the clinical outcome after a cardiovascular event. DeVan and colleagues demonstrated that traditional cardiovascular risk factors, such as advanced age, are associated with a greater magnitude and delayed recovery from endothelial IR-injury in humans [47]. Also experimental studies suggest that the presence of cardiovascular risk factors or disease is associated with exaggerated IR-injury [25, 95, 138], although some studies suggest otherwise [139, 140], including our previous work as presented in chapter 4. Accordingly, we examined the hypothesis that HF patients demonstrate an increased endothelial IR-injury compared to healthy peers *in vivo*.

Originally described in animals, ischaemic preconditioning (IPC; intermittent episodes of nonlethal ischaemia) is a powerful strategy to limit or even prevent IR-injury [4]. As described in chapter 3, previous human *in vivo* studies found that IPC effectively prevents endothelial IR-injury. Despite successful pre-clinical studies, clinical trials implementing IPC have demonstrated somewhat disappointing results [9], which was partly established in chapter 4, as IPC was only effective in young healthy individuals and not in the elderly.

Another potential explanation for the lack of clinical effects relates to the interaction between the efficacy of IPC and the presence of cardiovascular risk factors or disease. Indeed, some preclinical studies provide evidence that HF is associated with an attenuated efficacy of IPC to prevent injury [96, 141, 142]. Since no previous study in humans has explored this hypothesis, the second aim of the study is to examine the efficacy of IPC to prevent or attenuate endothelial IR-injury in patients with HF. To study these questions, we examined brachial artery flow-mediated dilation (FMD) before and after ischaemia (induced by 5-minute ischaemic handgrip exercise) and reperfusion (15-min) and used the reduction in FMD as a well-validated marker of endothelial injury.

METHODS

Participants

15 subjects with HF (67±10 years, NYHA-class II/III, ejection fraction ≤ 45%) and 15 healthy, age- and sex-matched older subjects (65±9 years) were included in this study. HF patients were recruited from the Department of Cardiology of the Radboud University Nijmegen Medical Centre. Exclusion criteria were set for pre-menopausal women (or women with hormone replacement therapy), subjects with diabetes mellitus type 1 or 2, hypertension (systolic ≥140 or diastolic ≥90 mmHg), chronic obstructive pulmonary disease and severe hepatic or renal insufficiency. Healthy control subjects were free of any chronic disease and did not use any type of medication known to interfere with the cardiovascular system. HF patients were categorised as New York Heart Association (NYHA) class II/III. Patients were on

stable optimised pharmacological therapy for ≥3 months. All subjects signed an informed consent and study procedures were approved by the local ethics committee and performed according to the Declaration of Helsinki (2000).

Experimental Design

Subjects attended our laboratory twice (separated by at least 7 days). Brachial artery endothelial function was measured with FMD in the right arm. Brachial artery FMD was measured before and after IR-injury. IR-injury was induced by a 5-minute ischaemic handgrip exercise stimulus followed by 15 minutes of reperfusion. Local ischaemia during handgrip exercise was induced with upper arm cuff inflation to 220 mmHg. The assessment of FMD before and after IR-injury was performed with or without the preceding ischaemic preconditioning stimulus (IPC-intervention). IPC consisted of 3 cycles of 5-minute upper arm cuff inflation to 220 mmHg, with 5 minutes reperfusion time after each occlusion.

Measurements

Body anthropometric data. Body mass (Seca 888 scale, Hamburg, Germany) and height were measured to calculate body mass index (in kg/m^2). A four-point skin fold thickness measurement (biceps, triceps, sub-scapular, supra-iliac) was obtained in order to calculate the lean body mass. Waist circumference was measured midway between the lower rib margin and iliac crest. Hip circumference was measured at the level of widest circumference over greater trochanters. Waist to-hip ratio was calculated as waist circumference divided by hip circumference. Resting HR and BP were measured twice in supine position, using a

manual sphygmomanometer after 5-min of rest. Finally, with a finger stitch a small amount of blood was collected in order to assess glucose and blood cholesterol levels.

Flow mediated dilation (FMD). All measurements were performed in a temperature-controlled room (22.5°C) and using recent guidelines of FMD [34], as described in more detail in chapter 3.

Brachial artery diameter and blood flow analysis

Analysis of the brachial artery diameter was performed using custom-designed edge-detection and wall-tracking software, as described in chapter 3.

Statistical analysis

All data were analysed using the Statistical Package for the Social Sciences (SPSS, version 20). Data are presented as mean±SD unless stated otherwise. Baseline characteristics between groups were compared using an unpaired Student's *t*-test. In order to evaluate the impact of IR on endothelial function (measured as FMD) between groups (primary aim), and whether IPC can (partially) prevent endothelial IR (secondary aim), we employed a linear mixed model analysis. For aim 1, FMD was analysed with random factor subject and 2 fixed factors: time (pre *versus* post) and group (HF *versus* control). The interaction-effect IR*group was used to examine our primary aim (i.e. examine whether IR injury was different between groups). To examine whether IPC can prevent the decline in FMD after IR in both groups (i.e. secondary aim), we repeated this analysis with the addition of 1 fixed factor: intervention (IPC *versus* control) and explored the interaction IR*group*IPC. When a significant interaction-effect was found, we adopted post-hoc analysis to identify differences. As

described in chapter 3, analysis for FMD were repeated using the allometric modelling solution [119]. The level of statistical significance was set at 0.05.

RESULTS

Baseline characteristics are presented in Table 1 and 2. Compared to controls, HF patients demonstrated a lower total cholesterol and a higher waist-to-hip ratio, whilst no differences between HF patients and controls were found for age, body mass, height, systolic BP, diastolic BP, fat percentage and fasting glucose.

Table 1: Subject characteristics in heart failure (n=15) and healthy, age- and sex-matched controls (n=15). Data is presented as mean±SD.

Variable	Heart failure	Controls	P-value
Age (yrs)	65±9	67±10	0.56
Sex (male:female)	13:2	11:4	
Body weight (kg)	92±18	82±15	0.13
Height (cm)	178±8	176±8	0.67
BMI (kg/m2)	29.2±6.4	26.2±3.5	0.12
Systolic blood pressure (mmHg)	114±14	121±7	0.08
Diastolic blood pressure (mmHg)	71±9	77±5	0.05
Waist-to-hip ratio	1.02±0.09	0.92±0.12	0.02
Fat percentage (%)	33.2±7.4	29.8±6.2	0.19
Cholesterol(mmol/L)	4.9±1.1	6.1±1.2	0.01
Glucose(mmol/L)	4.9±1.4	4.9±0.4	0.98

BMI; body mass index

Table 2: Heart failure patient characteristics (n=15).

	Heart failure
Ischaemic / non-ischaemic	12 / 3
Medication use	
ACE-inhibitors	8 (53 %)
Angiotensin II receptor antagonists	6 (40%)
Diuretics	10 (67%)
β-blockers	15 (100%)
Coumarin derivatives	7 (47%)
Statins	8 (53%)

Endothelial IR-injury

No significant differences between HF patients and healthy controls were found for baseline brachial artery diameter, brachial artery FMD (absolute (FMDmm)) or relative change from baseline (FMD%), time to peak diameter, or SR_{AUC} (all P>0.05, Table 3). IR resulted in a significant increase in resting diameter (Table 3). To control for the potential impact of the increase in diameter on FMD%, baseline diameter was included as a co-factor in the 2-factor statistical analysis (IR and group). This analysis revealed a significant interaction-effect (IR*group: *P*=0.01, Table 3). Subsequent analysis revealed that IR resulted in a significant decrease in brachial artery FMD in HF and controls (*P*=0.002 and 0.02, respectively, Figure 1). However, the magnitude of decrease in FMD after IR was larger in HF patients compared to controls (Table 3). Also when FMD was presented as the absolute change (in mm), the decrease in FMDmm was significantly larger after IR in HF patients than in controls (Table 3). When repeating the analysis for FMD using the allometric scaling approach, including

correction for the change in diameter, our initial observations of a larger decline in FMD in HF compared to controls after IR were confirmed (Table 3).

Table 3: Brachial artery characteristics before and after ischaemia-reperfusion (IR) when preceded by a control intervention or ischaemic preconditioning (IPC) in heart failure and controls (n=15).

	Heart failure		Controls		Two-way and three-way ANOVA					
	Baseline	*IR*	*Baseline*	*IR*	*IR*	*Group*	*IR*Group*	*IPC*	*IR*IPC*	*Group*IR*IPC*
FMD (%)	6.2±3.6	3.3±1.8*	4.9±2.1	4.1±2.0*	<0.001	0.79	0.01			
FMD (%), allometrically scaled	6.1±2.4	3.3±2.4*	4.8±2.4	4.0±2.3*	<0.001	0.73	0.01			
Diameter baseline (cm)	0.44±0.07	0.46±0.08	0.43±0.06	0.44±0.06	0.006	0.47	0.95			
Absolute FMD (mm)	0.26±0.14	0.15±0.09*	0.21±0.10	0.18±0.10*	<0.001	0.77	0.006			
Time-to-peak diameter (s)	51±19	58±29	45±13	48±15	0.299	0.13	0.76			
SR_{AUC} (s, 10^3)	22.2±8.1	23.0±8.5	22.7±8.9	22.7±1.0	0.74	0.98	0.74			
IPC										
FMD (%)	5.8±2.7	3.7±2.4*	4.2±1.5	4.0±2.0	<0.001	0.52	0.002	0.53	0.20	0.85
FMD (%), allometrically scaled	5.6±2.2	3.8±2.2*	4.0±2.2	4.1±2.2	<0.001	0.47	0.001	0.50	0.14	0.89
Diameter baseline (cm)	0.43±0.07	0.46±0.09*	0.42±0.05	0.46±0.06*	<0.001	0.65	0.86	0.74	0.15	0.81
Absolute FMD (mm)	0.24±0.12	0.16±0.10*	0.18±0.07	0.18±0.08	<0.001	0.54	0.001	0.40	0.22	0.93
Time to peak diameter (s)	55±22	57±26	66±20	54±26	0.93	0.72	0.22	0.04	0.17	0.42
SR_{AUC} (s, 10^3)	23.5±1.3	23.9±9.5	21.0±8.6	19.2±7.0	0.92	0.51	0.55	0.54	0.66	0.79

Data is presented as mean±SD. $SR_{AUC,}$ area under the shear rate curve; FMD, flow-mediated dilation.

*Post hoc significantly different from baseline

IPC and endothelial IR-injury

No differences were found in HF patients or in controls between both testing days for baseline brachial artery diameter, brachial artery FMD (absolute (FMDmm)) or relative change from baseline (FMD%), time to peak diameter, or SR_{AUC} (all P>0.05, Table 3). In line with the analysis above, it was statistically controlled for the potential impact of baseline diameter on the FMD%, by including these parameters as a co-factor in the statistical analysis. The 3-way ANOVA confirmed the presence of a decrease in brachial artery FMD after IR (*P*<0.001), which is significantly larger in HF patients compared to controls (*P*=0.01, Figure 1). Moreover, IPC did not significantly alter the decrease in FMD (Time*Group*IPC-interaction: *P*=0.85). Also when FMD was presented as the absolute change (in mm) or using allometric scaling, we found that the decrease in FMD after IR was not changed by IPC (Table 3). Post-hoc analysis revealed that IPC prevented the decline in FMD after IR in healthy controls, whilst IPC showed no effect in heart failure patients (Table 3).

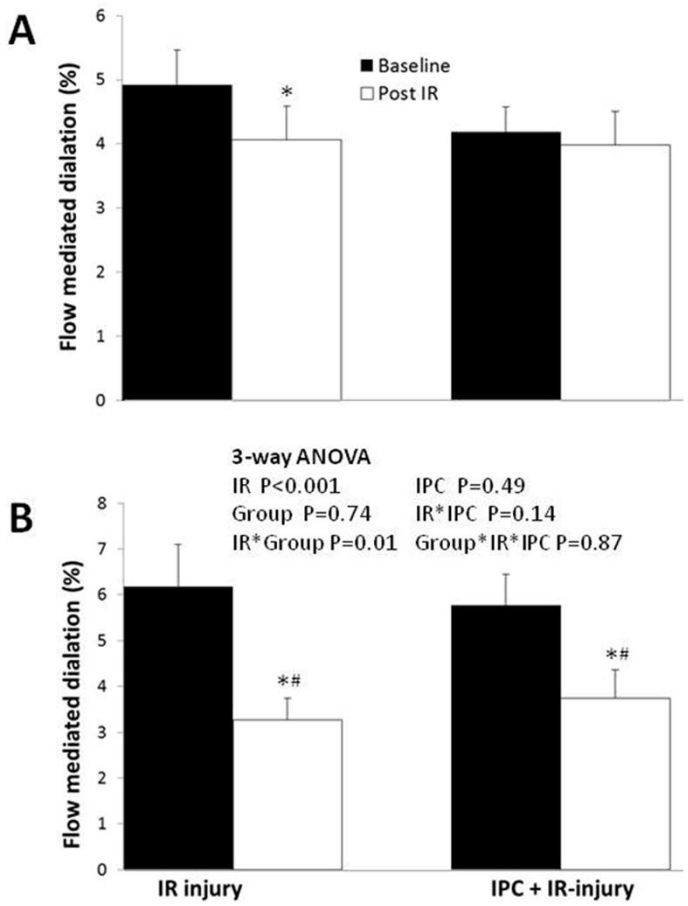

FIGURE 1. Brachial artery flow-mediated dilation before (baseline, black bars) and after endothelial ischaemia-reperfusion (Post IR, white bars) ischaemia-reperfusion (IR) injury and when preceded by ischaemic preconditioning (IPC + IR-injury) in healthy controls (A, n=15) and heart failure patients (B, n=15). A mixed model analysis revealed a statistically larger decline in FMD after IR in heart failure compared to controls (P=0.001), whilst IPC did not change the decline in FMD after IR in both groups (P=0.87). Error bars represent SE. *Post hoc significantly different from baseline at P<0.05. #Post hoc significantly larger change in FMD than in controls at P<0.05.

DISCUSSION

This study provides a number of novel findings. First, a significantly larger decline in brachial artery FMD after IR-injury in HF patients (~46%) compared with their healthy peers (~16%) was found. This indicates that in agreement with our hypothesis, HF patients demonstrate an exaggerated endothelial injury after IR compared to their healthy controls. Second, we found that IR-induced endothelial dysfunction cannot be prevented by ischaemic preconditioning in HF patients, a finding that is in line with the observation from chapter 4 in

which IR-induces endothelial dysfunction could not be prevented by ischaemic preconditioning with older age. Accordingly, the magnitude of decline in FMD after IR-injury when preceded with IPC, remains larger in HF patients than in their healthy age- and sex-matched controls. Therefore, our study revealed that HF is associated with an exaggerated decline in endothelial function after IR-injury, whilst IPC failed to protect against this decrease.

Endothelial IR-injury

In agreement with previous work, including chapter 4, it was found that IR-injury induces a transient, conduit artery endothelial dysfunction [109, 110]. A novel observation is that HF patients demonstrate an exaggerated decline in FMD after IR-injury compared with their healthy peers. To date, the impact of HF on IR-injury has only been examined in animal studies, which provided conflicting results ranging from an increased [139, 140] to a decreased tolerance [25, 95, 138] against prolonged ischaemia. Differences in the ischaemia-stimulus *within* and *between* studies may contribute to these conflicting results. In this study, both groups received the same ischaemic stimulus. Moreover, inter-species differences or the experimental procedures to induce HF may also have contributed to the conflicting results from animal studies. Nonetheless, our study provides support that, in humans, HF is associated with an exaggerated decline in endothelial function after endothelial IR-injury.

Although speculative, the larger decline in FMD in HF patients than controls after IR may relate to differences in antioxidative capacity between groups. Endothelial injury after IR is caused, at least partially, by excessive production of oxidative stress [143]. Whilst healthy individuals have a well-controlled balance between the production of reactive oxygen species (ROS) and antioxidative enzymes, patients with HF demonstrate less antioxidative capacity, resulting in increased oxidative stress [144, 145]. Nonetheless, our novel observations in HF patients warrants future research to better understand the potential underlying mechanisms that contribute to the exaggerated IR-injury in HF.

IPC and endothelial IR-injury

In the previous chapter, it was demonstrated that the well-established protective effects of IPC are abolished in healthy older men compared to younger control patients. In addition, the present study provides evidence that the protective effect of IPC to attenuate endothelial IR-injury is also abolished in HF patients. This latter observation is in line with data from animals, supporting an emerging hypothesis of a reduced efficacy of IPC associated with cardiovascular disease or risk factors [9, 45, 141]. For example, a recent animal study revealed the inability of preconditioning to protect the old diabetic heart against an ischaemic insult [146]. In line with these findings, preclinical studies in patients with HF demonstrated an impaired effect of preconditioning to prevent ischaemia-induced tissue damage [96, 141, 142].

In this chapter, IPC was effective in healthy age-matched controls, which is in contrast to chapter 4 in which it was established that IPC was not effective in healthy older humans.

Some important differences between both studies must be highlighted. First, subjects included in chapter 4 were somewhat older than chapter 5 (72 years vs 67 years). In addition, baseline FMD values were lower in chapter 4, while systolic BP was higher in chapter 5 (although still within normal range), suggesting that the older subjects in the previous chapter show somewhat impaired vascular function. At the other hand, cholesterol levels tended to be somewhat higher in the control group from this study. Finally, the model to induce ischaemia-reperfusion injury differed between studies (20-min ischaemia *versus* 5-min ischaemic handgrip exercise). Although speculative, these differences may relate to the different findings between chapter 4 and 5.

This study supports the detrimental findings on the efficacy of IPC in HF patients. We can only speculate about possible mechanisms to explain this finding. Preclinical studies suggest that the impaired efficacy of IPC in cardiovascular disease is linked to morphological and biochemical alterations, which may impact on signal transduction [45]. For instance, it was recently demonstrated that the presence of post-infarction cardiac remodelling is closely linked to an abolished effect of IPC [142]. Whilst this finding supports a role for morphological changes underlying our findings, others have provided support for biochemical alterations in patients with HF. Indeed, IPC in HF failed to induce protein kinase C-ε translocation [147], which represents an important step in the protection through preconditioning. Finally, mitochondrial defects in the genesis and progression of HF have also been proposed to contribute to the diminished effect of IPC. While mitochondria seem to serve as end-effectors of IPC, decreased enzyme activities of the electron transport seen in failing hearts may potentially negatively impact the efficacy of IPC [45].

Clinical implication. Our finding of a reduced efficacy of IPC in HF patients may have clinical implications. Various (non)pharmacological preconditioning interventions are currently applied in randomised controlled trials in patients, including those with HF. Despite some recent successful studies [112, 148], application of (remote) IPC in the clinical setting in general is often disappointing [126]. The increased cardiovascular death rate in post-infarcted, failing hearts suggests that endogenous protective mechanisms in HF against IR-injury may be lost or attenuated [45]; a finding which is in line with the present study. Possible reasons relate to the inclusion of relatively young animals in preclinical studies with a relatively short disease duration, whilst clinical trials mostly involve patient groups such as HF (e.g. in heart transplantation) [45]. Therefore the majority of preclinical studies do not adequately reflect the clinical setting in which patients are included with lower efficacy of IPC. This should be taken into consideration when examining the impact of IPC in clinical groups, such as HF.

Limitations. A number of limitations must be discussed. As discussed in chapter 4, we used the endothelium of the forearm as a surrogate marker. Given the aetiology of HF as a disease affecting the heart, studying the heart muscle tissue directly might reveal even more pronounced results on the magnitude of IR-injury. Secondly, in contrast to previous studies that adopted 15-20 minutes of ischaemia to induce endothelial IR-injury, our study used 5-min ischaemic handgrip. However, as described earlier, previous work demonstrated that 5-minutes of ischaemic handgrip exercise induces reperfusion that is at least similar to 15-minutes of ischaemia. Moreover, FMD decreased in both groups using this protocol.

Therefore, this approach is valid to examine endothelial IR-injury. Third, patients in our study continued their medication during testing. Continuing medication in HF patients may explain why we found no differences in FMD between groups. Indeed, our FMD-data matches with previous studies that included medicated HF patients [149]. We deliberately chose to continue (pharmacological) treatment, so that our results would reflect a 'real life' situation. This approach revealed, despite the intake of drugs with established preconditioning effects (i.e. statins), that an exaggerated endothelial IR-injury in HF patients could not be attenuated by IPC. These findings raise questions regarding the potential loss of preconditioning effects of statins with sustained intake, such as recently highlighted [150]. Finally, we only examined a single time-point after IR-injury. This limits insight into a potential difference between groups (or between interventions) in the time-course of restoration of FMD after IR injury. Such differences in time-course may have provided further insight to better understand our findings.

In conclusion, we provide data in humans *in vivo* that HF is associated with an exaggerated damage to the endothelium after an ischaemic insult, which cannot be prevented by IPC. These novel findings may contribute to the poor clinical outcome after cardiac injury in HF patients, and should be considered when examining the effects of traditional, non-pharmacological preconditioning in HF patients. This highlights the search for alternative protocols or application of IPC that may bypass these limitations, ultimately leading to the ability of HF patients (and other populations) to benefit from IPC. In the next chapters, a link is made between the effects of exercise and IPC. To explore the possible links between exercise and IPC, the next chapter will first explore the effects of IPC on exercise

performance. Therefore, in the next chapter, a study is presented, in which both acute and 24-IPC is investigated in runners performing a 5-km trial. A link between IPC and exercise would increase possible strategies to benefit from the cardioprotective, preconditioning effects of IPC.

6

Is delayed ischaemic preconditioning as effective on running performance during a 5-km time trial as acute IPC?

JOOST P.H. SEEGER, SILVIE TIMMERS, DANIQUE J.M. PLOEGMAKERS, N. TIMOTHY CABLE, MARIA T.E. HOPMAN, DICK H.J. THIJSSEN.

INTRODUCTION

IPC was originally described as an effective strategy to protect cardiac cells against a prolonged period of ischaemia. Most studies therefore have focused on the potential cardioprotective abilities, leading to large, multi-centre trials that established the potency of IPC to attenuate cardiac damage and improve clinical prognosis [112, 148, 151]. Interestingly, IPC is suggested to exert also beneficial effects when applied directly before an exercise session.

De Groot et al. were the first to explore the ability of IPC to enhance physical performance [16]. They found significant improvement in exercise performance when a maximal cycle test was preceded by IPC. Similar findings of performance enhancement were reported by others during swimming, running, cycling, rowing and handgrip exercise, [18, 101, 152-155], but not all [155-157] (i.e. involving running and cycling). Those studies were mainly designed to assess the direct effect of IPC on exercise performance, while the working mechanism of IPC on skeletal muscle oxygenation status is less explored.

All previous studies exploring the impact of IPC to exercise performance timed IPC in close proximity to the exercise event. This however, imposes practical limitations, as IPC cannot always be applied in close proximity to the start of an athletic event. As described in chapter 2, SWOP can be applied 24-72 hours before an event and may be effective in reducing the consequences of IR-injury (just like the benefits described for IPC).These observations raise the question whether enhanced performance is also present when IPC is applied 24-72 h

prior to the exercise event to match the event with SWOP. Practically, such timing would be preferred over the application of IPC immediately before an athletic event.

The primary aim of this study, therefore, was to assess the hypothesis of whether IPC applied *24-hours* before the running trial (i.e. timed together with the start of the SWOP) is equally effective in changing exercise performance compared to the application of IPC immediately before a running event in healthy volunteers. Secondly, it was explored whether the effect of (24-h) IPC is related to changes in local tissue oxygenation (measured with Near-infrared Spectroscopy (NIRS)) of the vastus lateralis muscle during running exercise and/or production of lactate at the end of exercise. Such insight may help to better understand the potential mechanisms contributing to the exercise benefits of (24-h) IPC in humans.

METHODS

Sample size calculation

The sample size calculation was based on a study performed by Baily et al. [18]. In this study, also moderately trained healthy subjects were included and performed a 5-km time trial with and without IPC. *Bailey* et al. found that IPC resulted in a significant decrease of 34 seconds to cover the 5 km ($p<0.05$), with a standard deviation of 28 seconds (σ). Therefore, a difference in finish time between the IPC and control condition of 30 seconds (d) was

considered relevant and realistic in our study population. Furthermore, a power (β) of 90% and an alpha (α) of 0.05 was used for the power calculation with the formula $(Z_{\alpha} + Z_{\beta})^2 \times \sigma^2))/ d^2$ with $\alpha = 0.05$ (two-sided, $Z_{\alpha} = 1.96$), power = 0.90 ($Z_{\beta} = 1.65$), $\sigma=28$ and $d=30$. According to nQuery, a total of 12 subjects is needed to detect a relevant effect of IPC on exercise performance.

Subjects

Adopting a randomised, cross-over study, 12 healthy participants volunteered to participate. Baseline characteristics are shown in Table 1. Subjects were moderate to well-trained amateur runners (Table 1), who exercised at least two hours a week, including a minimum of one hour running at moderate-to-high intensity. Older participants (>50 years) were excluded as well as subjects with cardiovascular disease or any other chronic disease effecting maximal performance as this may affect the efficacy of IPC as shown in chapters 4 and 5. Prior to participation, subjects were informed about the procedures of the study, but not about the rationale of the study to keep subjects naive about the potential effect of IPC as well as the timing of IPC. All subjects gave their written informed consent prior to participation. This study was approved by the local ethics committee of the Radboud University Medical Centre.

Experimental design

Subjects visited our laboratory at six different occasions (including 3 familiarization sessions to customise to the 5-km time trial), to perform a 5-km time trial on a treadmill. On the first day, participants were examined prior to testing by a physician, comprising an assessment of an electrocardiography under resting conditions. On all testing days, subjects refrained from alcohol, caffeine, tea, chocolate and (intensive) physical exercise for at least 24 h prior to testing as these factors may potentially influence exercise performance. Additionally, subjects were asked to adopt a similar eating pattern at all days of the measurements. Participants were tested at the same time of day to control for diurnal variation and its impact on exercise performance [158], while measurements were performed in a temperature-controlled testing room with the temperature set at 19ºC. Testing days were separated by at least 4 days, in order to prevent possible carry-over effects of the exercise bout and/or IPC.

First, participants performed three familiarization sessions on a treadmill. Participants were instructed to run a 5-km time trial on a motorised treadmill (EN-BO Systems, Bonte BV, the Netherlands) as fast as possible, while being blinded for time, speed and HR, but not running distance. Average group times were 1416 seconds for the first session, 1418 seconds for the second session and 1434 seconds for the last familiarisation session. The coefficient of variance was calculated between the first and second session (1.8%), and between the first and third session (1.6%). When being familiarised with running the 5-km trial, the experiment started. In a randomised order, participants received IPC, 24-IPC and SHAM. Participants were informed that all interventions, including SHAM, could potentially lead to an improved running performance to keep them naive. After the application of IPC, participants performed a standardised warm-up, followed by the 5-km time trial.

Interventions

Ischaemic preconditioning (IPC). IPC was performed in the supine position using bilateral arterial occlusion [16]. Occlusion cuffs were positioned proximally around the thigh (bilaterally) and inflated to 220 mmHg to block arterial inflow for 5 minutes, followed by a 5 minute deflation. This procedure was repeated 4 times, with each ischaemic episode separated by 5 minutes rest. For the IPC intervention, this procedure was started 1 hour before the time trial, whilst the procedure was timed exactly 24 hour prior to the time trial for the 24-IPC.

Control intervention (SHAM). The control intervention was performed under the same conditions as the intervention test, but this time the cuff was inflated to only 20 mmHg, which did not alter the arterial inflow. The Sham intervention was performed directly before exercise.

Measurements

5-km running trial. After the IPC/SHAM-intervention, subjects were seated for 5 minutes. Subsequently, subjects performed a 5-minute warm-up followed by another 5 minutes to stretch their muscles as preferred, after which the 5-km running time trial was started. The 5-km running trial was performed on a motorised treadmill. Main outcome parameter was finish time (Fisher Scientific, the Netherlands). The speed of the treadmill was set at 11 km/h, where after participants were allowed to alter running speed, but were kept blinded for running speed and running time. Participants were instructed to run 5-km as fast as

possible. The only information available to the participants during each time trial was total distance covered (m) as to adjust work-output to pace towards the known endpoint. No further information/feedback and/or encouragements were provided during the 5-km trial.

Near-infrared Spectroscopy (NIRS)

NIRS measurements were carried out on the belly of the vastus lateralis muscle 12 cm above the fibular head. To ensure the optodes and detector did not move relative to the participant's skin, the device was fixed into position using surgical tape. Quantitative NIRS measurements of muscle oxygenation (mVO_2) from O_2Hb were obtained during exercise and subsequently, tissue saturation index (TSI) was calculated as the percentage of O_2Hb/O_2Mb of total Hb and Mb. NIRS is described in more detail in chapter 3.

Secondary parameters – HR was measured continuously by a Polar chest band (Polar® RS 800) and recorded every 500m. BP was measured at the right arm before, during and after the IPC/SHAM-intervention and after the time trial. Furthermore, a finger capillary blood sample (Accutrend® Lactate, Roche Diagnostics, Mannheim, Germany) was taken before and after the IPC-/SHAM-intervention, but also after the 5-km time trial to measure blood lactate levels. In addition, the rate of perceived exertion was registered on a Borg-scale (6-20) during warm up, every 500 m and after the 5-km time trial.

Statistics

Data is presented as mean ± SD, unless stated otherwise. To examine differences in finish time between the interventions, one-way repeated measures ANOVA was used. To examine

whether IPC and 24-IPC lead to comparable changes in exercise performance (i.e. primary aim of the study), we used a Pearson correlation coefficient to relate changes in finish time after IPC *versus* 24-IPC. Using a 2-way ANOVA, we examined whether changes in TSI, HR, pace time and BORG during the time trial (every 500m; 'time') differs across the 3 conditions ('intervention'; SHAM *vs* IPC *vs* 24-IPC). Differences across the conditions were analysed by repeated measures ANOVA. Finally, to investigate which parameters contributed to the *change* in exercise performance after IPC or 24-IPC (i.e. ΔIPC-control, Δ24-IPC–control), a stepwise linear regression analysis was performed. Study parameters included in this model were presented as the *change* between IPC versus control or 24-IPC *versus* control. Differences were considered to be statistically significant at $P<0.05$.

RESULTS

Table 1. Baseline characteristics (n = 12). Values are presented as mean ± SD.

Parameter	Mean±Sd
Male/female	10/2
Age, years	31±6
Body mass index, kg/m^2	21.8±2.5
Running time/week, minutes	191±78
Total running experience, years	9±8

5-km time trial

No differences in finish time between IPC (1381±112s), 24-IPC (1385±112s) and SHAM (1400±105s), (P=0.30, Figure 1A) were found. Also no significant changes were observed

when calculating the change in finish time between IPC *versus* SHAM (-16±39s, *P*=0.14) or 24-IPC *versus* SHAM (-16±58s, *P*=0.29). However, when pooling the individual changes in finish time after IPC and 24-IPC, a trend was observed for improvement in finish time (*P*=0.10, Figure 1B). Interestingly, a significant positive relation appeared to be present between the change in finish time after IPC *versus* the change after 24-IPC (*P*=0.016; *r*=0.677, Figure 2).

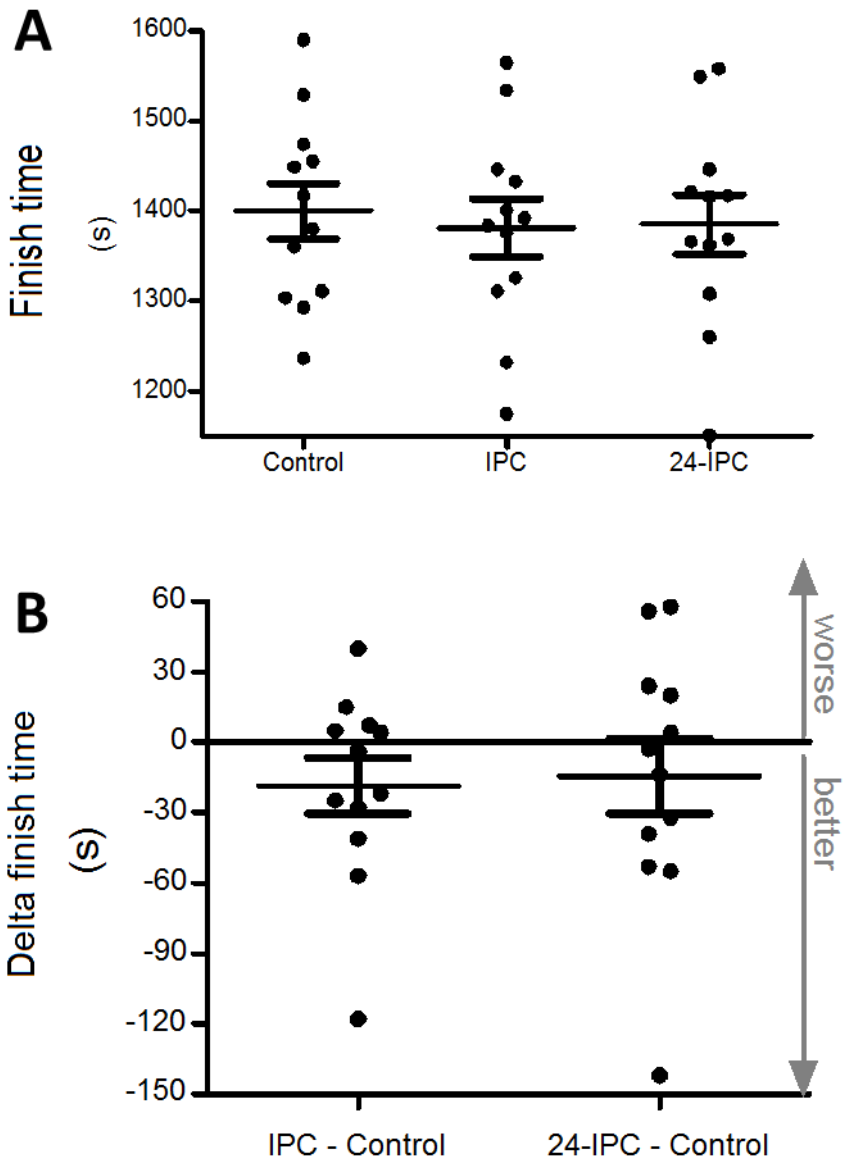

FIGURE 1. Individual presentation (n=12) of the finish time during the SHAM, IPC and 24-IPC intervention (A, in seconds) and the change in finish time compared to SHAM after application of IPC or 24-IPC (B, in seconds). A negative value in B

relates to a better finish time. Each dot represents a single participant. The horizontal line represents the average, with the error bars representing the SE.

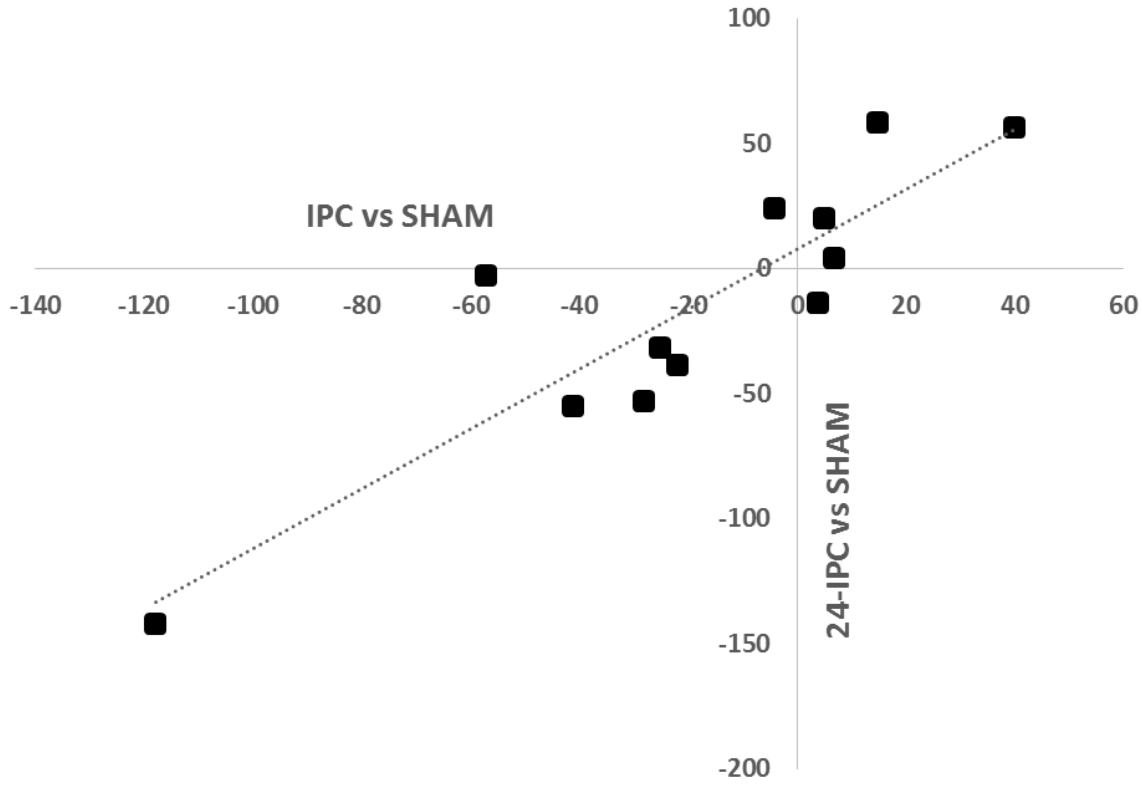

FIGURE 2: Correlation between the individual changes in finish time compared to SHAM after application of IPC (X-axis, in seconds) and the change in finish time between SHAM and 24-IPC (Y-axis, in seconds) in our participants (n=12). A negative value on both axes relates to a better finish time after IPC or 24-IPC. The dotted line represents the regression line from the Pearson's correlation coefficient

Exercise characteristics

HR gradually increased during the 5-km time trial, but these increases were similar among the different interventions (*P*=0.63). Pace slightly decreased during the 5-km time trial in the three conditions (figure 3), with a significant time*treatment effect for 24-IPC (*P*=0.016). Post-hoc analysis showed that subjects started at a higher pace during the 24-IPC time trial and ended with a somewhat lower pace compared to the other two conditions. All participants reached a similar level of exertion at the end of the 3 time trials (SHAM: 19 ± 2

BORG, IPC: 19 ± 2 BORG, 24-IPC: 19 ± 2 BORG; *P*=0.60)). Blood lactate levels significantly increased after the 5-km time trial, whilst no differences were found among the 3 trials in post-exercise lactate levels (SHAM: 6.4 ± 3.1 mmol/l, IPC: 8.0 ± 2.7 mmol/l, 24-IPC: 7.5 ± 2.2 mmol/l; P=0.30, *P*=0.24). Finally, TSI, as measured with NIRS on the vastus lateralis muscle, showed a rapid decrease in saturation upon the start of the 5-km time trial and remained stable thereafter. This time-dependent change was similar among all three conditions (*P*=0.14, Figure 4).

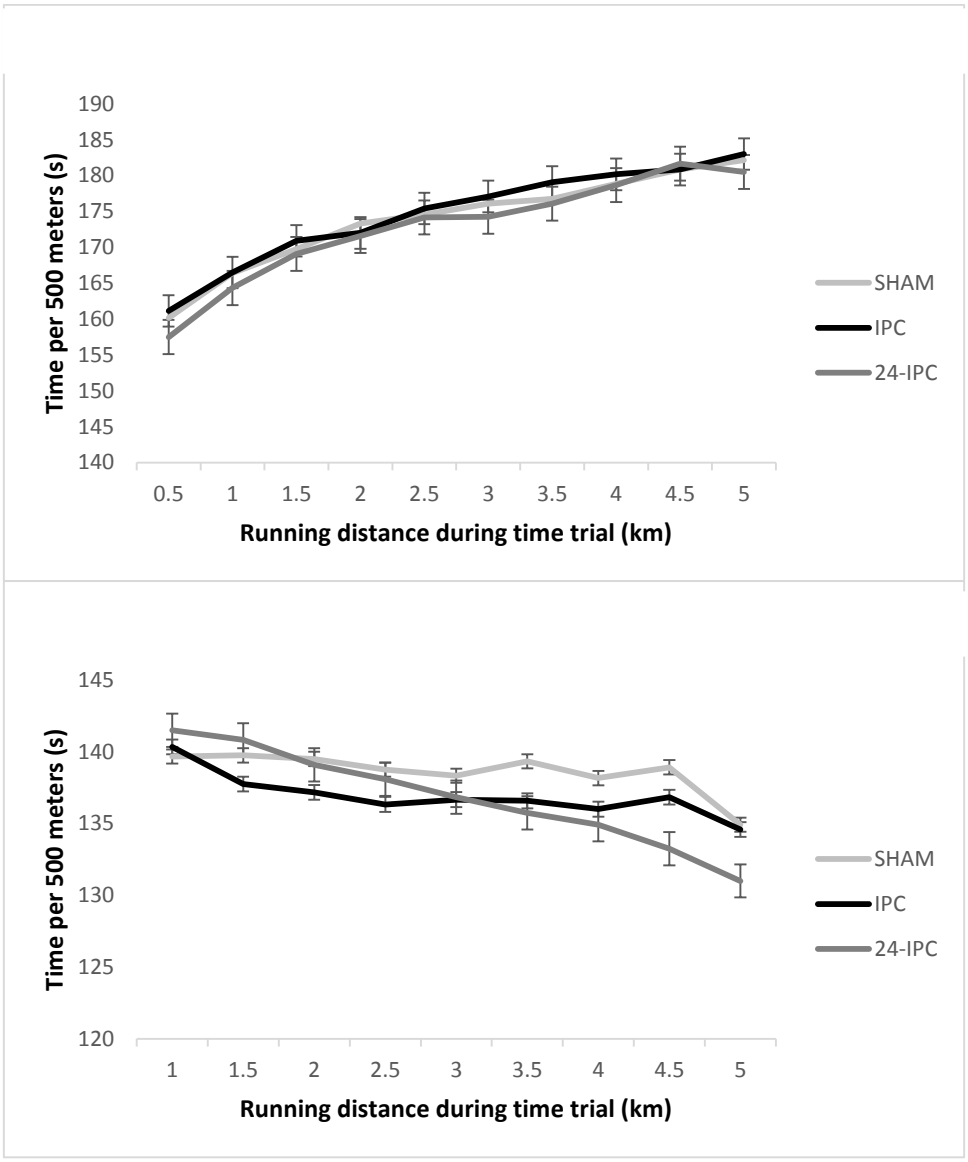

FIGURE 3. A) Average heart rate is shown for each intervention, for every 500 meters (n=12) A similar increase between interventions was found (*P*=0.63). B) Pacing time is shown for every 500 meters, with the exception of the first 500 meters (n-12). Error bars represent SE. Pace slightly decreased during the 5-km time trial in the three conditions (figure 3), with a significant time*treatment effect for 24-IPC (P=0.016).

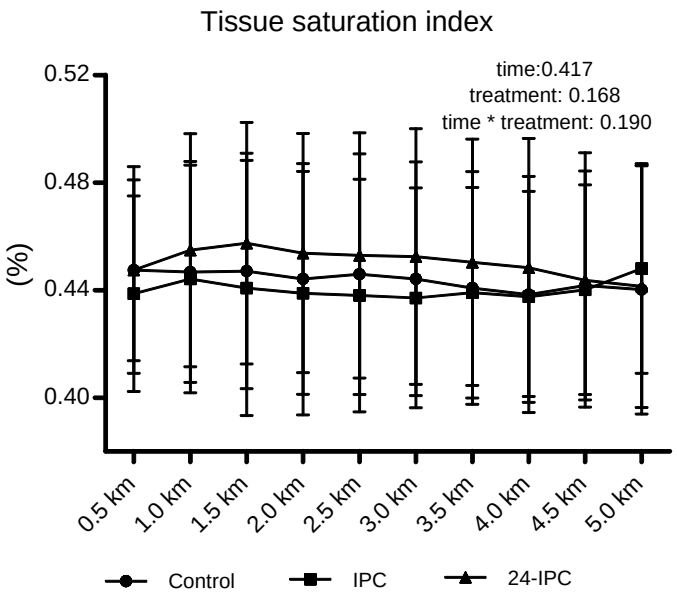

FIGURE 4 Tissue saturation index (TSI) derived from the near infra-red spectroscopy measurement on the vastus lateralis muscle during the 5-km time trial at 0.5-km intervals in healthy volunteers (n=9). The time trial was preceded by SHAM (closed circles), IPC (closed squares) or 24-IPC (closed triangles). Error bars represent SE.

Factors predicting change in finish time

Stepwise linear regression was performed to identify whether exercise characteristics could predict the change in finish time after IPC or 24-IPC. Post-exercise blood lactate concentration significantly contributed to the change in running time after IPC or 24-IPC (R^2=0.47, β=-0.687, *P*=0.007). More specifically, lower post-exercise blood lactate levels after IPC or 24-IPC were significantly related to an improvement in finish time. The number of training hours and differences between trials in maximal HR and TSI did not contribute to changes in finish time.

DISCUSSION

This is the first study to compare the impact of the application of IPC 24-hours before the exercise bout *versus* IPC immediately before exercise on performance. The following findings are presented. Overall, no significant effect of 24-IPC or IPC on exercise performance during a 5-km time trial in moderately-to-well trained athletes was found, despite the fact that the majority of the participants improved their running performance after IPC or 24-IPC. However, a strong and positive relationship existed, between the change in finish time when the 5-km trial was preceded by IPC *versus* 24-IPC. This suggests that, at an individual level, 24-IPC exerts a comparable effect on exercise performance compared to IPC during a 5-km running time trial. Finally, improvement in finish time was strongly related to a decrease in post-exercise blood lactate levels, but not to changes in vastus lateralis muscle oxygenation. Taken together, at an individual level, the effect sizes of 24-IPC and IPC during a 5-km time trial are strongly related.

No significant effect of IPC on exercise performance was found, which contrast with some [16, 18, 101, 152-155], but not all studies [155-157]. Although not significant, the effect size found in our study is 1.4% reduction in running time, which corresponds well with other studies that reported a 1- 4% improvement in peak workload [16, 152], peak oxygen consumption [16], improvement in finish time [18, 101, 154] or prolonged time to exhaustion [153, 155]. One potential explanation for the absence of a significant effect is that our self-selected exercise protocol resulted in a relatively large variation in running time within participants despite three familiarization sessions before the actual start. These types of exercise trials are importantly influenced by the athlete's experience and motivation [159-162]. As a result, the coefficient of variation of the self-paced 5-km time trial (1.6-1.8%) is

somewhat higher than *a priori* expected. Moreover, our sample size calculation was based on a study performed by Bailey et al. [18], in which participants ran faster and therefore showed a relatively larger effect of IPC than in the present study. Consequently, more subjects may be necessary to detect differences when adopting the self-paced 5-km time trial. In support of this hypothesis, previous work that reported an effect of IPC to improve exercise performance, adopted an exercise protocol where subjects performed exercise at maximal effort level. Indeed, these previous studies adopted exercise tests that caused substantially larger blood lactate concentration (~12-13 mmol/l) [16, 101] compared to our protocol (~6-8 mmol/l). Taken together, although we found no significant effect of IPC on exercise performance, the effect size of IPC is comparable to previous studies in this field.

The primary aim of our study was to explore whether 24-IPC and IPC would cause comparable changes in running time. In agreement with our hypothesis, a strong and positive correlation was found between the change in running time after IPC and 24-IPC. In other words, subjects who demonstrated an improved running performance after IPC were likely to show comparable benefits of 24-IPC. Similarly, those who did not show improvement in running time after IPC, also showed no change after 24-IPC. Although our study is the first in the literature to demonstrate that IPC and 24-IPC have comparable effects on exercise performance, these findings are largely in agreement with previous work on IPC and protection of cardiac damage by the SWOP. Previous work on the cardioprotective effects of IPC and 24-IPC found comparable protection against prolonged ischaemia [163-165], although some data is conflicting [166]. Taken together, our data

extends previous work in the area of cardiology, in that the effect of 24-IPC on exercise performance is comparable to that observed after IPC.

In an attempt to better understand the underlying mechanisms that contribute to the potential effects of IPC and 24-IPC on exercise performance, we explored tissue saturation of the vastus lateralis muscle during the time trial. Previous work in animals linked IPC to improved muscle oxygenation during exercise as well as after exercise performance in rats [167]. Studies that have measured the TSI using NIRS, a measure that reflects the ability of tissue to take up oxygen, report a 20% decrease in TSI after IPC relative to control [154, 168]. In our study, it was found that IPC and 24-IPC exerted a similar impact on TSI during exercise than SHAM [168]. This observation suggests that IPC and 24-IPC did not alter muscle oxygen delivery during exercise [169]. This is supported by the regression analysis, which excluded the change in TSI induced by IPC or 24-IPC as a potential predictor for a change in running time during the 5-km time trial.

An alternative explanation for the underlying mechanisms relates to blood lactate levels during exercise. Previous work demonstrated that changes in blood lactate concentration and mitochondrial capacity account for 68% of the variation in cycling time trial performance [170]. Furthermore, lower blood lactate concentrations at a given workload improves endurance exercise in various populations, including in highly trained [165]. These observations are somewhat in line with earlier observations from Bailey et al., who demonstrated that IPC lowers blood lactate levels during running exercise at submaximal intensity [18]. Interestingly, running speed associated with lower blood lactate levels after

IPC matched with the running speed of the 5-km time trial [18]. Therefore, our findings provide some further support that IPC may enhance exercise performance through changes in the lactate pathways. Alternatively, IPC and 24-IPC may enhance mitochondrial function [5]. During exercise, contracting skeletal muscles generate free radicals that cause cellular damage and impaired mitochondrial function [171]. Since mitochondrial oxidative capacity is strongly linked to exercise performance [172], the effects of IPC and 24-IPC may be related to the protective effects on mitochondrial function.

Limitations. The correlation we observed between the change in running time after IPC and 24-IPC may be confounded by the presence of collinearity, as both parameters are calculated using the SHAM-running trial. This must be considered when interpreting these findings. Although three familiarizations sessions were included prior to testing, the relatively high day-to-day variability of 1.6-1.8% in athlete's performance could have influenced our results, especially since the effect size of 1.4 % was somewhat smaller than initially anticipated. Interestingly, when all data from IPC and 24-IPC are pooled (n=24), we found a trend for a decline in the time trial (P=0.10). Most limitations of the present study relate to the protocol adopted and are discussed in more detail in chapter 3.

Perspectives

On an individual level, 24-IPC is as effective as acute IPC, which implies that both can be used preceding a contest. However, as there are high interindividual differences in response to

application of IPC, more research is needed in order to establish the interaction between the timing of IPC stimulus and exercise performance during a 5-km time trial.

In conclusion, our results suggest a small, non-significant effect of IPC and 24-IPC on exercise performance during a self-paced 5-km time trial in a group of healthy volunteers. In addition we found a strong relation between the effect of IPC *versus* 24-IPC, which suggests that IPC and 24-IPC demonstrate comparable effect sizes (at an individual level), although the results could be partly a result of collinearity . Consequently, IPC may be applied before the exercise event, which represents a more feasible and practical approach compared to the application of IPC immediately before the exercise event. However, based on the results from this study, it is too early to advice an individual athlete whether he or she would respond to 24-IPC. In order to better understand the effects of 24-IPC, more studies a needed, giving more insight into underlying mechanisms that could potentially influence whether someone responds to IPC or does not.

In this study we have investigated whether 24-IPC has got the same ability as acute IPC to enhance exercise performance in healthy individuals. Although we did not find a significant overall effect, we established that individuals who respond to acute IPC, likely also respond to 24-IPC. While this study adds to the current knowledge of research performed in healthy individuals, to date there has been no study published in which the effect of IPC on exercise performance was explored in clinical populations. This question is specifically relevant, since our and other work showed that IPC has reduced efficacy to prevent endothelial ischaemia-reperfusion injury in clinical populations (see chapters 4-5). Whether the exercise benefits of IPC are also reduced in clinical populations is unknown. Therefore, we wanted to know whether IPC could also be performed in subjects with a spinal cord lesion (chapter 7).

Therefore, in our follow-study, we investigated the effect of IPC and RIPC on performance in individuals with a complete spinal cord lesion.

7

Ischaemic preconditioning improves performance in spinal cord injured individuals

JOOST P.H. SEEGER, JAN T. GROOTHUIS, ILSE VAN NES, SILVIE TIMMERS, N. TIMOTHY CABLE, MARIA T.E. HOPMAN, DICK H.J. THIJSSEN

INTRODUCTION

IPC has the ability to enhance exercise performance (see chapter 6). Whilst the mechanisms are unclear, studies which have shown that IPC also provides protection of many organs and tissues, including the peripheral vasculature [110] and the skeletal muscle [57]. Studies have found that IPC prior to an exercise bout in healthy subjects improved finish time [18, 101], power output [102] and lowered lactate accumulation [18], but no change in oxygen consumption was found [18, 102, 156]. To date, studies examining the impact of IPC on exercise performance have focused exclusively on healthy young (trained) subjects.

Sport participation by spinal cord injured (SCI) individuals is associated with increased perceived quality of life and social integration [173, 174] and, as a result, has become increasingly popular, also at the competitive level [175]. This coincides with the interest in procedures to improve exercise performance in SCI [176]. In line with observations in able-bodied subjects, it is hypothesised that IPC will enhance arm crank exercise performance in SCI individuals. Therefore, the first aim of this study was to examine the impact of IPC, applied to the upper limbs, on exercise duration, maximal workload and maximal oxygen consumption during an incremental arm crank exercise test in SCI individuals.

Studies have described that, in addition to the local effects of IPC in the area exposed to repeated ischaemia, IPC can also exert beneficial effects in distant, remote areas [6, 177]. For example, IPC applied to a single coronary artery also protects a remote vascular bed of the myocardium supplied by another artery [6]. Also, IPC applied to another organ, such as

the kidney, reduces myocardial infarct size [177]. Subsequently, many others have confirmed the presence of RIPC in the protection of prolonged ischaemia [178-180]. Interestingly, a recent study provided evidence for improved exercise performance after the application of RIPC [153]. Whether RIPC also improves exercise performance in SCI is of special importance, since the activation of neural pathways have been suggested to partly explain the effect of RIPC [71]. As neural pathways from the legs are interrupted in SCI individuals with a complete thoracic lesion, we hypothesize that RIPC has no effect on exercise performance in SCI. Our second aim, therefore, was to study the impact of RIPC (i.e. stimulus applied to the legs) on exercise performance during an incremental arm crank test in SCI individuals.

METHODS

Subjects

12 SCI individuals (1 female) volunteered to participate in this study. All subjects had a traumatic motor and sensory complete thoracic spinal cord lesion (T4-T12); AIS A [181], except for one who had a motor complete but sensory incomplete lesion (AIS B). One spinal cord lesion originated from surgical removal of a benign tumour (table 1). All individuals were free of any cardiovascular disease and were not physically restricted to perform strenuous arm-crank exercise. Since older age may impact the efficacy of IPC (chapter 4), we selected subjects 18-60 years. Moreover, based on the results of chapter 5, we expect that individuals with a maximum age of 60 years old, do respond to IPC. Subject characteristics are summarised in Table 1. Prior to participation, all subjects gave their written informed

consent. The study was performed according to the Declaration of Helsinki (2000) and approved by the ethics committee of the Radboud university medical center.

Experimental design

In this randomised, single-blind, crossover study, subjects visited our laboratory at 3 different occasions to perform an incremental maximal arm crank exercise test. Randomization was done before the first intervention by means of a computer-generated randomization list (SPSS, version 22). Testing days were separated by at least 7 days, in order to prevent possible carry-over effects. On the first day, participants were examined prior to testing by a physician, including assessment of an electrocardiography under resting conditions. Prior to all testing days, subjects refrained from alcohol, caffeine, and (intensive) physical exercise for at least 24 hours, as these factors are known to potentially influence exercise performance. During the 3 measurement days, subjects were tested at the same time of day, while measurements were performed in a temperature-controlled testing room with the temperature set at 19ºC.

The incremental maximal arm crank exercise test was preceded with: 1. Repeated, 5-minute arterial occlusions of 220 mmHg on the arms (IPC), 2. Repeated, 5-minute arterial occlusions of 220 mmHg on the legs (RIPC), or 3. SHAM-intervention, in which the cuffs were repeatedly inflated for 5-minute cycles of 20 mmHg on the legs (Figure 1). Given the nature of the lesions, SCI subjects noticed no differences between the RIPC and SHAM-condition and were naïve about the purpose of these testing trials. Cuff inflation to 20 mmHg does not alter blood flow to the leg [182], and therefore represents a valid SHAM-condition.

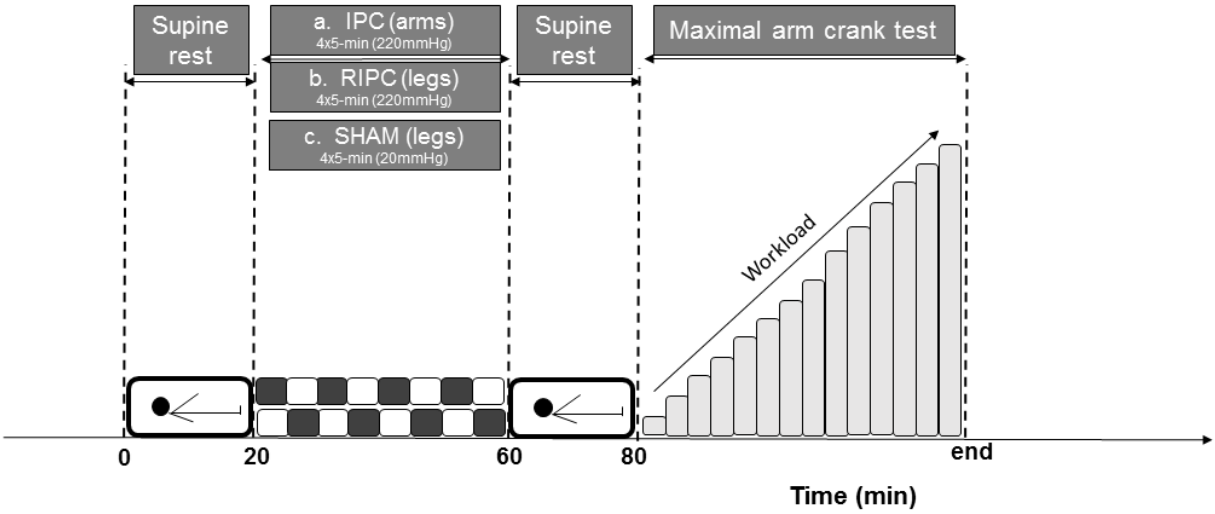

FIGURE 1. Protocol of the study. Interventions were randomised. IPC, ischaemic preconditioning; RIPC, remote ischaemic preconditioning.

Measurements

Body anthropometric data. Body mass was measured in the seated position on a customised weighing chair, while height was reported by the subjects in order to calculate body mass index (in kg/m^2). Resting HR and BP were measured twice in the supine position on the left arm, using a manual sphygmomanometer after 5 minutes of rest.

Incremental maximal arm crank exercise test. The incremental maximal arm crank test was performed as described in chapter 3. We recorded total exercise duration in seconds and total workload in wattage. Oxygen consumption, respiratory quotient (RQ) and HR were represented as the mean of the last 30 seconds of each minute. In addition, we recorded the exercise time necessary to exceed RQ >1.00. Resting values were averaged over a 2-minute

period. The peak values were measured as the mean of the last 30 seconds in which arm crank exercise was performed.

Blood lactate and BORG – Blood lactate level was measured with a finger stick (Lactate Pro LT-1710, Arkray), before and after the intervention and within 2-3 minutes after completing the maximal arm crank exercise test. Immediately after the maximal exercise test, subjects were asked to indicate their perceived exertion on a BORG 6-20 scale.

Blood pressure - Using a manual sphygmomanometer, BP was measured every second occlusion during the IPC intervention, prior to exercise.

Interventions

IPC was performed in the supine position using bilateral arterial occlusion of both arms, similarly to de Groot et al. [16]. The automated occlusion cuffs (E20 rapid cuff inflator, Hokanson, USA) were positioned proximally around the upper limbs and alternately inflated to 220 mmHg for 5 min. This procedure allows for a complete blockade of the arterial inflow in the upper limb throughout these 5 minutes. This ischaemic procedure was repeated 4 times, each separated by 5 min of reperfusion (during which the contra-lateral upper limb was occluded). On another day, RIPC was applied using the same protocol as described above for the IPC, except that the occlusion cuffs were now placed proximally around the lower limbs. On a third occasion, subjects followed a SHAM-intervention, which was identical to the RIPC procedure with the occlusion cuffs being inflated to 20 mmHg.

Statistics

Data is presented as mean ± SD, unless stated otherwise. In order to assess whether IPC enhances performance in SCI, a Students' paired *t*-test was used to calculate the difference between the IPC and SHAM-session to answer the first aim of our study. Secondly, to study whether a remote effect was present (i.e. the second aim of our study), a Students' paired *t*-test was used in order to compare the RIPC with SHAM-session. Differences were considered to be statistically significant at P=0.05.

RESULTS

Impact of local IPC on exercise performance

Baseline characteristics are presented in Table 1. We found a significantly longer total exercise duration when the incremental arm crank exercise was preceded by IPC compared to the SHAM-intervention (P=0.05; Figure 2). Workload tended to be higher after IPC compared to SHAM (P=0.06). No significant differences were found in maximal oxygen consumption between the exercise tests preceded by IPC or SHAM-intervention. Moreover, no differences in RQ, HR, perceived exhaustion and time to RQ >1.00 were measured (Table 2).

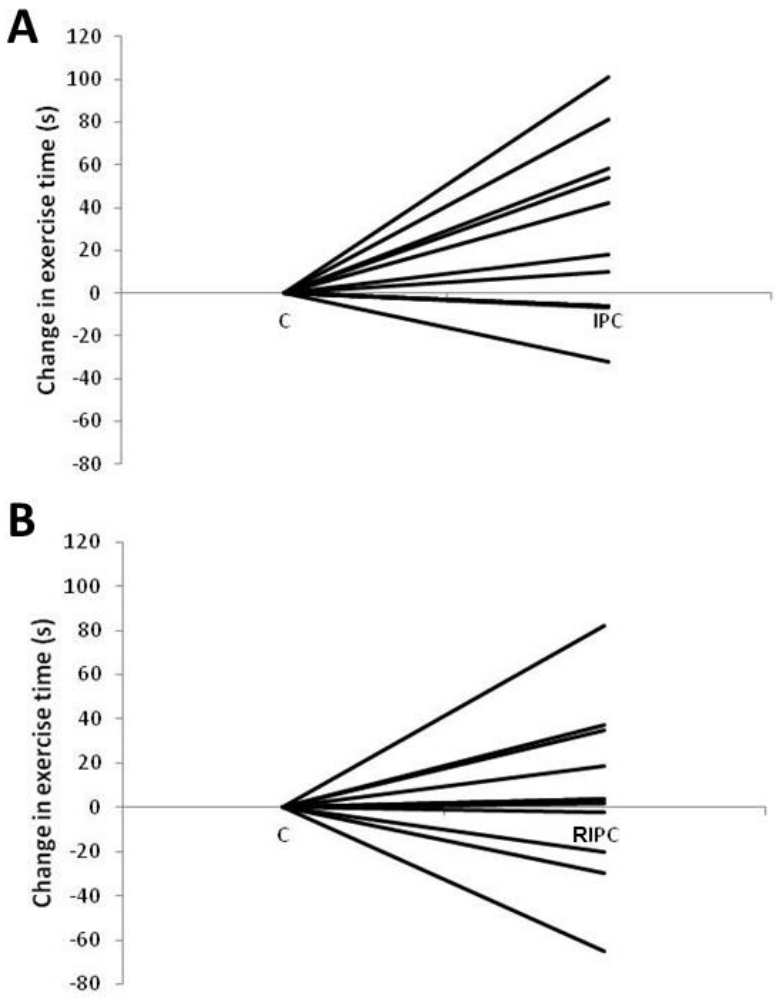

FIGURE 2. Change in exercise time (in seconds) during an incremental maximal arm crank exercise test after IPC of the upper limbs (A) or RIPC of the lower limbs (B) compared to a control trial in 12 spinal cord injured-individuals. Changes in exercise time are provided for all 12 individuals. Paired Students' *t*-tests indicate a significant improvement in exercise time after IPC (*P*=0.05), but not after RIPC (*P*=0.60)

Table 1. Baseline characteristics of spinal cord injured (SCI) individuals included in the present study (n=12). Values are presented as mean ± SD.

Subject	*Sex*	*Age (years)*	*Body mass (kg)*	*BMI (kg/m²)*	*MAP (mmHg)*	*Level of lesion (years)*	*Time since injury (years)*	*Sport (h/wk)*	*Medication*
1	male	51	97	28	91	T10	11	5	-
2	male	38	58	19	80	T11	10	1	microlax
3	male	31	72	22	97	T4	13	2	-
4*	male	28	78	23	82	T5	8	1	melatonin
5	male	45	78	25	85	T4	16	1	-
6	male	45	69	21	95	T6-	22	2	microlax
7#	male	25	70	21	82	T11	9	4	-
8	male	26	92	26	105	T4	7	4	sodium picosulfate
9	male	23	78	23	93	T12	9	1	movicolon, solifenacin, levocetirizine
10	female	46	62	26	90	T10	43	2	benzodiazepine, lamotrigine, clomipramine, promethazine, propranolol, fluimucil, transipeq
11	male	57	86	29	93	T8	25	3	-
12	male	32	72	24	82	T10	15	2	microlax
Average	**11/1**	**37**	**76±11**	**24±3**	**90±8**	**n/a**	**16±10**	**2±1**	**n/a**

#Incomplete thoracic sensory lesion, AIS B.*SCI due to surgical removal benign tumour

Table 2. Characteristics of the maximal arm crank exercise test in spinal cord injured individuals (n=12) when preceded by SHAM (4X5-min 20 mmHg on both legs), local IPC (4X5-min arterial occlusion to 220 mmHg on both arms), and remote IPC (4X5-min arterial occlusion to 220 mmHg on both legs). Values are presented as mean±SD. P-value represents a Student's paired t-test between SHAM and IPC (i.e. local IPC), or between SHAM and RIPC (i.e. remote IPC).

Parameter	*SHAM*	*IPC*	*RIPC*	**P-value (SHAM vs IPC)**	**P-value (SHAM vs RIPC)**
Exercise time (s)	636±184	662±176	642±182	**0.05**	0.60
Maximal load (W)	106±31	110±29	106±30	0.06	0.74
VO_{2max} (ml/min per kg)	25.2±6.5	25.3±5.7	24.1±5.5	0.94	0.23
Lactate, mmol/L	8.0±2.8	9.3±2.9	8.9±3.0	0.41	0.26
BORG-score	18±3	18±3	17±3	0.28	0.43
Peak heart rate (bpm)	176±19	173±17	178±19	0.08	0.93
Respiratory quotient	1.14±0.12	1.15±0.12	1.19±0.13	0.73	**0.04**
Time to RQ >1.00 (s)	455±214	440±155	427±164	0.76	0.42

IPC, ischaemic preconditioning; RIPC, remote ischaemic preconditioning; RQ, respiratory quotient

Impact of remote IPC on exercise performance

No significant differences were found between the RIPC-intervention and the SHAM-intervention for total exercise time or maximal workload (Table 2). In addition, no differences were found for maximal oxygen consumption, HR, time to RQ >1.00 and perceived exhaustion among the two tests, while the RQ was significant higher after the arm crank exercise test preceded by RIPC compared to SHAM ((P= 0.04) Table 2).

DISCUSSION

In this study the local and remote effect of IPC on arm crank exercise performance in a group of SCI individuals was examined. The following findings are presented. First, this study demonstrated that IPC applied to the upper limbs (i.e. IPC) in a group of SCI individuals with a motor complete thoracic spinal cord lesion can significantly improve exercise performance during an incremental maximal arm crank exercise test. This effect of IPC on exercise performance was not accompanied by an increase in maximal oxygen consumption. Secondly, no impact of IPC was found when the cuffs were applied to the lower limbs (i.e. RIPC) preceding an arm crank exercise bout. This study suggests that local IPC successfully improves exercise performance in SCI individuals (in line with previous work in able-bodied healthy volunteers, including in chapter 6), whereas RIPC does not.

This study revealed that a single session of IPC (4 repeated arterial occlusions of the upper arm) that precedes a maximal arm crank exercise test improves upper body exercise performance in SCI individuals. The average improvement in maximal performance of 4% is in line with previous studies that reported improvement in cycle, running and/or swimming performance of 1-4% after application of IPC [16, 18, 101, 154]. However, the present study design importantly differs from these previous studies. A first important difference is that, in contrast to previous studies that included predominantly lower limb exercise (e.g. cycling, running, rowing), an arm crank exercise protocol was adopted. Therefore, the study suggests that the impact of IPC on exercise performance is not simply related to (lower limb) large muscle exercise and/or application of IPC to the lower limbs. A second important difference with previous studies is that subjects with a SCI rather than healthy, able-bodied subjects were included. Presence of SCI commonly leads to a relatively physical inactive lifestyle which has unfavourable consequences for general health and physical fitness [183, 184]. The significant improvement in maximal workload suggests that the impact of IPC on exercise performance is not restricted to the able-bodied population only.

In contrast with one previous study in able-bodied subjects [153], no improvement in exercise performance after RIPC (i.e. IPC applied to the lower limbs) in SCI individuals was found. Cardioprotective effects of IPC are, at least partly, mediated through neural pathways [178]. Interruption of neural pathways from the lower limbs, as in SCI individuals with a complete lesion, may explain the absence of an effect of RIPC on arm crank exercise performance in our study. Alternatively, the amount of muscle mass occluded during RIPC may also contribute to our observations. Loukogeorgakis et al. [113] demonstrated that 2

cycles of RIPC on the contralateral arm did not protect the injured arm after IR-injury, while 2 cycles of RIPC applied to both legs (i.e. a significantly larger muscle mass) was sufficient to protect the arm against ischaemia-reperfusion injury. Participants with a chronic (complete) thoracic lesion demonstrate severe muscle atrophy of the lower limbs [185]. Consequently, our RIPC stimulus affects a relatively small muscle mass, possibly leading to a too small 'dose' to initiate beneficial effects of IPC. Therefore, possible explanations for the observation of a lack of RIPC in SCI individuals relates to the interruption of neural signalling and/or the volume of muscle mass available for the RIPC stimulus.

When explaining the absence of an effect of RIPC in SCI above, an important assumption is made that RIPC improves exercise performance in the able-bodied population. Despite some initial findings that support this assumption [101, 153], it should be emphasised that these previous studies were not directly set-up to compare IPC and RIPC and involved a single intervention-arm only (i.e. IPC on non-active area). Moreover, one of these studies explored "RIPC" by applying IPC to the arms, followed by a swim time trial [101]. Since both arms and legs are used during swimming, it is unlikely that a true remote IPC stimulus was applied. Therefore, we believe it is highly speculative whether RIPC improves exercise performance in the able-bodied population. Hence, at this stage, we can only speculate whether the lack of an effect of RIPC on exercise performance in our study is a general finding, or specifically relates to the population of SCI individuals.

The present observations raise questions about the potential underlying mechanisms. In line with most [18, 102, 156], but not all [16], previous studies, no differences were found for

maximum oxygen uptake after application of IPC. Therefore, it is unlikely that a larger maximal oxygen uptake explains the larger maximum performance in our study. Furthermore, no differences for other parameters that were examined during the incremental arm crank exercise test were found, like maximum RQ, time to RQ >1.00 and maximum HR. In a previous study, it was established that IPC impairs lactate accumulation during submaximal exercise, without affecting oxygen consumption [18]. Changes in metabolism may therefore contribute to these findings. To support this hypothesis, Schneider et al. have shown that paraplegic subjects have a delayed anaerobic threshold, accompanied by an increased rate of lipid utilization, in their upper body musculature compared to able-bodied subjects [186]. Furthermore, it has been shown that IPC is associated with muscle energy preservation [57, 187]. More specifically, these studies found that IPC maintained a higher muscle content of ATP and energy charge potential, and decreased muscle lactate content during ischaemia. In a follow-up study [188], the authors showed that opening of the mitochondrial ATP-sensitive potassium channels is likely contributing to the ATP-sparing effect induced by IPC. Furthermore, a study by Andreas et al. have shown that IPC positively influences muscle metabolism during reperfusion as evidenced by an increased production of phosphocreatine (Pcr) [189]. However, as we did not find any differences in maximal oxygen consumption and were not able to measure some of the suggested local mechanisms, we can only speculate about the potential causes for the observed performance enhancement in SCI after IPC.

Clinical Relevance. As sport participation in SCI has become increasingly popular on competitive level [175], SCI individuals have tried to enhance their exercise performance

with varying stimuli, including boosting [176]. 'Boosting' is defined as the intentional induction of autonomic dysreflexia and while boosting can lead to a significant increase in improvement in race time, it can be detrimental for health and is therefore forbidden [176]. IPC importantly differs from 'boosting' as IPC relates to a physiological stimulus (rather than pathological stimulus). In addition, we measured HR and BP during all three different interventions (unpublished data) to control for a 'boosting' like stimulus. In this study, no differences between the interventions or during the interventions were found, that would be present during a 'boosting' like stimulus. Therefore, IPC is likely to serve as a legal, non-harmful stimulus to enhance physical performance.

Limitations. A potential limitation of our study is that we did not include an able-bodied control group. Although performance enhancement IPC in able-bodied individuals is already well studied and our results are in line with these studies, based on our results, we cannot make conclusions about whether RIPC is possible in relation to exercise enhancement due to the lack of a control group. A second limitation is that upper body muscle tissue in able-bodied persons differs from SCI. Indeed, adaptations include an increase in the oxidative capacity of the upper-body musculature with a reduction in glycocogenolysis and a higher rate of lipid utilization [186]. Finally, we adopted a maximal incremental test, which represents a reliable test. However, it does not reflect a "real world" situation for the athlete during a sporting event. Consequently, our results cannot be simply extrapolated to a sport performance benefit for the individual athlete.

In conclusion, it was found that local IPC (applied to the upper limbs) significantly improved arm crank exercise performance during an incremental exercise test in subjects with a

complete thoracic SCI. This beneficial effect of IPC on exercise performance cannot be explained through changes in (sub)maximal oxygen uptake. Furthermore, and in marked contrast to IPC, no effect of RIPC (applied to the lower limbs) on arm crank exercise performance in SCI individuals was found. Whether these latter results also apply to healthy individuals should be subject of future research.

In chapters 4 and 5, it was established that IPC is effective in protecting the endothelium against IR-injury in healthy, young individuals. Our observations from chapter 6 and 7 provide further evidence for a strong link between IPC can exercise (performance). Interestingly, both IPC and exercise share the stimulus of repeated exposure to (local) ischaemia and reperfusion. To further focus on this link, it is also suggested that exercise exert similar preconditioning effects as IPC, with the consequence that (acute) exercise can precondition tissue and prevent or attenuate the impact of IR-injury. Therefore, in the next study, it is investigated whether exercise has similar preconditioning effects as IPC.

8

Interval exercise, but not endurance exercise, prevents endothelial ischaemia-reperfusion injury in healthy subjects

JOOST P.H. SEEGER, CHARLOTTE J. LENTING, TIM H.A. SCHREUDER, THIJS R.J. LANDMAN, N. TIMOTHY CABLE, MARIA T.E. HOPMAN, DICK H.J. THIJSSEN

AMERICAN JOURNAL OF PHYSIOLOGY. HEART AND CIRCULATORY PHYSIOLOGY 2015 FEB 15 ;308(4):H351-7

INTRODUCTION

Despite significant improvement in (pharmacological) treatment in recent decades, cardiovascular diseases remain the world's leading cause of death. As described in detail in previous chapters, IPC has been suggested as an effective strategy to reduce the negative consequences of IR-injury. Moreover, it is hypothesised that IR-injury of the endothelium contributes to cardioprotection and, subsequently, contributes to the outcomes in patients with coronary heart disease [190].

Exercise may also have preconditioning effects that reduces endothelial IR-injury, especially since some types of exercise have similar characteristics as IPC (i.e. short, repeated bouts of exercise/ischaemia). Preliminary data from animal studies revealed that (acute) exercise reduces cardiovascular injury associated with prolonged (potentially lethal) ischaemia [191-193]. Recently, Michelsen and co-workers provided further support that exercise may possess preconditioning effects [17]. They found that interval running exercise (4x2-min) and IPC (4x5-min) in humans, followed by blood withdrawal and perfused through isolated rabbit hearts, were similarly effective in reducing infarct size in the rabbit hearts [17]. These effects of exercise on cardiac IR-injury may also be present in humans adopting an *in vivo* model of endothelial IR-injury.

To date, little is known about the possible preconditioning effects of (acute) exercise in humans. Therefore, the aim of this study was to investigate the ability of an acute bout of exercise to prevent endothelial IR-injury in healthy, young humans. To study endothelial IR-injury, we adopted a frequently used and validated human *in vivo* model, similar to chapter 4 and chapter 5. Secondly, it was hypothesised that a single bout of interval exercise represents a more potent preconditioning stimulus than endurance exercise, as interval

exercise would lead to short periods of local deoxygenation of the working muscle mass, thereby mimicking 'mechanical' IPC application. Moreover, based on studies in which different modalities of exercise were studied [194], interval exercise presumably leads to different shear patterns, which lead to distinct shear patterns working on the endothelial layer. Therefore, the second aim was to compare the ability of a single bout of interval exercise *versus* endurance exercise to prevent endothelial IR-injury in healthy, young humans.

METHODS

Subjects

A total of 17 healthy volunteers were included to participate in this study (Table 1). All subjects performed regular physical activity, with an average sport participation of 6.7±5.0 h (range 1.5 to 17.5 h). Subjects were non-smokers, free of any cardiovascular disease, diabetes mellitus, hypertension (diastolic >90 and/or systolic BP >140 mmHg) and hypercholesterolemia (total cholesterol >6.5 mmol/L). In addition, obese subjects (BMI ≥30 kg/m^2) and those on medication potentially influencing the cardiovascular system were excluded. The study was conducted according to the Declaration of Helsinki (2000) and approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre.

Table 1. Baseline characteristics (n = 17)

Subject characteristics	Subjects
Male/female	10/7
Age, yr	23 ± 4
Body mass index, kg/m^2	21.8 ± 1.5
SBP, mmHg	121 ± 9
DBP, mmHg	73 ± 8
Heart rate, bpm	57 ± 9
Cholesterol, mmol/l	4.3 ± 0.5
Glucose, mmol/l	3.6 ± 0.5
Maximal Incremental cycling test	
Maximum oxygen uptake, ml/kg/min	52.4 ± 8.8
Maximum heart rate, bpm	193 ± 11
Maximum workload, wattage	309 ± 74
Post-exercise blood lactate, mmol/l	13.0 ± 1.6
Maximum respiratory exchange ratio	1.20 ± 0.06

Values are presented as mean ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure.

Experimental design

First, subjects performed an incremental cycling test to determine maximum oxygen consumption (VO_2max) and maximum workload (wattage). Subsequently, participants visited our laboratory on 3 different occasions (separated by at least 7 days, with a maximum of 1 month, Figure 1). During these visits, brachial artery endothelial function (using FMD) was examined under resting conditions, after the intervention (*i.* interval exercise, *ii.* endurance exercise and *iii.* control), and after IR (20-minutes of arm ischaemia, 20-minutes of reperfusion). In this study, 20 minutes reperfusion was chosen. This represents a small adjustment compared to the protocol adopted in chapter 4, since it was

found that 15 minutes of reperfusion was not sufficient for vessel diameter to return to baseline values. To examine local tissue oxygenation in the physically active lower limbs during the exercise bouts, near infrared spectrometry (NIRS) was placed on the thigh during exercise. All measurements for a subject were performed at the same time of day, whilst the order of testing (interval exercise, endurance exercise, and control) was randomised between subjects. The randomization procedure was performed using the Statistical Package for the Social Sciences (SPSS, version 20).

Day 1: Incremental cycling test

Subjects completed an incremental exercise test on a stationary bike as described in chapter 3. All tests included in our study met at least 3 out of 4 most often used quality assessment points: ±10 beats/min of the predicted maximum HR (208-age*0.7), levelling off VO_2 (<150 mlO_2 during last minute), post-exercise RER >1.1, and post-exercise lactate of >8.0 mmol/L [122].

Day 2-3-4: Experimental protocol

Subjects were instructed to abstain from caffeine, chocolate, alcohol and high doses of vitamin C for at least 18 hours before testing and were instructed to not perform strenuous exercise 24 hours before testing. Before each experiment, subjects refrained from food ingestion for at least 4 hours and were instructed to have a standardised meal on the measurement days (sandwiches with jam without butter). All subjects were tested at the same time of day to prevent diurnal variation in FMD response, while the measurements were performed in a temperature-controlled testing rooms and using recent guidelines of

FMD [34] and as described in chapter 3. Women were consistently tested in the luteal phase of their menstrual cycle [195].

The experimental protocol started with a 15-minute rest period in the supine position, followed by assessment of BP using a manual sphygmanometer (Welch Allyn pressostabil, The Netherlands) at the left upper arm. Subsequently, brachial artery endothelial function was examined using the FMD on the right arm. This was followed by a 43-minute intervention that consisted of: *i.* interval exercise, *ii.* endurance exercise, or *iii.* control period of rest in the supine position. Both exercise bouts were isocaloric and workload was individually calculated based on the maximum incremental test on day 1. This means that the total work performed by the individuals was the same between the endurance and interval exercise bout, excluding the possibility that differences in caloric expenditure could affect our outcomes. During exercise, we continuously monitored HR and local oxygenation of the right thigh using NIRS. Blood lactate concentration was measured immediately before and after the exercise bouts. Ratings of perceived exertion were obtained at the end of the exercise intervention, just before the cool-down period using a BORG 6-20 scale. After exercise, subjects rested for 30 minutes in the supine position in which another FMD was performed at the end of this period. Then 20 minutes of upper limb *ischaemia* and 20 minutes of *reperfusion* were applied, where after brachial artery FMD was measured again in order to determine the impact of IR-injury on the right arm.

Ischaemia-reperfusion injury

A rapid inflation/deflation pneumatic cuff (E10 rapid cuff inflator, Hokanson, USA) was positioned proximally around the right upper arm. IR was induced by 20 minutes of occlusion (cuff inflation to 220 mmHg) of the brachial artery, followed by 20 minutes of reperfusion.

This method represents a method to assess endothelial changes to IR and was already used in chapter 4 and chapter 5. As the vascular diameter did not return to baseline values after 15 minutes of reperfusion in the study conducted in older subjects (chapter 4), a 20 minutes reperfusion period was adopted to ensure diameter to return to baseline. Assessment of prolonged ischaemia (40-60 minutes), as applied in animal studies to the myocardium, is for obvious reasons not possible in humans

Interventions

Interval exercise

After a 10-minutes warm-up period at 30% of maximum workload, subjects performed ten 1-minute cycle exercise bouts at 100% of maximum workload. These 100%-bouts were separated by 2-minute recovery periods at with cycle exercise at 25% of maximum workload. The interval exercise session was finished with a 5-minute cool-down period at 30% of maximum workload.

Endurance exercise.

Cycle endurance exercise consisted of a 10-minute warm-up period at 30% of maximum workload, followed by a 28-minute exercise at 50% of maximum workload. The cycle endurance exercise session concluded with a 5-minute cool-down period at 30% of maximum workload. We ensured that subjects performed the same amount of total workload during both exercise bouts.

Control

Subjects rested in supine position for 43 minutes.

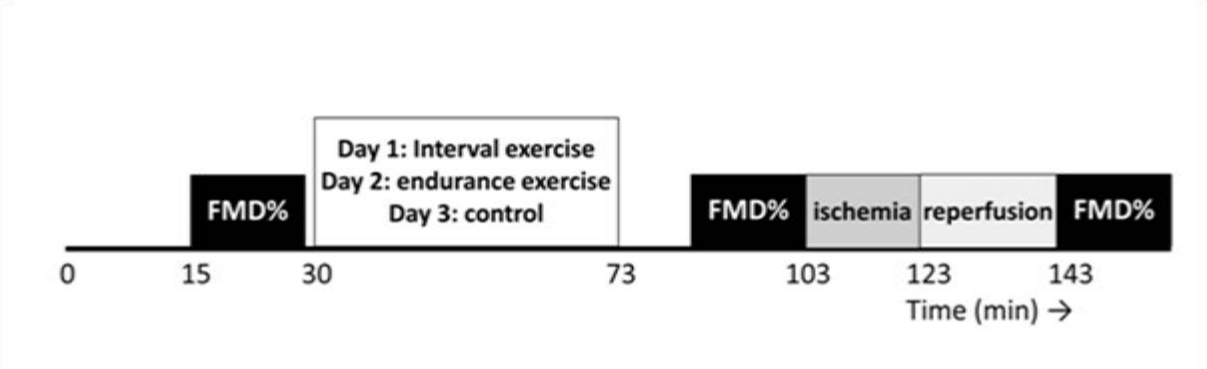

Figure 1. Experimental design (FMD: flow-mediated dilation).

Measurements

Flow-mediated dilation

All measurements were performed in a temperature-controlled room (22.5°C) and using recent guidelines of FMD [34], as described in more detail in chapter 3.

Near-infrared spectroscopy

NIRS was used to measure local oxygenation in the lower limb tissue during exercise by assessing regional concentration changes in oxyhaemoglobin (O_2Hb) and deoxyhaemoglobin (HHb) using a continuous-wave near-infrared spectrophotometer (OXYMON, Artinis Medical Systems, the Netherlands). The NIRS optodes were positioned on the right thigh, twelve centimetre proximal to the fibular head, on the vastus lateralis muscle parallel to the long

axis of the muscle. NIRS measurements were performed continuously. The changes in absorption measured by NIRS are converted into estimates of concentration changes of O_2Hb and HHb. The sum of O_2Hb and HHb reflects changes in blood volume, represented by the total haemoglobin signal (tHb) (Figure 2).

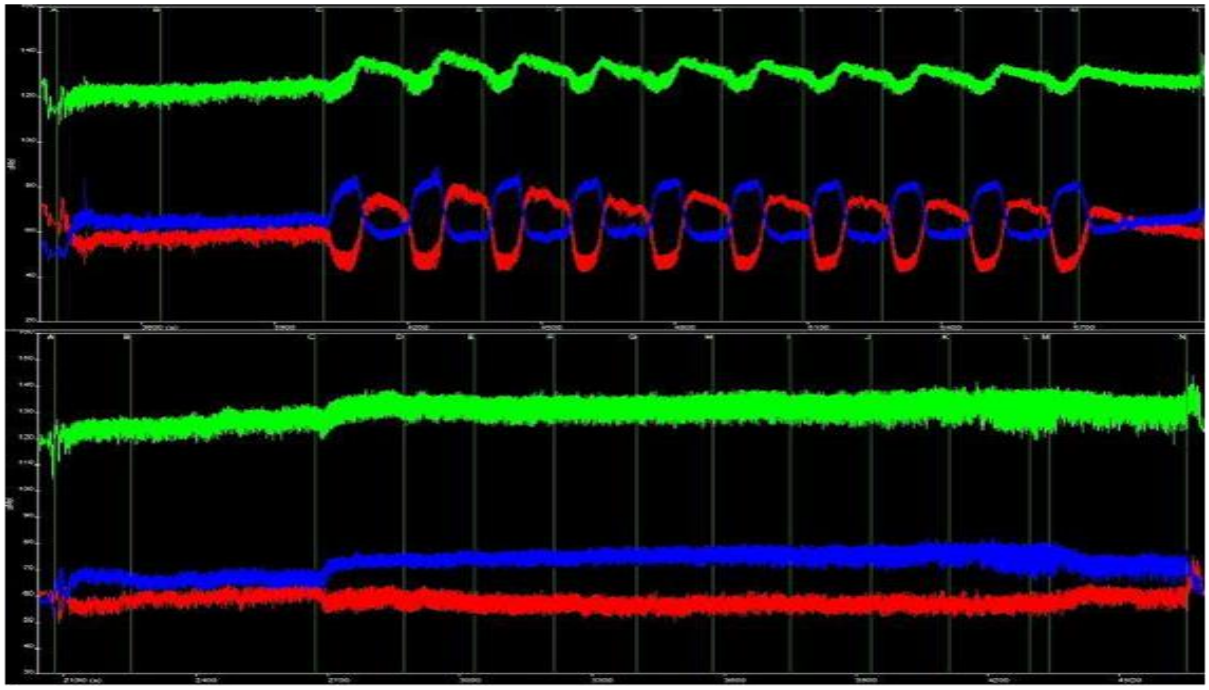

Figure 2. Near-infrared spectroscopy data for 1 subject. Upper graph (A) indicates interval exercise and bottom graph (B) indicates endurance exercise. Green line, total haemoglobin(tHb); Blue line, deoxyhaemoglobin(HHb); Red line, oxyhaemoglobin(O2Hb); X-axis, time(s); Y-axis, Hb-concentration(µMol); Vertical lines represent markers given every 3 minutes for additional data-analysis afterwards.

Near-infrared spectroscopy analysis

Baseline O_2Hb level was determined by averaging the 7-minute baseline period during warm-up. This typically represented a stable recording of O_2Hb (Figure 2). Subsequently, for each intervention total O_2Hb area-under-the-curve (HbO_2 total area) was calculated. Secondly, local oxygenation status was determined by calculating the positive area-under-the-curve above baseline ('positive area') and local deoxygenation/hypoxia as the negative area-under-the-curve below baseline ('negative area').

Statistical analysis

All statistical analyses were performed using SPSS 20 (SPSS, Chicago, IL, USA) software. Data were reported as mean±SD unless stated otherwise. Statistical significance was set at $P<0.05$. Baseline characteristics were compared with a paired Student's *t*-tests. To evaluate the effect of (different types of) exercise on endothelial IR-injury, we used a linear mixed model (LMM) with random factor 'subject' and fixed factors 'intervention' (interval exercise, endurance exercise, or control), 'time' (pre-intervention, post-intervention and post-IR) and the interaction 'intervention*time'. This differs from the analyses that were used in chapter 4 and chapter 5 where we adopted a 3-way ANOVA. The LMM represents a more advanced method that allows for controlling for various factors using this study design. This strategy was not available to us when performing the previous studies. In an additional analysis, shear rate area-under-the-curve and baseline diameter were added as covariates. and the analysis for FMD were repeated using allometric modelling [119], for reasons described in chapter 3.

Results

Interval exercise resulted in a higher post-exercise blood lactate, average HR and post-exercise BORG-score compared to endurance exercise (Table 2). There were no significant differences in (pre and post-exercise) BP between both exercise bouts (Table 2). During the endurance exercise bout, HR and estimated VO_2-uptake were 152±19 bpm and 33.9±10.6

mLO_2/kg/min (i.e. 64±14% of VO_{2peak}). During high-intensity exercise, HR and estimated VO_2 uptake of the first bout of high-intensity exercise was 167±11 bpm and 38.8±9.7 mLO_2/kg/min (i.e. 73±11% of VO_{2peak}). The last bout of high-intensity exercise was performed at 188±11 bpm and 49.4±9.7 mLO_2/kg/min (i.e. 95±6% of VO_{2peak}). HR and estimated VO_2 uptake during the rest intervals, was 147±14 bpm and 30.0±8.7 mLO_2/kg/min (i.e. 58±13% of VO_{2peak}).

Table 2. Exercise characteristics

Parameter	**Endurance exercise**		**P-value**	**Interval exercise**		**P-value**
	Pre	*Post*		*Pre*	*Post*	
SBP, mmHg	114 ± 10	109 ± 11	0.020	113 ± 9	108 ± 8	0.008
DBP, mmHg	70 ± 11	70 ± 10	0.887	68 ± 7	69 ± 7	0.771
Lactate, mmol/L	2.4 ± 0.8	3.3 ± 2.2	0.141	2.7±1.2	14.0 ± 2.7*	0.000
BORG-score		15 ± 2			18 ± 2*	
Peak heart rate		160±18			188±11*	

Values are presented as mean±SD. P-value represents a Student's paired t-test between pre and post-exercise. *Significantly different between endurance and interval exercise at P<0.05 (Student's paired t-test). SBP, systolic blood pressure; DBP, diastolic blood pressure.

Impact of exercise on endothelial IR

A significant interaction effect for the change in brachial artery FMD% after IR was found (P<0.001, Table 3). Whilst post-hoc analysis revealed no change in brachial artery FMD immediately after the intervention for all 3 conditions, a significant decrease was observed in FMD after IR during the endurance and control condition (LMM 'time', P<0.001). In marked contrast, the decline in FMD after IR was prevented by interval exercise (Post-hoc P=0.56, Figure 3). Repeating this analysis using allometric scaling to control for the potential impact of (inter- and intra-individual differences in) baseline diameter confirmed the

presence of a significant intervention (P=0.034), time (P<0.001) and intervention*time-effect (P<0.001).

The IR-procedure did not change brachial artery diameter (Table 3). No change in diameter was found after the control and endurance exercise session, whilst a significant increase in diameter was observed immediately after interval exercise (P<0.05, Table 3). The eliciting shear rate stimulus SR_{AUC} was significantly higher after interval exercise (Table 3), but returned to baseline levels during the post-IR measurement. The control and endurance exercise intervention showed no changes in $SR_{AUC.}$ To statistically control for the potential influence of these parameters, the analyses were repeated with the linear mixed model analysis with baseline diameter and SR_{AUC} as covariates. The analysis confirmed our earlier findings, and revealed a significant interaction-effect between time and intervention (P<0.001), with a decline in FMD after IR for the control and endurance exercise sessions, but not when interval exercise preceded IR.

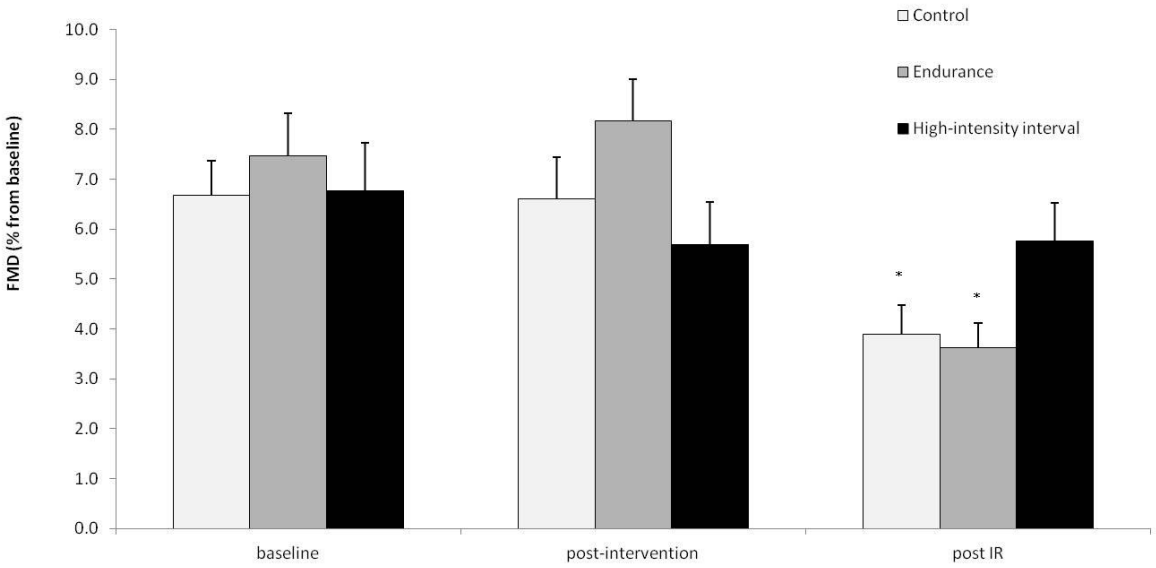

Figure 3. Flow mediated dilation ((FMD) % from baseline) at baseline, post-intervention and post ischaemia-reperfusion (IR) in control-, endurance- and interval intervention (n=17). Error bars represent standard error of the mean. *P<0.05.

Table 3. Brachial artery characteristics pre, post-intervention and post-IR in the control-, endurance- and interval intervention (n=17).

		'time'			LMM		
		Pre	*Post-Control*	*Post-IR*	'time'	'intervention'	Time*intervention
Baseline diameter, mm	*Control*	3.65±0.55	3.62±0.54	3.75±0.64	0.20	0.29	0.11
	Endurance	3.59±0.64	3.61±0.66	3.69±0.65			
	Interval	3.62±0.68	3.81±0.69	3.67±0.62			
FMD, mm	*Control*	0.26±0.08	0.25±0.11	0.16±0.09*	**<0.001**	**0.021**	**<0.001**
	Endurance	0.27±0.10	0.30±0.09	0.14±0.06*			
	Interval	0.27±0.11	0.25±0.11	0.25±0.09			
FMD, %	*Control*	7.1±2.3	6.9±3.0	4.3±2.3*	**<0.001**	**0.036**	**<0.001**
	Endurance	7.8±3.1	8.5±2.8	3.8±1.7*			
	Interval	7.7±3.1	7.0±3.4	7.2±3.1			
SR_{AUC}, s (10^3)	*Control*	29.9±6.9	28.1±10.3	26.5±11.7	**0.004**	**0.001**	0.32
	Endurance	31.8±7.1	33.8±6.9	28.0±8.1			
	Interval	32.5±9.8	38.0±7.1*	30.6±8.5			
Time to peak diameter, s	*Control*	49±15	53±21	46±25	**0.001**	0.25	0.37
	Endurance	48±19	60±20*	43±15			
	Interval	47±20	65±23*	53±25			

Values are presented as mean ± SD. IR, ischaemia-reperfusion; FMD, flow-mediated dilation; SR_{AUC}, area under the shear rate curve. *Post-hoc significantly different from Pre at $P < 0.05$.

Impact of exercise on local oxygenation status

NIRS data showed no significant differences between the endurance and interval exercise bout for the total area-under-the-curve for HbO_2 (63311±18048 and 60008±27941 A.U., respectively, P=0.21) and the area below baseline (-1.24±1.33 and -1.72±0.96 A.U, respectively, P=0.13). Endurance exercise was associated with a significantly smaller area above baseline compared to the interval exercise bout (1.28±1.67 and 3.72±2.38 A.U, respectively, P=0.03). Nonetheless, clear differences in the pattern of local HbO_2 can be observed between the endurance and interval exercise bout (Figure 2).

DISCUSSION

To the best of our knowledge, this is the first study examining whether an acute bout of exercise is able to protect the vascular endothelium against IR-injury in humans *in vivo*, and whether such preconditioning effect of exercise depends on the modality of exercise. The major finding of this study is that a single bout of interval exercise is able to protect the brachial artery endothelium against IR-injury *in vivo* in healthy young subjects. The observation that lower limb exercise (i.e. cycling) induced protective effects in the upper limb (i.e. brachial artery), suggests the presence of a *remote* preconditioning effect of interval exercise. Interestingly, when IR was preceded by a single bout of moderate-intensity endurance exercise, post-IR brachial artery endothelial function was impaired to a similar extent compared to the control condition. These findings indicate that a single, short-

duration bout of interval exercise, but not endurance exercise, possesses remote preconditioning effects in healthy, young subjects.

In this study, IR resulted in a significant decrease of ~40% in brachial artery endothelial function, measured as FMD. This reduction of 40% during the control condition is in line with chapter 4 and chapter 5, as well as those from others who adopted a similar protocol (38-65%) [107, 109, 113, 114]. A decrease in brachial artery FMD after IR was observed, which may reflect changes in tissue damage after ischaemia reperfusion. Furthermore, these results indicate that interval exercise is able to prevent endothelial IR-injury, which suggests that interval exercise induces preconditioning effects. An interesting observation is that exercise was performed using the lower limbs, whilst protection against endothelial IR was observed in the upper limbs. This observation provides support for a remote preconditioning effect of interval exercise, which suggests that also other vascular beds are protected against IR-injury.

In contrast to the observations during interval exercise, we found that the reduction in FMD after endothelial IR-injury remains present after performance of a bout of moderate-intensity endurance exercise in healthy subjects. Since the total workload was similar between both exercise bouts, the distinct impact on endothelial IR-injury may relate to differences in tissue oxygenation. Although not directly measured, another explanation could relate to differences in flow pattern, leading to potentially more shear stress during interval exercise. More specifically, it was hypothesised that interval exercise in contrast to endurance exercise would reveal similarities with 'mechanical' IPC, as both interval exercise

and IPC induce repeated, short periods of tissue de-oxygenation. Whilst our NIRS data revealed no differences in *total* deoxygenation between interval and endurance exercise, we indeed demonstrated that interval exercise, but not endurance exercise, induced repeated, short periods of ischaemia (Figure 2). This suggests that the *pattern*, rather than the total amount, of oxygenation contributes to the protective effects of a single bout of exercise on endothelial IR-injury. Future studies are warranted to better understand these potential underlying protective mechanisms of exercise on endothelial IR.

A recent paper studying the effects of exercise preconditioning, found that preconditioning effects of exercise are mediated by a blood born factor [17]. Moreover, they found that the preconditioning effects of a single bout of (interval) exercise are, at least partly, mediated through opioid receptors [17]. Exercise induces release of β-endorphins, especially when performed at higher intensity levels that lead to significant elevations of blood lactate [196-198]. Since β-endorphins contribute to the cardioprotective effects of IPC through their effects on opiod receptors, distinct release of β-endorphins between endurance and interval exercise (such as evident for blood lactate levels, Table 2) may contribute to these findings. Future studies are warranted to explore the potential role of β-endorphins and opioid-receptors in the preconditioning effects of exercise.

Another explanation for the preconditioning effects of interval exercise may relate to the NO-pathway. Although endurance exercise training improves eNOS expression [199], some recent evidence suggests a superior upregulation of the NO-pathway after interval training [200]. Another explanation may relate to the rapid depletion of ATP during interval exercise

compared to endurance exercise. Depletion of ATP activates the ATP-dependent potassium (K-ATP) channels in order to maintain the resting potential of the cells and to prevent cell death [201, 202]. The mitochondrial K-ATP channels are activated via a molecular cascade in which adenosine stimulates a G-coupled receptor protein transmitting the signal to a protein kinase C and, more importantly, are suggested to play a significant role in IPC [5, 202]. Whether (interval) exercise has protective preconditioning effects through eNOS and/or K-ATP channels remains unclear and should be subject of future research.

Study limitations

A limitation of our study is that there were no measures included to explore the potential mechanisms involved underlying the protective effects of (high-intensity interval) exercise on endothelial IR. However, the study was designed to explore whether (different forms of) excise could prevent endothelial IR-injury. Another limitation is that only healthy young individuals were included, and did not assess exercise bouts of different duration and/or intensity. Ischaemic events typically occur in an elderly population with cardiovascular risk and/or diseases. Whether exercise has similar preconditioning effects in groups with cardiovascular disease or risk is currently unknown and should be subject for future research. In this light, it is important to realize that the previous chapters showed that 'traditional' IPC in older subjects (chapter 4) and heart failure patients (chapter 5) fails to protect against the negative consequences of IR-injury. Exercise may represent a suitable, effective and safe alternative as a preconditioning stimulus in these groups. Furthermore, previous work has highlighted the impact of duration and intensity of exercise on the vasculature. Therefore, we cannot exclude the possibility that endurance exercise of

different intensity (i.e. above lactate threshold) or duration has preconditioning effects. Finally, we used the brachial artery endothelial function as a model to study IR-injury in the forearm. Although this model is frequently used by others as stated before, caution should be taken when extrapolating our findings to other vascular beds, such as the coronary circulation. Future studies are recommended to further validate this technique.

Clinical relevance

Exercise training has strong cardioprotective effects. Approximately 40% of the beneficial effects of exercise training can be explained by improvement in cardiovascular risk factors [31]. Possibly, the preconditioning effects of a *single* bout of (interval) exercise may contribute to the cardioprotective effects of exercise. Moreover, *repeatedly* performing this type of exercise (and therefore preconditioning) may protect against ischaemia-reperfusion injury. Previous work found that habitual endurance [47] or resistance [97] exercise (partly) prevented endothelial ischaemia-reperfusion injury. A potential implication of our results is that exercise training may possess preconditioning effects that ultimately protect against ischaemia-reperfusion injury. Future studies are needed to explore this hypothesis.

Conclusions

This study showed the ability of interval exercise to prevent endothelial IR-injury in a group of healthy young subjects, whilst this effect of exercise was absent when IR-injury was preceded by a single bout of short-duration, moderate-intensity endurance exercise. These protective effects of lower limb interval exercise were observed in the upper limb arteries,

which suggests that the preconditioning effects represent a 'remote', rather than a local, effect of interval exercise. These findings may have potential clinical relevance for the protection of the heart and other tissues against IR-injury by (interval) exercise.

9

Synthesis

Ischaemic preconditioning, a brief historical overview

Almost 30 years ago, Murry and co-workers [4] described the protective effect of IPC after a prolonged period of oxygen deprivation in the heart of dogs [4]. Their landmark study demonstrated that IPC prior to IR-injury resulted in a marked reduction in cardiac cellular damage of about 75%. Shortly after, others found that IPC also induced protective effects in other organs, such as the kidney [203] and the liver [204],which was confirmed by others who used different animal species and diverse organs sites [5]. In 1993 Przyklenk and her group described for the first time that IPC was also effective in areas that were not directly related to the IPC-intervention site [6]. This phenomenon is commonly referred to as RIPC and is of particular importance as it has marked advances when translating IPC to clinical care [112, 205]

IPC and the difficulties into translating the results into the clinic

In early research, primarily young, healthy animals were used to explore the benefits of IPC to improve understanding of the effects and mechanism of IPC. Although this work was very important, questions arose whether these models reflected the average cardiovascular patient and, subsequently, research was performed in diseased animals or in animals possessing certain cardiovascular risk factors [206]. Szilvassy et al. [207] reported for the first time that hypercholesterolemia, a well-known risk factor for developing cardiovascular disease, negatively influenced the responses to IPC [207]. It was established that hypercholesterolemia blocked the preconditioning effects in a rabbit model, which was irrespective of the development stage of atherosclerosis. This study was of major importance, as it provided the first evidence that cardiovascular disease/risk may negatively

affect efficacy of IPC (in animal models). In 1998 the same group published a review in which it was questioned whether IPC was a “healthy heart phenomenon” [208]. In this review, the complicated relation between disease state and preconditioning was discussed. Furthermore, it was concluded that clinically relevant pathological models were needed to assess the typical consequences of a clinically relevant disease state on efficacy of IPC. Unfortunately, to date, still a relatively small sample of research in the field of IPC is dedicated to gain insight into the impact of cardiovascular disease/risk on efficacy of IPC [209].

Diabetes is known to be a major risk factor for the development of cardiovascular disease [210]. Hence, a substantial number of patients with cardiovascular disease, and therefore potentially eligible for IPC-based interventions, are diagnosed with type 2 diabetes. Interestingly, an extended Pubmed research by Przyklenk [211] showed that diabetes was only included in 2 percent of the used models to assess the effect of IPC. Similarly, the impact of ageing, i.e. an important risk factor for cardiovascular disease and highly present in clinical populations that may benefit from IPC, was included in only 4% of the papers published [211]. Consequently, it was demonstrated that the vast majority (>90%) of IPC-related studies has been conducted using healthy, juvenile or adult populations that do not manifest the risk factors and comorbid conditions typically seen in patients with cardiovascular disease [212]. Therefore, only a few studies have contributed to the translation of IPC into the clinical setting. This is of special importance since the benefits of (traditional models and protocols of) IPC may be reduced in patients with cardiovascular disease/risk [213]. The lack of well-designed, clinical studies is an important factor for the difficulties of implementing IPC in daily practice [212, 213].

Another important limitation of previous work examining the IPC-effect in animals, is that animals often display a different pathological response to ischaemia or cardiac damage when compared to humans. These species differences further complicate the extrapolation of the results to clinical practice [209]. For example, animals respond differently to (the duration of) ischaemia, which is a major determinant of (cardiac) damage. Moreover, substantial between-species differences have even been reported for the impact of duration *and* location of infarction [214]. Another factor such as collateralization likely contributes to differences in response to ischaemia between species [214]. This highlights the need for studies in humans to better understand the impact and mechanisms of IPC as no animal model reflects the complexity of the human (coronary) circulation.

In our studies we included both older humans as well as patients with heart failure; two clinically relevant groups that are likely to represent populations that could benefit from IPC-interventions in *real life* situations. Unfortunately, we were not able to study the effects of IR-injury on the myocardium. Instead, we used a surrogate model, which involved assessment of vascular function of the brachial artery.

In order to observe the effects of IR-injury in these groups, the FMD of the brachial artery as a surrogate marker was used, as is described in chapter 3. Although the consequences of IR-injury and IPC could be observed, there are some clear limitations about the methods we have used. First, although there exists a strong correlation between the functional capacity of the coronary arteries and the brachial artery as described before, we still have to be careful into translating our results to the coronary arteries. In addition, we were limited to the time of ischaemia that could be given to our subjects. However, we were able to detect

differences between groups and between the given interventions using this protocol, which is similar to others who have used the same protocol[47, 110]. The studies presented in chapter 4 and chapter 5 were mainly observational and could benefit from extra analysis, as for example neutrophils count after IR-injury, measurements giving more insight into possible mechanisms, measurements with acetylcholine to control for endothelial function and other non-invasive vascular measurements, as for example Pulsed-wave Doppler.

Furthermore, and for obvious reasons, we are limited to the time of occlusion of the forearm. In our study we used an occlusion period of 20 minutes, which is not as long as the typical ischaemic period during a myocardial infarction [215]. Although the ischaemic period was relatively short, it proved to be long enough to find changes in endothelial function between healthy subjects and those with CV disease/risk. This model of 20-minutes of ischaemia leading to impaired endothelial function (but not necrosis) is clearly different to the clinical situation of a prolonged (~60 minutes) ischaemia of the heart, leading to impaired endothelial function and necrosis. Although much shorter, this model of 20-minutes of forearm ischaemia induces reduction in endothelial function. Furthermore, the duration of ischaemia may be inferior to the presence of reperfusion. It was for example found in cats, that a 90 minutes period of ischaemia alone, did not lead to detectable endothelium damage, while a short period of reperfusion after 90 minutes of ischaemia led to attenuated endothelial functioning [51]. The negative impact of reperfusion was greater after 20 minutes, than after only 2.5 minutes, which is suggestive a pivotal role for (the time of) reperfusion in relation to structural and functional damage to the endothelium. Despite the obvious dissimilarities with a myocardial infarction, our model is relevant in that it induces endothelial damage due to ischaemia-reperfusion injury. This study shows once more that the combination of ischaemia and reperfusion causes the detrimental changes in

endothelial health and this likely explains why impaired vascular function is present after a relatively short period of ischemia.

As vascular function ultimately returns to normal, our findings are therefore presumably based on the consequences of 'stunning', which is described as the mechanical dysfunction that persists after the restoration of spontaneous circulation [216]. Although stunning does reflect mechanical dysfunction, rather than actual tissue damage, in the myocardium stunning is the leading cause for early death after successful resuscitation [217] and therefore of great clinical importance.

Taken together, future studies could benefit from better mechanical insight. That way, it would be easier to translate the results. In addition, future studies should further explore the impact of different protocols, like an increase of the number of cycles or days that IPC will be given.

An important limitation of most clinical studies, is that ischaemia was planned, rather than spontaneous (such as present in clinical conditions with a myocardial infarction). Therefore, most clinical data relies on studies with elective interventions, as for example coronary artery bypass grafting, in which the number of participants studies are typically low [214]. Although there is some evidence that (R)IPC could exert beneficial effects in some type of patients, as for example shown in a meta-analysis performed by [218], most studies show disappointing results, as pointed out in two major reviews [219, 220]. They found no evidence that RIPC reduces mortality associated with ischaemic events, or led to a reduction in major adverse cardiovascular events [212]. Furthermore, the study performed by Healy and al. stated that data from pilot trials cannot confirm that RIPC has any significant effect on clinically relevant end-points, as for example: death, renal failure and stroke [221]. It was

discussed that studied populations consisted of heterogeneous groups, which likely affected the results. Hence, large clinical trials are needed and it is therefore worth mentioning that, recently two huge clinical trials have finished this year [222, 223].

The study performed by Meybohm et al. [222] represents a large multicentre trial in which almost 1,400 patients were analysed. These patients underwent a cardiopulmonary bypass and were treated with RIPC or SHAM. No differences in for example death, myocardial infarction and other and points were found and the authors concluded therefore that upper-limb RIPC did not show a relevant benefit among patients undergoing elective cardiac surgery. A second large multicentre trial was conducted in over 1,600 patients [223]. Patients undergoing coronary-artery bypass graft received either RIPC or SHAM and again no differences were found between the groups. These studies again stresses the importance about better clinical trials, to understand better how to transfer the preclinical knowledge into functioning clinical treatment. Although it must be emphasises that the use of specific drugs in these trials may have importantly confounded the negative study outcomes, as certain drugs can importantly interfere with the potential benefits of RIPC

In conclusion, although the effects of IPC are well determined in healthy, young preclinical models, translating the evidence into clinical practice is challenging and, at times, disappointing [219]. It is clear that the optimal design in IPC-related research does not exist, and therefore most evidence should be treated as ‘a pieces of a (very) complicated puzzle’. Future studies should focus more on typical clinical populations, and investigate IPC-interventions preferably in large clinical trials. Besides, more research should be done in specific groups, aiming for a better understanding of an optimized individualized IPC-protocol, as an increasing body of knowledge, including work presented in chapter 4 and

chapter 5, shows that there exists a lot of differences in responsiveness to the application of IPC.

Protocols

Although IPC has been studied extensively, the optimal IPC-protocol is largely unknown and represents a topic that is not frequently explored [218, 221]. Therefore most studies use a typical design in which an artery is blocked for 3-4 times for 5 minutes, interspersed with 5 minutes of reperfusion. Multiple variations to this 'default' protocol are possible, as one can vary in the number of cycles of occlusion, occlusion time, time before IR-injury, pressure of the cuff (when the arterial is occluded from outside), location of occlusion cuff, size of the ischaemic area, and the frequency of IPC (Figure 1). Additionally, IPC can be applied both locally as well as remotely, whilst studies have revealed that the benefits of (R)IPC are present immediately after application, but reappear 24-72 hours post-stimulus [7, 72]. Finally, application of repeated, brief periods of vascular occlusion has also been extensively studies *at the onset* of reperfusion (i.e. post-ischaemia rather than pre-ischaemia); a phenomenon which is termed 'ischaemic post-conditioning' [224]. To make things even more difficult, an optimal protocol may vary related to the endpoints being studied [225]. Taken together, these various factors make it very difficult to choose an optimal approach. Although some work suggests that there is no typical dose-response relationship in IPC, some studies tend to show better outcomes with different protocol that provide longer, more frequent or larger doses of ischaemia [226, 227]. These suggestions are of special importance for the clinical population that may demonstrate a smaller or attenuated efficacy of the traditional IPC protocol that consists of 3/4 cycles of ischaemia.

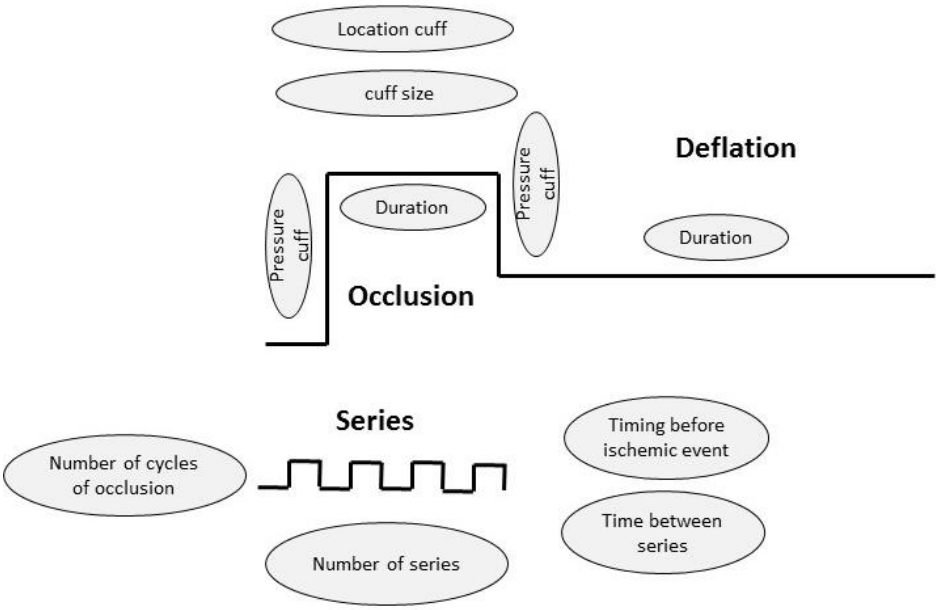

Figure 1. *Some possible variations to consider, when adopting an IPC-protocol. Figure is inspired by Buchheit et al.[228]*

Is IPC trainable?

Based on the potential impact of IPC, a very interesting question relates to the trainability of IPC. A single session of IPC is a safe and easy intervention to apply, but the effect of multiple IPC sessions within a certain time frame is currently largely unknown. In 2013, an *in vivo* study in humans was published in which it was demonstrated that daily episodes of IPC provided *sustained* protection from IR-injury [229]. Another recent, *in vivo* study, performed in young, healthy individuals, found improved vascular function after a 7-day daily exposure of the arm to IPC (4X5 minutes) [230]. Moreover, improved vascular function was established in both the treated arm as well as in the contralateral non-treated arm, suggesting that these effects of repeated IPC is trainable and works remotely. The same group performed a similar experiment with a longer duration of 8 weeks. Once more, improved vascular function was found, while no changes were observed in the control group [231]. Although these results in healthy young subjects are promising and provocative, once

again the question arises whether patients at increased cardiovascular risk would benefit from such a protocol. Interestingly, in a recent published study, the effect of intense RIPC - training (4X5 minutes brachial artery occlusion, 3 times a day; for a total duration of 20 consecutive days) on endothelial function in patients with coronary heart disease was studied [232]. In agreement with previous work in healthy volunteers, a significant increase in endothelial function was found after continued IPC application. Furthermore, another study explored the impact of 300-days of repeated, twice-daily IPC in stroke survivors. Remarkably, this study found that repeated IPC resulted in a better rehabilitation post-stroke, improved cerebral perfusion and significantly fewer stroke-recurrences [233]. This suggests that, somewhat in contrast to the application of single IPC in clinical groups, repeated IPC may have more potential to improve endothelial function and alter efficacy of IPC in subjects with cardiovascular risk/disease.

IPC and performance

In 2010, de Groot et al. were the first to report the beneficial effects of IPC in relation to exercise performance [16]. In their study, young healthy persons performed a maximal test after a control session or application of IPC. As hypothesised, they found improved exercise performance, as both the maximum oxygen uptake and the total work load improved after application of IPC. Inspired by these findings, others conducted similar experiments with varying protocols reporting mainly positive effects of IPC regarding exercise improvement as reported by Salavdor et al. in their meta-analysis [234].

Protocols

Studying the true effect of IPC on exercise enhancement is difficult as this intervention may introduce a placebo effect which is difficult to control for. Although the goal of most investigations is to keep the participants naïve, it remains difficult to implement a proper SHAM or a blinded control session as participants will feel the difference between the IPC (high pressure cuff, causing some discomfort) and the control of SHAM session (typically cuff inflation to low or no pressure). One could argue, therefore, that the effect of IPC could be based on a placebo-effect rather than a 'real' treatment effect.

Placebo has proven to be a very powerful in various circumstances. A nice example is provided by a study performed by Sihvonen et al. [235]. In this study, patients with symptoms of a degenerative medial meniscus tear and no knee osteoarthritis were included and randomly assigned to either an arthroscopic partial meniscectomy (normal care) or sham surgery. They followed these patients over a period of 12 months and found no differences in functional outcome and pain score. Also in sports it is well known that the performing athlete can extend performance limits and/or diminish fatigue perception under placebo conditions [236], and it is therefore not surprising that a recent paper boldly stated that IPC may not be a real but a placebo-effect [237]. As a counter-argument for a placebo effect, Bailey found that IPC altered the lactate kinetics during (sub)maximal running exercise, which strongly related to the improvement in 5-km running time (36-s). It seems unlikely that a placebo can alter biochemical processes underlying (sub)maximal running exercise. Nonetheless, these data highlight the importance that studying the impact of IPC on exercise performance should involve a well-designed protocol.

When summarizing studies that examined exercise improvement after application of IPC, a typical increase of 1-4% in workload, time to fatigue or other 'performance' parameters is observed. Studies presenting much higher values, as for example the study of Barbosa et al. (increase in time to task failure of about 11%), therefore should be interpreted with caution. In our study performed in spinal cord individuals (chapter 7), we found a small but significant increase in total time to exhaustion during a maximal incremental test on a hand bike. This improvement was within the expected range. Importantly, we found similar peak HR levels and BORG-scores between the interventions, which suggests that differences in performance were not motivational driven. Furthermore, in our 5-km time trial in able-bodied individuals (chapter 6), we investigated whether local *acute* IPC was as effective as local *delayed* (24-hours before running) in order to improve exercise performance. In this study two remarkable findings were made. First, it appeared that both acute and delayed IPC was effective in some, but not in all participants. It was remarkable to observe that participants who performed well after application of acute IPC, also performed well after the application of delayed IPC. In contrast, individuals who showed poorer results after acute IPC, also showed poorer results of delayed IPC. First, these data suggest that inter-individual differences exist regarding the impact of IPC on exercise performance, with strong relation between the effect of acute and delayed IPC. Secondly, one could argue that a placebo-effect is less likely, as the change in running time of acute IPC corresponded well with the change in running time after delayed (i.e. 24 hours) IPC.

Exercise related protocols

In chapter 6, a 5-km time trial was used. The main reasons for choosing the 5-km protocol instead of a maximal cycle test, was that a time trial better reflects an athlete performance.

In addition, Bailey et al. already successfully conducted a 5 km-trial in relation to IPC [18]. In order to minimize variance between 3 familiarisation sessions were performed before the actual measurement started, which resulted in a CV of 1.6%. These results are comparable to a study conducted by Laursen et al. [123]. In this study, it was investigated whether a time-to-exhaustion and time-trial exercise would differ in reliability. They found a greater level of absolute reliability (less variability) of time-trial compared with time-to-exhaustion running tests.

Although a 5km-trial was the best option in relation the research question of chapter 6, there are obviously a few limitations using this protocol. First, subjects can chose different pacing strategies, which might have an impact on the time trial. In order to minimize difference between the trials, first 3 trials were conducted, prior to testing in order to find an optimal strategy. In addition, to correct for this, pacing strategy was measured. Another main limitation relates to the fact, that the study presented in chapter 6 was mainly observational, although TSI, HR and end lactate were measured. As finish time was the main outcome, no gas analysing equipment was used, because this can influence the performance during running.

In contrast to chapter 6, the subjects in chapter 7 were tested using a maximal exercise incremental protocol. Like the study in chapter 6, also a time trial in the subjects with a SCI was preferred, but as the hand bike did not automatically correct for total workload, a time trial was not possible. Therefore a maximal incremental test was chosen, and has the advantage that it is well-validated and which has the ability to perform oxygen kinetics analysis [238].

In conclusion, one can state that there exists nog optimal protocol and that IPC testing which relates to performance enhancement is difficult, especially as the intervention itself is also

hard to control for. The optimal protocol for running would be on an indoor track with mobile equipment, but there are only few laboratory's that are able to conduct these tests under these circumstances.

(Proposed) mechanisms

Several potential mechanisms are discussed in literature. In this section, the most relevant mechanisms will be highlighted. A few studies focused on central hemodynamics and differences in maximal oxygen consumption. An increase in maximal oxygen consumption was found by de Groot and co-workers [16] after application of IPC. However, these results were not confirmed by others [18] [152, 155], including our study in spinal cord individuals, presented in chapter 7. Possible explanations for these conflicting results relate to differences in individuals, protocols and exercise types. In addition, Clevidence et al. investigated oxygen uptake during varying intensity levels up to 90% of the maximum intensity attained during a maximum test [156]. Again, no differences in oxygen consumption were found. Taken together, based on the currently available literature, it seems unlikely that a potential effect of ICP to enhance exercise performance is related to changes in oxygen uptake kinetics.

Bailey et al. postulated that IPC-related improvement in blood flow, could lead to a more optimal oxygen supply and faster removal of lactate [18]. In addition, it was suggested that IPC also improves muscle contraction efficiency, as for example established by Pang et al. [57]. These mechanisms may result in lower lactate levels. Indeed, Bailey and co-workers found that lactate levels during submaximal exercise showed an attenuated increase across incremental levels of submaximal running exercise after the application of IPC. In addition,

the same group also demonstrated that IPC prevented vascular impairment measured directly after exercise [103]. These findings favour a possible effect in the vasculature and may reflect a systemic rather than a local effect, as brachial vascular impairment was prevented after IPC application on the legs. In our 5-km study (chapter 6.), we were not able to measure lactate levels during exercise, but we measured lactate directly after exercise. Interestingly, we found that improvement in finish time was strongly related to a decrease in post-exercise blood lactate levels. In some respect, this finding confirms earlier observations from Bailey and co-workers, in that IPC may affect lactate metabolism and, subsequently, lead to changes in exercise performance.

In chapter 8, we also explored whether changes in local muscle oxygenation contribute to changes in exercise performance after IPC. This hypothesis is based on the fact that IPC-induced improvement in exercise performance may result from peripheral adaptations rather than from central cardiovascular adaptations. In our study, we were not able to detect differences in local muscle oxygenation of the quadriceps muscle during running exercise. This finding is in contrast with others, who found a marked decrease in local tissue oxygenation after the application of IPC [153, 154, 239]. An explanation for these conflicting results could relate to the fact that we did not find an overall improvement in exercise performance in all subjects. Barbosa et al. did not find a decrease in local oxygen consumption at relative intensity, but did find a difference in local oxygen uptake at different absolute intensities, most like caused by the longer duration of the IPC treated group [153]. Another explanation could relate to the fact that we were unable to measure baseline values before the trial, as participants were already performing a warming-up. Therefore, we were unable to correct for potential differences at baseline between groups. However, as the

previous mentioned studies found an overall lower oxygenation pattern after IPC, it is less likely that differences in baseline values explain our findings.

Taken together, IPC-related exercise performance enhancement remains a relatively poorly understood phenomenon. Studies so far, suggest favourable effects of IPC in relation to (some types of) exercise performance. Based on the heterogeneity of the studies, and the relatively short lifespan of this field of research, it is difficult to validly inform athletes about the use of IPC. Future research should therefore focus on better understanding of the potential underlying mechanisms, better (and more practical) protocols, and exploring the optimal conditions and/or protocols to apply IPC.

Exercise preconditioning

Exercise can serve as interesting therapeutic intervention in order to reduce the consequences of IR-injury. Already in 1978, it was described that chronic swim training in rats led to a marked reduction in infarct size after coronary artery occlusion [191]. This finding was suggested to be, at least partly, caused by improvement in the vasculature that resulted from this 5-week swim training. Possibly, these investigators demonstrated the powerful effects of (exercise) preconditioning. First evidence for 'exercise preconditioning' was provided a decade later, when researchers found the heart to be protected irrespective of adaptations within the heart [240]. Since these initial studies , some evidence supports the presence of an exercise preconditioning effect on the vasculature [241]. Therefore 'Exercise preconditioning', given the systemic nature and cardioprotective effects, may be

driven by a systemic, blood-borne factor. Michelsen and co-workers were the first to explore this hypothesis in humans [17]. They found that rabbits' hearts were protected against IR-injury when perfused with blood drawn from humans immediately after IPC, whilst a similar protection was found when blood was taken after intense, intermittent cycling exercise. Interestingly, the same authors demonstrated that these beneficial, protective effects of IPC *and* exercise were abolished when it was co-infused with an opioid receptor-blocker. This data, to our knowledge, provides the first evidence in humans that exercise can have preconditioning effects that are mediated through a blood-borne factor that works through opioid receptors.

As described in chapter 2, the onset of IPC can be triggered by multiple substances causing a signalling cascade that ultimately leads to (cardio)protection. Based on that principle, multiple researchers have focused on identifying pharmacological substances that can mimic this effect, without leading to 'factor X' that can be used to mimic IPC. Based on the results from Michelsen et al. [17], it seems interesting to further explore the role of opioids. A recent review explored whether opioids could be prescribed as preconditioning medication for myocardial ischaemia [242]. Opioids can function on 4 different type of groups of receptors: Mu, Delta, Kappa, and Nociceptin receptor [243]. When aiming for cardioprotection, the delta receptor seems the most logical target as these receptors are found in the human heart. The delta receptor responds best to the endogenous peptide enkephalin, but can also be stimulated by β-endorphins [244]. Interestingly, β-endorphins are released during (intense) exercise and, therefore, may represent a logical target in (exercise) preconditioning. Moreover, acute intravenous infusion of β-endorphins are also associated with improved left ventricular function and reduced systemic vascular resistance

in patients with mild to moderate chronic heart failure [245]. Although evidence is still small, β-endorphin release may represent an important target to explain preconditioning.

In an attempt to investigate whether exercise would lead to preconditioning *in vivo*, in chapter 8, we investigated the effect of two different modalities of exercise on brachial artery dysfunction. In this study, we revealed some interesting findings. First, high-intensity interval exercise successfully prevented the brachial artery from vascular injury after an IR-stimulus, while moderate intensity exercise exerted no effect at all. Second, the observed protective effect of high-intensity exercise proved to function systemically, as brachial artery dysfunction was prevented by doing leg exercise.

High intensity interval exercise

In chapter 8 we discussed the ability of high-intensity interval exercise in preventing brachial artery damage. We found that moderate intensity exercise did not exert similar beneficial effects. This finding is important for several reasons. First, it clearly shows the ability of high-intensity interval exercise to prevent brachial artery dysfunction after a longer duration of oxygen shortage. As exercise is a standard prescription in many patients with cardiovascular disease in the Netherlands, future research should focus on the ability of different types of exercise to prevent IR-injury. It could be an extra goal, in addition to goals as improving physical fitness and improving functional ability.

The observation that different types of exercise, although time– and workload-matched, exert different effects is important. Given the significant body of evidence regarding the strong and potent beneficial effects of exercise in improving health and in the prevention of

the development of chronic diseases, it is remarkable to notice that many of the mechanisms that explain this effect are poorly understood. As stated in chapter 2, we can only explain up to 60% of the beneficial effects provided by exercise [31].

Duration or intensity?

Although it was not the goal of this thesis to determine the effects of different types of exercise for a longer period, it is interesting to speculate whether to use endurance training or high-intensity interval training in daily live. For endurance training, it is suggested that the optimal training program consists of both high-intensity (short-duration) exercise training and low-intensity (high-volume) exercise training [246]. Both types of training exert different effects, but to date, several aspects are unclear how to optimize these components in order to achieve optimal exercise performance in well-trained athletes. High-intensity interval exercise consists of repeated, relatively short bouts of high-intensity exercise interspersed with recovery periods [247]. Potentially, not only endurance athletes could benefit from high-intensity interval exercise, but also individuals who are seeking alternatives compared to traditional forms of exercise for improving health. Interestingly, the last decade, increasing evidence suggests that low-volume, high-intensity interval exercise may represent a time-efficient strategy to induce health benefits that are normally only associated with endurance training [248]. Moreover, some evidence even suggests that high-intensity interval exercise is perceived as more enjoyable in healthy man [249], but also in overweight and obese individuals [250]. Moreover, possibly because high-intensity interval exercise is perceived as more enjoyable, overall adherence is suggested to be better than traditional moderate in training in for example subjects with prediabetes [251].

Furthermore, adherence has also found to be promising in cardiac rehabilitation [252]. Nonetheless, it is important to emphasize that no previous study provided strong evidence that high-intensity interval exercise leads to better adherence than traditional forms of exercise.

Is high-intensity interval exercise superior in patients with chronic disease?

High-intensity interval exercise is suggested to be a good alternative for traditional endurance training performed at moderate intensity, especially in specific type of patients who are expected to present with superior health effects compared to regular endurance training. One of the most cited studies in this area is the study performed by Wisloff and colleagues [99]. In summary, it was founds that high-intensity interval exercise led to an higher increase in physical fitness, vascular function and even left ventricular ejection fraction compared to moderate exercise training in a group with heart failure [99]. In support of these results, others found that high-intensity interval exercise has the ability to improve insulin sensitivity and glycaemic control in patients diagnosed with type 2 diabetes [253] and in obese man [254]..

While high-intensity interval exercise is mostly described as being superior to moderate intensity training, some recent papers investigating the effects of both protocols in cardiac rehabilitation found comparable improvements. For example, Cocks et al. [254] did not find a superior effect of high-intensity interval exercise in diabetic patients. Although they did not find a superior effect, they concluded that high-intensity interval exercise is more time efficient and therefore represents a nice alternative. Another example originates from a large multicentre trial, in which high-intensity interval exercise was investigated and

compared with moderate intensity training in patients with coronary artery disease. In contrast to Wisloff et al [99], similar improvements were found in exercise capacity and endothelial function [255]. These results were also found by others, who adopted the same exercise training protocols in patients with coronary artery disease [256]. Based on these results, one could argue that differences between the studies may be explained by differences in the selected patient population. However, from a recent study from the Department of Physiology at the Radboud University Medical Center in heart failure patients also revealed no differences between both exercise training protocols [257]. Possibly, and somewhat driven by the observation of large inter-individual differences in improvement after exercise training, it is speculated that treatment should be more individualised. In this line, inter-individual differences may contribute to differences in responses to different types of exercise training between individuals.

In conclusion, current evidence suggests that high-intensity interval exercise is a safe [258], enjoyable and time-effective way to perform exercise. However, more research is needed to specify the exact effects in different type of groups, but also aim to better understand why this type of exercise may lead to superior effects compared to traditional forms of exercise training. Moreover, as evidence is improving, one should carefully consider whether current guidelines regarding the total amount of exercise are still up-to-date, and maybe it is time to give *intensity* a more prominent share, next to the duration of physical activity.

Conclusion

In this thesis, we have tested IPC in various circumstances, using different subjects, with diverse objectives. In addition we have tested whether exercise might have similar beneficial outcomes. We have shown that IPC is a very promising intervention, but has also some clear restrictions, as for example seen in some specific groups. The contents of this thesis contribute to further understand this complicated phenomenon.

References

1. Roth, G.A., et al., *Global and Regional Patterns in Cardiovascular Mortality From 1990 to 2013.* Circulation, 2015. **132**(17): p. 1667-78.
2. Eltzschig, H.K. and T. Eckle, *Ischemia and reperfusion--from mechanism to translation.* Nat Med, 2011. **17**(11): p. 1391-401.
3. Jennings, R.B., et al., *Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog.* Arch Pathol, 1960. **70**: p. 68-78.
4. Murry, C.E., R.B. Jennings, and K.A. Reimer, *Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium.* Circulation, 1986. **74**(5): p. 1124-36.
5. Yellon, D.M. and J.M. Downey, *Preconditioning the myocardium: from cellular physiology to clinical cardiology.* Physiol Rev, 2003. **83**(4): p. 1113-51.
6. Przyklenk, K., et al., *Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion.* Circulation, 1993. **87**(3): p. 893-9.
7. Kuzuya, T., et al., *Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia.* Circ Res, 1993. **72**(6): p. 1293-9.
8. Baxter, G.F., et al., *Adenosine receptor involvement in a delayed phase of myocardial protection 24 hours after ischemic preconditioning.* Circulation, 1994. **90**(6): p. 2993-3000.
9. Ovize, M., H. Thibault, and K. Przyklenk, *Myocardial conditioning: opportunities for clinical translation.* Circ Res, 2013. **113**(4): p. 439-50.
10. Abete, P., et al., *Ischemic preconditioning in the younger and aged heart.* Aging Dis, 2011. **2**(2): p. 138-48.
11. Downey, J.M. and M.V. Cohen, *Why do we still not have cardioprotective drugs?* Circ J, 2009. **73**(7): p. 1171-7.
12. Bahjat, F.R., R. Gesuete, and M.P. Stenzel-Poore, *Steps to translate preconditioning from basic research to the clinic.* Transl Stroke Res, 2013. **4**(1): p. 89-103.
13. Lakatta, E.G., *Age-associated cardiovascular changes in health: impact on cardiovascular disease in older persons.* Heart Fail Rev, 2002. **7**(1): p. 29-49.
14. Passantino, A., et al., *Predicting mortality in patients with acute heart failure: Role of risk scores.* World J Cardiol, 2015. **7**(12): p. 902-11.
15. Granfeldt, A., D.J. Lefer, and J. Vinten-Johansen, *Protective ischaemia in patients: preconditioning and postconditioning.* Cardiovasc Res, 2009. **83**(2): p. 234-46.
16. de Groot, P.C., et al., *Ischemic preconditioning improves maximal performance in humans.* Eur J Appl Physiol, 2010. **108**(1): p. 141-6.
17. Michelsen, M.M., et al., *Exercise-induced cardioprotection is mediated by a bloodborne, transferable factor.* Basic Res Cardiol, 2012. **107**(3): p. 260.
18. Bailey, T.G., et al., *Effect of ischemic preconditioning on lactate accumulation and running performance.* Med Sci Sports Exerc, 2012. **44**(11): p. 2084-9.
19. Kavazis, A.N., *Exercise preconditioning of the myocardium.* Sports Med, 2009. **39**(11): p. 923-35.
20. Nichols, M., et al., *Cardiovascular disease in Europe 2014: epidemiological update.* Eur Heart J, 2014. **35**(42): p. 2929.
21. Leal, J., et al., *Economic burden of cardiovascular diseases in the enlarged European Union.* Eur Heart J, 2006. **27**(13): p. 1610-9.
22. Leening, M.J., et al., *Heart disease in the Netherlands: a quantitative update.* Neth Heart J, 2014. **22**(1): p. 3-10.
23. Engelfriet PM, H.R., Poos MJJC, Blokstra A, van Baal PHM, Verschuren WMM, *Heart failure: epidemiology, risk factors and future.* 2012.
24. British Heart Foundation, *Heart statistics.* 2014.

25. Boengler, K., R. Schulz, and G. Heusch, *Loss of cardioprotection with ageing.* Cardiovasc Res, 2009. **83**(2): p. 247-61.
26. Wang, M., R.E. Monticone, and E.G. Lakatta, *Arterial aging: a journey into subclinical arterial disease.* Curr Opin Nephrol Hypertens, 2010. **19**(2): p. 201-7.
27. Lloyd-Jones, D., et al., *Heart disease and stroke statistics--2010 update: a report from the American Heart Association.* Circulation, 2010. **121**(7): p. e46-e215.
28. Morris, J.N., et al., *Coronary heart-disease and physical activity of work.* Lancet, 1953. **265**(6796): p. 1111-20; concl.
29. Tanasescu, M., et al., *Exercise type and intensity in relation to coronary heart disease in men.* JAMA, 2002. **288**(16): p. 1994-2000.
30. Myers, J., et al., *Physical activity and cardiorespiratory fitness as major markers of cardiovascular risk: their independent and interwoven importance to health status.* Prog Cardiovasc Dis, 2015. **57**(4): p. 306-14.
31. Mora, S., et al., *Physical activity and reduced risk of cardiovascular events: potential mediating mechanisms.* Circulation, 2007. **116**(19): p. 2110-8.
32. Green, D.J., et al., *Exercise and cardiovascular risk reduction: time to update the rationale for exercise?* J Appl Physiol, 2008. **105**(2): p. 766-8.
33. Halcox, J.P., et al., *Endothelial function predicts progression of carotid intima-media thickness.* Circulation, 2009. **119**(7): p. 1005-12.
34. Thijssen, D.H., et al., *Assessment of flow-mediated dilation in humans: a methodological and physiological guideline.* Am J Physiol Heart Circ Physiol, 2011. **300**(1): p. H2-12.
35. Eltzschig, H.K. and C.D. Collard, *Vascular ischaemia and reperfusion injury.* Br Med Bull, 2004. **70**: p. 71-86.
36. Varadarajan, R., et al., *Nitric oxide in early ischaemia reperfusion injury during human orthotopic liver transplantation.* Transplantation, 2004. **78**(2): p. 250-6.
37. Parks, D.A. and D.N. Granger, *Contributions of ischemia and reperfusion to mucosal lesion formation.* Am J Physiol, 1986. **250**(6 Pt 1): p. G749-53.
38. Hausenloy, D.J. and D.M. Yellon, *Clinical translation of cardioprotective strategies : report and recommendations of the Hatter Institute 5th International Workshop on Cardioprotection.* Basic Res Cardiol, 2008. **103**(5): p. 493-500.
39. Yellon, D.M. and D.J. Hausenloy, *Myocardial reperfusion injury.* N Engl J Med, 2007. **357**(11): p. 1121-35.
40. Sanada, S., I. Komuro, and M. Kitakaze, *Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures.* Am J Physiol Heart Circ Physiol, 2011. **301**(5): p. H1723-41.
41. Pike, M.M., et al., *NMR measurements of Na+ and cellular energy in ischemic rat heart: role of Na(+)-H+ exchange.* Am J Physiol, 1993. **265**(6 Pt 2): p. H2017-26.
42. Piper, H.M., Y. Abdallah, and C. Schafer, *The first minutes of reperfusion: a window of opportunity for cardioprotection.* Cardiovasc Res, 2004. **61**(3): p. 365-71.
43. Halestrap, A.P., *A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection.* Biochem Soc Trans, 2010. **38**(4): p. 841-60.
44. Murphy, E. and C. Steenbergen, *Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury.* Physiol Rev, 2008. **88**(2): p. 581-609.
45. Ferdinandy, P., R. Schulz, and G.F. Baxter, *Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning.* Pharmacol Rev, 2007. **59**(4): p. 418-58.
46. Cines, D.B., et al., *Endothelial cells in physiology and in the pathophysiology of vascular disorders.* Blood, 1998. **91**(10): p. 3527-61.
47. Devan, A.E., et al., *Endothelial ischemia-reperfusion injury in humans: association with age and habitual exercise.* Am J Physiol Heart Circ Physiol, 2011. **300**(3): p. H813-9.
48. Kharbanda, R.K., et al., *Transient limb ischemia induces remote ischemic preconditioning in vivo.* Circulation, 2002. **106**(23): p. 2881-3.

49. Chan, W., et al., *Usefulness of transient and persistent no reflow to predict adverse clinical outcomes following percutaneous coronary intervention.* Am J Cardiol, 2012. **109**(4): p. 478-85.
50. Asiedu-Gyekye, I.J. and A. Vaktorovich, *The "no-reflow" phenomenon in cerebral circulation.* Medical science monitor : international medical journal of experimental and clinical research, 2003. **9**(11): p. BR394-7.
51. Tsao, P.S., et al., *Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat.* Circulation, 1990. **82**(4): p. 1402-12.
52. Le Page, S. and F. Prunier, *Remote ischemic conditioning: Current clinical perspectives.* J Cardiol, 2015. **66**(2): p. 91-6.
53. Gori, T. and S. Forconi, *The role of reactive free radicals in ischemic preconditioning--clinical and evolutionary implications.* Clin Hemorheol Microcirc, 2005. **33**(1): p. 19-28.
54. Jaffe, M.D. and N.K. Quinn, *Warm-up phenomenon in angina pectoris.* Lancet, 1980. **2**(8201): p. 934-6.
55. Yoshizumi, T., et al., *Amelioration of liver injury by ischaemic preconditioning.* Br J Surg, 1998. **85**(12): p. 1636-40.
56. Stenzel-Poore, M.P., et al., *Effect of ischaemic preconditioning on genomic response to cerebral ischaemia: similarity to neuroprotective strategies in hibernation and hypoxia-tolerant states.* Lancet, 2003. **362**(9389): p. 1028-37.
57. Pang, C.Y., et al., *Acute ischaemic preconditioning protects against skeletal muscle infarction in the pig.* Cardiovasc Res, 1995. **29**(6): p. 782-8.
58. Jabs, A., et al., *Ischemic and non-ischemic preconditioning: Endothelium-focused translation into clinical practice.* Clin Hemorheol Microcirc, 2010. **45**(2-4): p. 185-91.
59. Cohen, M.V., X.M. Yang, and J.M. Downey, *Conscious rabbits become tolerant to multiple episodes of ischemic preconditioning.* Circ Res, 1994. **74**(5): p. 998-1004.
60. Przyklenk, K. and R.A. Kloner, *Preconditioning: a balanced perspective.* Br Heart J, 1995. **74**(6): p. 575-7.
61. Barbosa, V., et al., *Preconditioning ischemia time determines the degree of glycogen depletion and infarct size reduction in rat hearts.* Am Heart J, 1996. **131**(2): p. 224-30.
62. Schulz, R., et al., *Ischemic preconditioning in pigs: a graded phenomenon: its relation to adenosine and bradykinin.* Circulation, 1998. **98**(10): p. 1022-9.
63. Morris, S.D. and D.M. Yellon, *Angiotensin-converting enzyme inhibitors potentiate preconditioning through bradykinin B2 receptor activation in human heart.* J Am Coll Cardiol, 1997. **29**(7): p. 1599-606.
64. Riksen, N.P., P. Smits, and G.A. Rongen, *Ischaemic preconditioning: from molecular characterisation to clinical application--part I.* Neth J Med, 2004. **62**(10): p. 353-63.
65. Gross, G.J. and J.A. Auchampach, *Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs.* Circ Res, 1992. **70**(2): p. 223-33.
66. Gross, G.J. and J.N. Peart, *KATP channels and myocardial preconditioning: an update.* Am J Physiol Heart Circ Physiol, 2003. **285**(3): p. H921-30.
67. Tapuria, N., et al., *Remote ischemic preconditioning: a novel protective method from ischemia reperfusion injury--a review.* J Surg Res, 2008. **150**(2): p. 304-30.
68. Lim, S.Y. and D.J. Hausenloy, *Remote ischemic conditioning: from bench to bedside.* Front Physiol, 2012. **3**: p. 27.
69. Tokuno, S., et al., *Spontaneous ischemic events in the brain and heart adapt the hearts of severely atherosclerotic mice to ischemia.* Arterioscler Thromb Vasc Biol, 2002. **22**(6): p. 995-1001.
70. Takaoka, A., et al., *Renal ischemia/reperfusion remotely improves myocardial energy metabolism during myocardial ischemia via adenosine receptors in rabbits: effects of "remote preconditioning".* J Am Coll Cardiol, 1999. **33**(2): p. 556-64.
71. Hausenloy, D.J. and D.M. Yellon, *Remote ischaemic preconditioning: underlying mechanisms and clinical application.* Cardiovasc Res, 2008. **79**(3): p. 377-86.

72. Marber, M.S., et al., *Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction.* Circulation, 1993. **88**(3): p. 1264-72.
73. Onody, A., et al., *Effect of classic preconditioning on the gene expression pattern of rat hearts: a DNA microarray study.* FEBS Lett, 2003. **536**(1-3): p. 35-40.
74. Woo, K.S. and H.D. White, *Factors affecting outcome after recovery from myocardial infarction.* Annu Rev Med, 1994. **45**: p. 325-39.
75. Kannel, W.B., *Incidence and epidemiology of heart failure.* Heart Fail Rev, 2000. **5**(2): p. 167-73.
76. Hausenloy, D.J., et al., *Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations.* Basic Res Cardiol, 2010. **105**(6): p. 677-86.
77. Ebrahim, Z., D.M. Yellon, and G.F. Baxter, *Ischemic preconditioning is lost in aging hypertensive rat heart: independent effects of aging and longstanding hypertension.* Exp Gerontol, 2007. **42**(8): p. 807-14.
78. Fenton, R.A., et al., *Aging reduces the cardioprotective effect of ischemic preconditioning in the rat heart.* J Mol Cell Cardiol, 2000. **32**(7): p. 1371-5.
79. Boengler, K., et al., *Loss of ischemic preconditioning's cardioprotection in aged mouse hearts is associated with reduced gap junctional and mitochondrial levels of connexin 43.* Am J Physiol Heart Circ Physiol, 2007. **292**(4): p. H1764-9.
80. He, Z., et al., *Aging blunts ischemic-preconditioning-induced neuroprotection following transient global ischemia in rats.* Curr Neurovasc Res, 2005. **2**(5): p. 365-74.
81. Przyklenk, K., G. Li, and P. Whittaker, *No loss in the in vivo efficacy of ischemic preconditioning in middle-aged and old rabbits.* J Am Coll Cardiol, 2001. **38**(6): p. 1741-7.
82. Dai, W., B.Z. Simkhovich, and R.A. Kloner, *Ischemic preconditioning maintains cardioprotection in aging normotensive and spontaneously hypertensive rats.* Exp Gerontol, 2009. **44**(5): p. 344-9.
83. Bartling, B., et al., *Ischemic preconditioning is not cardioprotective in senescent human myocardium.* Ann Thorac Surg, 2003. **76**(1): p. 105-11.
84. Wenzel, P., et al., *Manganese superoxide dismutase and aldehyde dehydrogenase deficiency increase mitochondrial oxidative stress and aggravate age-dependent vascular dysfunction.* Cardiovasc Res, 2008. **80**(2): p. 280-9.
85. Savitha, S., et al., *Oxidative stress on mitochondrial antioxidant defense system in the aging process: role of DL-alpha-lipoic acid and L-carnitine.* Clin Chim Acta, 2005. **355**(1-2): p. 173-80.
86. Sivonova, M., et al., *Relationship between antioxidant potential and oxidative damage to lipids, proteins and DNA in aged rats.* Physiol Res, 2007. **56**(6): p. 757-64.
87. Kleinbongard, P., et al., *Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans.* Free Radic Biol Med, 2006. **40**(2): p. 295-302.
88. Abete, P., et al., *Preconditioning does not prevent postischemic dysfunction in aging heart.* J Am Coll Cardiol, 1996. **27**(7): p. 1777-86.
89. Tani, M., et al., *Direct activation of mitochondrial K(ATP) channels mimics preconditioning but protein kinase C activation is less effective in middle-aged rat hearts.* Cardiovasc Res, 2001. **49**(1): p. 56-68.
90. Fenton, R.A., E.W. Dickson, and J.G. Dobson, Jr., *Inhibition of phosphatase activity enhances preconditioning and limits cell death in the ischemic/reperfused aged rat heart.* Life Sci, 2005. **77**(26): p. 3375-88.
91. Abete, P., et al., *Ischemic preconditioning in the aging heart: from bench to bedside.* Ageing Res Rev, 2010. **9**(2): p. 153-62.
92. Abete, P., et al., *A four-year-old rabbit cannot be considered the right model for investigating cardiac senescence.* J Am Coll Cardiol, 2002. **39**(10): p. 1701; author reply 1701-2.

93. Wever, K.E., et al., *Determinants of the Efficacy of Cardiac Ischemic Preconditioning: A Systematic Review and Meta-Analysis of Animal Studies.* PLoS One, 2015. **10**(11): p. e0142021.
94. Solomon, S.D., et al., *Influence of nonfatal hospitalization for heart failure on subsequent mortality in patients with chronic heart failure.* Circulation, 2007. **116**(13): p. 1482-7.
95. Murray, A.J., et al., *Insulin resistance, abnormal energy metabolism and increased ischemic damage in the chronically infarcted rat heart.* Cardiovasc Res, 2006. **71**(1): p. 149-57.
96. Miki, T., et al., *Cardioprotective mechanism of ischemic preconditioning is impaired by postinfarct ventricular remodeling through angiotensin II type 1 receptor activation.* Circulation, 2000. **102**(4): p. 458-63.
97. DeVan, A.E., et al., *Habitual resistance exercise and endothelial ischemia-reperfusion injury in young adults.* Atherosclerosis, 2011. **219**(1): p. 191-3.
98. Tjonna, A.E., et al., *Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: a pilot study.* Circulation, 2008. **118**(4): p. 346-54.
99. Wisloff, U., et al., *Superior cardiovascular effect of aerobic interval training versus moderate continuous training in heart failure patients: a randomized study.* Circulation, 2007. **115**(24): p. 3086-94.
100. Michelsen, M.M., et al., *Exercise-induced cardioprotection is mediated by a bloodborne, transferable factor.* Basic research in cardiology, 2012. **107**(3): p. 1-9.
101. Jean-St-Michel, E., et al., *Remote preconditioning improves maximal performance in highly trained athletes.* Med Sci Sports Exerc, 2011. **43**(7): p. 1280-6.
102. Crisafulli, A., et al., *Ischemic preconditioning of the muscle improves maximal exercise performance but not maximal oxygen uptake in humans.* J Appl Physiol, 2011. **111**(2): p. 530-6.
103. Bailey, T.G., et al., *Remote ischemic preconditioning prevents reduction in brachial artery flow-mediated dilation after strenuous exercise.* Am J Physiol Heart Circ Physiol, 2012. **303**(5): p. H533-8.
104. Bailey, T.G., et al., *Effect of Ischemic Preconditioning on Lactate Accumulation and Running Performance.* Med Sci Sports Exerc, 2012.
105. Naylor, L.H., et al., *Measuring peripheral resistance and conduit arterial structure in humans using Doppler ultrasound.* J Appl Physiol, 2005. **98**(6): p. 2311-5.
106. Liuni, A., et al., *Loss of the preconditioning effect of rosuvastatin during sustained therapy: a human in vivo study.* American journal of physiology. Heart and circulatory physiology, 2012. **302**(1): p. H153-8.
107. Loukogeorgakis, S.P., et al., *Role of NADPH oxidase in endothelial ischemia/reperfusion injury in humans.* Circulation, 2010. **121**(21): p. 2310-6.
108. Wouters, C.W., et al., *Short-term statin treatment does not prevent ischemia and reperfusion-induced endothelial dysfunction in humans.* J Cardiovasc Pharmacol, 2012. **59**(1): p. 22-8.
109. van den Munckhof, I., et al., *Aging attenuates the protective effect of ischemic preconditioning against endothelial ischemia-reperfusion injury in humans.* Am J Physiol Heart Circ Physiol, 2013. **304**(12): p. H1727-32.
110. Kharbanda, R.K., et al., *Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo.* Circulation, 2001. **103**(12): p. 1624-30.
111. Anderson, T.J., et al., *Close relation of endothelial function in the human coronary and peripheral circulations.* J Am Coll Cardiol, 1995. **26**(5): p. 1235-41.
112. Botker, H.E., et al., *Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial.* Lancet, 2010. **375**(9716): p. 727-34.
113. Loukogeorgakis, S.P., et al., *Remote ischemic preconditioning provides early and late protection against endothelial ischemia-reperfusion injury in humans: role of the autonomic nervous system.* J Am Coll Cardiol, 2005. **46**(3): p. 450-6.

114. Loukogeorgakis, S.P., et al., *Transient limb ischemia induces remote preconditioning and remote postconditioning in humans by a K(ATP)-channel dependent mechanism.* Circulation, 2007. **116**(12): p. 1386-95.
115. Walsh, S.R., et al., *Ischaemic preconditioning during cardiac surgery: systematic review and meta-analysis of perioperative outcomes in randomised clinical trials.* Eur J Cardiothorac Surg, 2008. **34**(5): p. 985-94.
116. Woodman, R.J., et al., *Improved analysis of brachial artery ultrasound using a novel edge-detection software system.* J Appl Physiol (1985), 2001. **91**(2): p. 929-37.
117. Black, M.A., et al., *Impact of age, sex, and exercise on brachial artery flow-mediated dilatation.* Am J Physiol Heart Circ Physiol, 2009. **297**(3): p. H1109-16.
118. Thijssen, D.H., et al., *Retrograde flow and shear rate acutely impair endothelial function in humans.* Hypertension, 2009. **53**(6): p. 986-92.
119. Atkinson, G., et al., *A new approach to improve the specificity of flow-mediated dilation for indicating endothelial function in cardiovascular research.* J Hypertens, 2013. **31**(2): p. 287-91.
120. Brunnekreef, J.J., et al., *Forearm blood flow and oxygen consumption in patients with bilateral repetitive strain injury measured by near-infrared spectroscopy.* Clin Physiol Funct Imaging, 2006. **26**(3): p. 178-84.
121. Tew, G.A., A.D. Ruddock, and J.M. Saxton, *Skin blood flow differentially affects near-infrared spectroscopy-derived measures of muscle oxygen saturation and blood volume at rest and during dynamic leg exercise.* Eur J Appl Physiol, 2010. **110**(5): p. 1083-9.
122. Midgley, A.W., et al., *Criteria for determination of maximal oxygen uptake: a brief critique and recommendations for future research.* Sports Med, 2007. **37**(12): p. 1019-28.
123. Laursen, P.B., et al., *Reliability of time-to-exhaustion versus time-trial running tests in runners.* Med Sci Sports Exerc, 2007. **39**(8): p. 1374-9.
124. Settergren, M., et al., *L-arginine and tetrahydrobiopterin protects against ischemia/reperfusion-induced endothelial dysfunction in patients with type 2 diabetes mellitus and coronary artery disease.* Atherosclerosis, 2009. **204**(1): p. 73-8.
125. Riksen, N.P., P. Smits, and G.A. Rongen, *Ischaemic preconditioning: from molecular characterisation to clinical application--part II.* Neth J Med, 2004. **62**(11): p. 409-23.
126. Hausenloy, D.J., et al., *Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations.* Basic research in cardiology, 2010. **105**(6): p. 677-86.
127. He, Z., et al., *Aging blunts ischemic-preconditioning-induced neuroprotection following transient global ischemia in rats.* Current neurovascular research, 2005. **2**(5): p. 365-74.
128. Liuni, A., et al., *Rosuvastatin prevents conduit artery endothelial dysfunction induced by ischemia and reperfusion by a cyclooxygenase-2-dependent mechanism.* J Am Coll Cardiol, 2010. **55**(10): p. 1002-6.
129. Boengler, K., et al., *Loss of ischemic preconditioning's cardioprotection in aged mouse hearts is associated with reduced gap junctional and mitochondrial levels of connexin 43.* American journal of physiology. Heart and circulatory physiology, 2007. **292**(4): p. H1764-9.
130. Abete, P., et al., *Angina-induced protection against myocardial infarction in adult and elderly patients: a loss of preconditioning mechanism in the aging heart?* J Am Coll Cardiol, 1997. **30**(4): p. 947-54.
131. Lee, T.M., et al., *Loss of preconditioning by attenuated activation of myocardial ATP-sensitive potassium channels in elderly patients undergoing coronary angioplasty.* Circulation, 2002. **105**(3): p. 334-40.
132. Black, M.A., et al., *Impact of age, sex, and exercise on brachial artery flow-mediated dilatation.* American journal of physiology. Heart and circulatory physiology, 2009. **297**(3): p. H1109-16.
133. Kunuthur, S.P., et al., *The Akt1 isoform is an essential mediator of ischaemic preconditioning.* Journal of cellular and molecular medicine, 2012. **16**(8): p. 1739-49.

134. Wojtovich, A.P., et al., *Ischemic preconditioning: the role of mitochondria and aging.* Exp Gerontol, 2012. **47**(1): p. 1-7.
135. Zhu, J., et al., *Cardioprotection of the aged rat heart by GSK-3beta inhibitor is attenuated: age-related changes in mitochondrial permeability transition pore modulation.* American journal of physiology. Heart and circulatory physiology, 2011. **300**(3): p. H922-30.
136. Halestrap, A.P., et al., *Mitochondria and cell death: a pore way to die?* Symp Soc Exp Biol, 2000. **52**: p. 65-80.
137. Koopman, C., et al., *Shifts in the age distribution and from acute to chronic coronary heart disease hospitalizations.* European journal of preventive cardiology, 2014.
138. Seal, J.B. and B.L. Gewertz, *Vascular dysfunction in ischemia-reperfusion injury.* Ann Vasc Surg, 2005. **19**(4): p. 572-84.
139. Hoskins, D.E., et al., *Myocardial infarct size is smaller in dogs with pacing-induced heart failure.* Cardiovasc Res, 1996. **32**(2): p. 238-47.
140. Sharikabad, M.N., et al., *Cardiomyocytes from postinfarction failing rat hearts have improved ischemia tolerance.* Am J Physiol Heart Circ Physiol, 2009. **296**(3): p. H787-95.
141. Ghosh, S., N.B. Standen, and M. Galinianes, *Failure to precondition pathological human myocardium.* J Am Coll Cardiol, 2001. **37**(3): p. 711-8.
142. Andersen, A., et al., *Right ventricular hypertrophy and failure abolish cardioprotection by ischaemic pre-conditioning.* Eur J Heart Fail, 2013. **15**(11): p. 1208-14.
143. Di Lisa, F., et al., *Mitochondria and reperfusion injury. The role of permeability transition.* Basic Res Cardiol, 2003. **98**(4): p. 235-41.
144. Kaul, N., et al., *Free radicals and the heart.* J Pharmacol Toxicol Methods, 1993. **30**(2): p. 55-67.
145. MacCarthy, P.A. and A.M. Shah, *Oxidative stress and heart failure.* Coron Artery Dis, 2003. **14**(2): p. 109-13.
146. Whittington, H.J., et al., *Cardioprotection in the aging, diabetic heart: the loss of protective Akt signalling.* Cardiovasc Res, 2013. **99**(4): p. 694-704.
147. Miki, T., et al., *Interruption of signal transduction between G protein and PKC-epsilon underlies the impaired myocardial response to ischemic preconditioning in postinfarct remodeled hearts.* Mol Cell Biochem, 2003. **247**(1-2): p. 185-93.
148. Thielmann, M., et al., *Cardioprotective and prognostic effects of remote ischaemic preconditioning in patients undergoing coronary artery bypass surgery: a single-centre randomised, double-blind, controlled trial.* Lancet, 2013. **382**(9892): p. 597-604.
149. Colombo, P.C., et al., *Endothelial cell activation in patients with decompensated heart failure.* Circulation, 2005. **111**(1): p. 58-62.
150. Liuni, A., et al., *Loss of the preconditioning effect of rosuvastatin during sustained therapy: a human in vivo study.* Am J Physiol Heart Circ Physiol, 2012. **302**(1): p. H153-8.
151. Hausenloy, D.J. and D.M. Yellon, *Myocardial ischemia-reperfusion injury: a neglected therapeutic target.* J Clin Invest, 2013. **123**(1): p. 92-100.
152. Crisafulli, A., et al., *Ischemic preconditioning of the muscle improves maximal exercise performance but not maximal oxygen uptake in humans.* J Appl Physiol (1985), 2011. **111**(2): p. 530-6.
153. Barbosa, T.C., et al., *Remote ischemic preconditioning delays fatigue development during handgrip exercise.* Scand J Med Sci Sports, 2014.
154. Kjeld, T., et al., *Ischemic preconditioning of one forearm enhances static and dynamic apnea.* Med Sci Sports Exerc, 2014. **46**(1): p. 151-5.
155. Kido, K., et al., *Ischemic preconditioning accelerates muscle deoxygenation dynamics and enhances exercise endurance during the work-to-work test.* Physiol Rep, 2015. **3**(5).
156. Clevidence, M.W., R.E. Mowery, and M.R. Kushnick, *The effects of ischemic preconditioning on aerobic and anaerobic variables associated with submaximal cycling performance.* Eur J Appl Physiol, 2012. **112**(10): p. 3649-54.

157. Tocco, F., et al., *Muscle Ischemic Preconditioning does not Improve Performance during Self-Paced Exercise.* Int J Sports Med, 2015. **36**(1): p. 9-15.
158. Reilly, T. and J. Waterhouse, *Sports performance: is there evidence that the body clock plays a role?* Eur J Appl Physiol, 2009. **106**(3): p. 321-32.
159. Noakes, T.D., *Time to move beyond a brainless exercise physiology: the evidence for complex regulation of human exercise performance.* Appl Physiol Nutr Metab, 2011. **36**(1): p. 23-35.
160. Noakes, T.D., *The Central Governor Model in 2012: eight new papers deepen our understanding of the regulation of human exercise performance.* Br J Sports Med, 2012. **46**(1): p. 1-3.
161. Noakes, T.D., A. St Clair Gibson, and E.V. Lambert, *From catastrophe to complexity: a novel model of integrative central neural regulation of effort and fatigue during exercise in humans.* Br J Sports Med, 2004. **38**(4): p. 511-4.
162. Tucker, R., *The anticipatory regulation of performance: the physiological basis for pacing strategies and the development of a perception-based model for exercise performance.* Br J Sports Med, 2009. **43**(6): p. 392-400.
163. Wu, Y.N., et al., *Noninvasive delayed limb ischemic preconditioning attenuates myocardial ischemia-reperfusion injury in rats by a mitochondrial K(ATP) channel-dependent mechanism.* Physiol Res, 2011. **60**(2): p. 271-9.
164. Guo, Y., et al., *Demonstration of an early and a late phase of ischemic preconditioning in mice.* Am J Physiol, 1998. **275**(4 Pt 2): p. H1375-87.
165. Lucia, A., et al., *Which laboratory variable is related with time trial performance time in the Tour de France?* Br J Sports Med, 2004. **38**(5): p. 636-40.
166. Qiu, Y., et al., *The early and late phases of ischemic preconditioning: a comparative analysis of their effects on infarct size, myocardial stunning, and arrhythmias in conscious pigs undergoing a 40-minute coronary occlusion.* Circ Res, 1997. **80**(5): p. 730-42.
167. Saito, T., et al., *Ischemic preconditioning improves oxygenation of exercising muscle in vivo.* J Surg Res, 2004. **120**(1): p. 111-8.
168. Patterson, S.D., et al., *The effect of ischemic preconditioning on repeated sprint cycling performance.* Med Sci Sports Exerc, 2015.
169. Buchheit, M. and P. Ufland, *Effect of endurance training on performance and muscle reoxygenation rate during repeated-sprint running.* Eur J Appl Physiol, 2011. **111**(2): p. 293-301.
170. Jacobs, R.A., et al., *Determinants of time trial performance and maximal incremental exercise in highly trained endurance athletes.* J Appl Physiol (1985), 2011. **111**(5): p. 1422-30.
171. Lee, S., et al., *Strenuous exercise induces mitochondrial damage in skeletal muscle of old mice.* Biochem Biophys Res Commun, 2015. **461**(2): p. 354-60.
172. Hepple, R.T., J.L. Hagen, and D.J. Krause, *Oxidative capacity interacts with oxygen delivery to determine maximal O(2) uptake in rat skeletal muscles in situ.* J Physiol, 2002. **541**(Pt 3): p. 1003-12.
173. McVeigh, S.A., S.L. Hitzig, and B.C. Craven, *Influence of sport participation on community integration and quality of life: a comparison between sport participants and non-sport participants with spinal cord injury.* J Spinal Cord Med, 2009. **32**(2): p. 115-24.
174. Gioia, M.C., et al., *Psychological impact of sports activity in spinal cord injury patients.* Scand J Med Sci Sports, 2006. **16**(6): p. 412-6.
175. Blauwet, C. and S.E. Willick, *The Paralympic Movement: using sports to promote health, disability rights, and social integration for athletes with disabilities.* PM R, 2012. **4**(11): p. 851-6.
176. Blauwet, C.A., et al., *Testing for boosting at the Paralympic games: policies, results and future directions.* Br J Sports Med, 2013. **47**(13): p. 832-7.
177. Mcclanahan, T.B., et al., *Brief Renal Occlusion and Reperfusion Reduces Myocardial Infarct Size in Rabbits.* Faseb Journal, 1993. **7**(3): p. A118-A118.

178. Gho, B.C., et al., *Myocardial protection by brief ischemia in noncardiac tissue.* Circulation, 1996. **94**(9): p. 2193-200.
179. Birnbaum, Y., S.L. Hale, and R.A. Kloner, *Ischemic preconditioning at a distance: reduction of myocardial infarct size by partial reduction of blood supply combined with rapid stimulation of the gastrocnemius muscle in the rabbit.* Circulation, 1997. **96**(5): p. 1641-6.
180. Oxman, T., et al., *Limb ischemia preconditions the heart against reperfusion tachyarrhythmia.* Am J Physiol, 1997. **273**(4 Pt 2): p. H1707-12.
181. Kirshblum, S.C., et al., *International standards for neurological classification of spinal cord injury (revised 2011).* J Spinal Cord Med, 2011. **34**(6): p. 535-46.
182. Groothuis, J.T., et al., *Venous cuff pressures from 30 mmHg to diastolic pressure are recommended to measure arterial inflow by plethysmography.* J Appl Physiol (1985), 2003. **95**(1): p. 342-7.
183. van den Berg-Emons, R.J., J.B. Bussmann, and H.J. Stam, *Accelerometry-based activity spectrum in persons with chronic physical conditions.* Arch Phys Med Rehabil, 2010. **91**(12): p. 1856-61.
184. van Koppenhagen, C.F., et al., *Wheelchair exercise capacity in spinal cord injury up to five years after discharge from inpatient rehabilitation.* J Rehabil Med, 2013. **45**(7): p. 646-52.
185. de Groot, P.C., et al., *Rapid and extensive arterial adaptations after spinal cord injury.* Arch Phys Med Rehabil, 2006. **87**(5): p. 688-96.
186. Schneider, D.A., et al., *VO2peak and the gas-exchange anaerobic threshold during incremental arm cranking in able-bodied and paraplegic men.* Eur J Appl Physiol Occup Physiol, 1999. **80**(4): p. 292-7.
187. Pang, C.Y., et al., *Role of ATP-sensitive K+ channels in ischemic preconditioning of skeletal muscle against infarction.* Am J Physiol, 1997. **273**(1 Pt 2): p. H44-51.
188. Moses, M.A., et al., *Mitochondrial KATP channels in hindlimb remote ischemic preconditioning of skeletal muscle against infarction.* Am J Physiol Heart Circ Physiol, 2005. **288**(2): p. H559-67.
189. Andreas, M., et al., *Effect of ischemic preconditioning in skeletal muscle measured by functional magnetic resonance imaging and spectroscopy: a randomized crossover trial.* J Cardiovasc Magn Reson, 2011. **13**: p. 32.
190. Powers, S.K., et al., *Ischemia-reperfusion-induced cardiac injury: a brief review.* Med Sci Sports Exerc, 2007. **39**(9): p. 1529-36.
191. McElroy, C.L., S.A. Gissen, and M.C. Fishbein, *Exercise-induced reduction in myocardial infarct size after coronary artery occlusion in the rat.* Circulation, 1978. **57**(5): p. 958-62.
192. Bowles, D.K., R.P. Farrar, and J.W. Starnes, *Exercise training improves cardiac function after ischemia in the isolated, working rat heart.* Am J Physiol, 1992. **263**(3 Pt 2): p. H804-9.
193. Brown, D.A., et al., *Exercise training preserves coronary flow and reduces infarct size after ischemia-reperfusion in rat heart.* J Appl Physiol (1985), 2003. **95**(6): p. 2510-8.
194. Thijssen, D.H., et al., *Brachial artery blood flow responses to different modalities of lower limb exercise.* Med Sci Sports Exerc, 2009. **41**(5): p. 1072-9.
195. Williams, M.R., et al., *Variations in endothelial function and arterial compliance during the menstrual cycle.* J Clin Endocrinol Metab, 2001. **86**(11): p. 5389-95.
196. de Vries, W.R., et al., *Dynamic exercise discloses different time-related responses in stress hormones.* Psychosom Med, 2000. **62**(6): p. 866-72.
197. de Meirleir, K., et al., *Beta-endorphin and ACTH levels in peripheral blood during and after aerobic and anaerobic exercise.* Eur J Appl Physiol Occup Physiol, 1986. **55**(1): p. 5-8.
198. Schwarz, L. and W. Kindermann, *Changes in beta-endorphin levels in response to aerobic and anaerobic exercise.* Sports Med, 1992. **13**(1): p. 25-36.
199. Hambrecht, R., et al., *Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase.* Circulation, 2003. **107**(25): p. 3152-8.

200. Rognmo, O., et al., *Endothelial function in highly endurance-trained men: effects of acute exercise.* J Strength Cond Res, 2008. **22**(2): p. 535-42.
201. Yellon, D.M., A.M. Alkhulaifi, and W.B. Pugsley, *Preconditioning the human myocardium.* Lancet, 1993. **342**(8866): p. 276-7.
202. Lalonde, F., et al., *Exercise-induced ischemic preconditioning detected by sequential exercise stress tests: A meta-analysis.* Eur J Prev Cardiol, 2013.
203. Cochrane, J., et al., *Ischemic preconditioning attenuates functional, metabolic, and morphologic injury from ischemic acute renal failure in the rat.* Ren Fail, 1999. **21**(2): p. 135-45.
204. Hardy, K.J., D.N. McClure, and S. Subwongcharoen, *Ischaemic preconditioning of the liver: a preliminary study.* Aust N Z J Surg, 1996. **66**(10): p. 707-10.
205. Przyklenk, K. and P. Whittaker, *Remote ischemic preconditioning: current knowledge, unresolved questions, and future priorities.* J Cardiovasc Pharmacol Ther, 2011. **16**(3-4): p. 255-9.
206. Przyklenk, K., T.H. Sanderson, and M. Huttemann, *Clinical benefits of remote ischemic preconditioning: new insights...and new questions.* Circ Res, 2014. **114**(5): p. 748-50.
207. Szilvassy, Z., et al., *The loss of pacing-induced preconditioning in atherosclerotic rabbits: role of hypercholesterolaemia.* J Mol Cell Cardiol, 1995. **27**(12): p. 2559-69.
208. Ferdinandy, P., Z. Szilvassy, and G.F. Baxter, *Adaptation to myocardial stress in disease states: is preconditioning a healthy heart phenomenon?* Trends Pharmacol Sci, 1998. **19**(6): p. 223-9.
209. McCafferty, K., et al., *The challenge of translating ischemic conditioning from animal models to humans: the role of comorbidities.* Dis Model Mech, 2014. **7**(12): p. 1321-33.
210. Kannel, W.B. and D.L. McGee, *Diabetes and cardiovascular risk factors: the Framingham study.* Circulation, 1979. **59**(1): p. 8-13.
211. Przyklenk, K., *Efficacy of cardioprotective 'conditioning' strategies in aging and diabetic cohorts: the co-morbidity conundrum.* Drugs Aging, 2011. **28**(5): p. 331-43.
212. Wider, J. and K. Przyklenk, *Ischemic conditioning: the challenge of protecting the diabetic heart.* Cardiovasc Diagn Ther, 2014. **4**(5): p. 383-96.
213. Hausenloy, D.J., 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.* Cardiovasc Res, 2013. **98**(1): p. 7-27.
214. Heusch, G., *Cardioprotection: chances and challenges of its translation to the clinic.* Lancet, 2013. **381**(9861): p. 166-75.
215. Bagai, A., et al., *Reperfusion strategies in acute coronary syndromes.* Circ Res, 2014. **114**(12): p. 1918-28.
216. Chalkias, A. and T. Xanthos, *Pathophysiology and pathogenesis of post-resuscitation myocardial stunning.* Heart Fail Rev, 2012. **17**(1): p. 117-28.
217. Ruiz-Bailen, M., et al., *Reversible myocardial dysfunction after cardiopulmonary resuscitation.* Resuscitation, 2005. **66**(2): p. 175-81.
218. Pei, H., et al., *Remote ischemic preconditioning reduces perioperative cardiac and renal events in patients undergoing elective coronary intervention: a meta-analysis of 11 randomized trials.* PLoS One, 2014. **9**(12): p. e115500.
219. Brevoord, D., et al., *Remote ischemic conditioning to protect against ischemia-reperfusion injury: a systematic review and meta-analysis.* PLoS One, 2012. **7**(7): p. e42179.
220. Remote Preconditioning Trialists, G., et al., *Remote preconditioning and major clinical complications following adult cardiovascular surgery: systematic review and meta-analysis.* Int J Cardiol, 2014. **176**(1): p. 20-31.
221. Healy, D.A., et al., *Remote ischaemic preconditioning as a method for perioperative cardioprotection: concepts, applications and future directions.* Int J Surg, 2014. **12**(10): p. 1093-9.
222. Meybohm, P., et al., *A Multicenter Trial of Remote Ischemic Preconditioning for Heart Surgery.* N Engl J Med, 2015. **373**(15): p. 1397-407.

223. Hausenloy, D.J., et al., *Remote Ischemic Preconditioning and Outcomes of Cardiac Surgery.* N Engl J Med, 2015. **373**(15): p. 1408-17.
224. Zhao, Z.Q., et al., *Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning.* Am J Physiol Heart Circ Physiol, 2003. **285**(2): p. H579-88.
225. Tomai, F., et al., *Ischemic preconditioning in humans: models, mediators, and clinical relevance.* Circulation, 1999. **100**(5): p. 559-63.
226. Zahir, T.M., et al., *Ischemic preconditioning of musculocutaneous flaps: effects of ischemia cycle length and number of cycles.* Ann Plast Surg, 1998. **40**(4): p. 430-5.
227. Kocman, E.A., et al., *Effects of ischemic preconditioning protocols on skeletal muscle ischemia-reperfusion injury.* J Surg Res, 2015. **193**(2): p. 942-52.
228. Buchheit, M. and P.B. Laursen, *High-intensity interval training, solutions to the programming puzzle: Part I: cardiopulmonary emphasis.* Sports Med, 2013. **43**(5): p. 313-38.
229. Luca, M.C., et al., *Daily ischemic preconditioning provides sustained protection from ischemia-reperfusion induced endothelial dysfunction: a human study.* J Am Heart Assoc, 2013. **2**(1): p. e000075.
230. Jones, H., et al., *Seven-day remote ischemic preconditioning improves local and systemic endothelial function and microcirculation in healthy humans.* Am J Hypertens, 2014. **27**(7): p. 918-25.
231. Jones, H., et al., *Impact of eight weeks of repeated ischaemic preconditioning on brachial artery and cutaneous microcirculatory function in healthy males.* Eur J Prev Cardiol, 2015. **22**(8): p. 1083-7.
232. Liang, Y., et al., *Long-term, regular remote ischemic preconditioning improves endothelial function in patients with coronary heart disease.* Braz J Med Biol Res, 2015. **48**(6): p. 568-76.
233. Meng, R., et al., *Upper limb ischemic preconditioning prevents recurrent stroke in intracranial arterial stenosis.* Neurology, 2012. **79**(18): p. 1853-61.
234. Salvador, A.F., et al., *Ischemic Preconditioning and Exercise Performance: A Systematic Review and Meta-Analysis.* Int J Sports Physiol Perform, 2015.
235. Sihvonen, R., et al., *Arthroscopic partial meniscectomy versus sham surgery for a degenerative meniscal tear.* N Engl J Med, 2013. **369**(26): p. 2515-24.
236. Pollo, A., E. Carlino, and F. Benedetti, *Placebo mechanisms across different conditions: from the clinical setting to physical performance.* Philos Trans R Soc Lond B Biol Sci, 2011. **366**(1572): p. 1790-8.
237. Marocolo, M., et al., *Are the Beneficial Effects of Ischemic Preconditioning on Performance Partly a Placebo Effect?* Int J Sports Med, 2015.
238. Kofsky, P.R., et al., *Field testing: assessment of physical fitness of disabled adults.* Eur J Appl Physiol Occup Physiol, 1983. **51**(1): p. 109-20.
239. Patterson, S.D., et al., *The Effect of Ischemic Preconditioning on Repeated Sprint Cycling Performance.* Med Sci Sports Exerc, 2014.
240. Brinkman, C.J., et al., *Assessment of hemodynamic function and tolerance to ischemia in the absence or presence of calcium antagonists in hearts of isoproterenol-treated, exercise-trained, and sedentary rats.* Eur J Cardiothorac Surg, 1988. **2**(6): p. 448-52.
241. Starnes, J.W. and R.P. Taylor, *Exercise-induced cardioprotection: endogenous mechanisms.* Med Sci Sports Exerc, 2007. **39**(9): p. 1537-43.
242. Ishii, H., *Cardioprotection with opioids-- trusted old friends -clinical science.* Curr Pharm Des, 2014. **20**(36): p. 5794-8.
243. Dragasis, S., et al., *The role of opioid receptor agonists in ischemic preconditioning.* Eur J Pharmacol, 2013. **720**(1-3): p. 401-8.
244. Selley, D.E. and J.M. Bidlack, *Effects of beta-endorphin on mu and delta opioid receptor-coupled G-protein activity: low-Km GTPase studies.* J Pharmacol Exp Ther, 1992. **263**(1): p. 99-104.

245. Cozzolino, D., et al., *Acute effects of beta-endorphin on cardiovascular function in patients with mild to moderate chronic heart failure.* Am Heart J, 2004. **148**(3): p. E13.
246. Laursen, P.B., *Training for intense exercise performance: high-intensity or high-volume training?* Scand J Med Sci Sports, 2010. **20 Suppl 2**: p. 1-10.
247. Billat, L.V., *Interval training for performance: a scientific and empirical practice. Special recommendations for middle- and long-distance running. Part II: anaerobic interval training.* Sports Med, 2001. **31**(2): p. 75-90.
248. Gibala, M.J., *High-intensity interval training: a time-efficient strategy for health promotion?* Curr Sports Med Rep, 2007. **6**(4): p. 211-3.
249. Bartlett, J.D., et al., *High-intensity interval running is perceived to be more enjoyable than moderate-intensity continuous exercise: implications for exercise adherence.* J Sports Sci, 2011. **29**(6): p. 547-53.
250. Smith-Ryan, A.E., *Enjoyment of high-intensity interval training in an overweight/obese cohort: a short report.* Clin Physiol Funct Imaging, 2015.
251. Jung, M.E., et al., *High-intensity interval training as an efficacious alternative to moderate-intensity continuous training for adults with prediabetes.* J Diabetes Res, 2015. **2015**: p. 191595.
252. Aamot, I.L., et al., *Long-term Exercise Adherence After High-intensity Interval Training in Cardiac Rehabilitation: A Randomized Study.* Physiother Res Int, 2015.
253. Little, J.P., et al., *Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes.* J Appl Physiol (1985), 2011. **111**(6): p. 1554-60.
254. Cocks, M., et al., *Sprint interval and moderate-intensity continuous training have equal benefits on aerobic capacity, insulin sensitivity, muscle capillarisation and endothelial eNOS/NAD(P)Hoxidase protein ratio in obese men.* J Physiol, 2015.
255. Conraads, V.M., et al., *Aerobic interval training and continuous training equally improve aerobic exercise capacity in patients with coronary artery disease: the SAINTEX-CAD study.* Int J Cardiol, 2015. **179**: p. 203-10.
256. Tschentscher, M., et al., *High-intensity interval training is not superior to other forms of endurance training during cardiac rehabilitation.* Eur J Prev Cardiol, 2014.
257. Benda, N.M., et al., *Effects of High-Intensity Interval Training versus Continuous Training on Physical Fitness, Cardiovascular Function and Quality of Life in Heart Failure Patients.* PLoS One, 2015. **10**(10): p. e0141256.
258. Rognmo, O., et al., *Cardiovascular risk of high- versus moderate-intensity aerobic exercise in coronary heart disease patients.* Circulation, 2012. **126**(12): p. 1436-40.