Translating organ conditioning

On the use of remote ischaemia and helium

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TABLE OF CONTENTS

Chapter 1

General introduction and outline of this thesis

ISCHAEMIA AND REPERFUSION

Interruption of blood flow leads to tissue injury and cell death in a process called ischaemia. This interruption can have multiple causes, such as an acute occlusion of a blood vessel, a state of systemic hypotension or low cardiac output. Treatment of ischaemia is therefore focused on correcting the underlying condition, for example by reopening an occluded blood vessel or restoring blood pressure and cardiac function.

On a cellular level, ischaemia is characterised by the inability to sustain adenosine triphosphate (ATP) production in the mitochondria. This impairs cell homeostasis and disturbs intracellular ion levels, as well as regulation of intracellular signal transduction pathways.

Unfortunately, restoration of local blood flow does not immediately stop the process of ongoing tissue injury, but in fact, might even aggravate it. During reperfusion intracellular calcium increases whilst the low pH that inhibited cell activation during ischaemia is now quickly raised. These changes cause hypercontracture in myocardial muscle cells and agitation in neurons. In addition, the re-supply of oxygen leads to the formation of free radicals. Intracellular signal transduction is influenced by regulation of intracellular kinases, e.g. by activation, phosphorylation or translocation. All these processes destabilise the mitochondrial membrane, further disturbing cellular energy production and ultimately leading to cell death.[1] Since there is not only tissue injury during ischaemia, but also during reperfusion as well, this is commonly referred to as ischaemia-reperfusion injury.

Clinical settings

Ischaemia-reperfusion injury is not a disease, but rather a common endpoint in different clinical situations. In the next section we will discuss a few of these settings. The common denominator will be cardiovascular disease, which is the most common cause of death in the Netherlands.

Acute myocardial infarction

Acute myocardial infarction causes 94 hospitalisations daily in the Netherlands, and is the cause of death of 14 patients per day.[2] Most infarctions are a complication of coronary artery disease and are caused by an acute occlusion of a coronary artery due to plaque rupture and subsequent clot formation, which obstructs blood flow and renders the downstream myocardium ischaemic. Swift reopening of the blood vessel by a percutaneous coronary intervention (PCI) is the treatment of choice in most cases, since it reduces the size of the infarction which leads to reduced mortality and morbidity.

Cardiac surgery

15.000 cardiac surgical procedures are performed yearly in the Netherlands alone, mostly as treatment for coronary artery disease or valvular heart disease. In most procedures it is necessary to arrest the heart to facilitate surgery. This is commonly achieved by interrupting the blood supply to the heart by cross-clamping the aorta and injecting a cardioplegic solution into the coronary arteries. This inherently causes an ischaemic insult to the heart and could lead to myocardial stunning or infarction.

Cardiac arrest

There were approximately 8000 cases of out-of-hospital cardiac arrest in 2018 in the Netherlands, with an overall survival of only 23%.[2] When cardiopulmonary resuscitation is successful, a dramatic form of ischaemia-reperfusion injury is seen. Even after successful resuscitation patients can still suffer from the consequences of ischaemia-reperfusion injury to the whole body sustained during the period of no-flow or low-flow and subsequent reperfusion. The brain is especially vulnerable and brain injury is the major cause of death and disabilities after out-of-hospital cardiac arrest.[3]

The benefit of a potential treatment for reperfusion injury should be clear. As mentioned before, the spectrum of ischaemia-reperfusion injury is broad. In this thesis, we will only focus on three specific scenarios; induced forearm ischaemia in healthy volunteers, myocardial ischaemia during cardiac surgery and post cardiac arrest treatment.

PROTECTION BY CONDITIONING

Ischaemic conditioning

For more than 30 years it has been known that ischaemia-reperfusion injury can indeed be reduced. However, until today, no form of conditioning has moved from the experimental setting into clinical practice. The golden standard in experimental settings has been ischaemic preconditioning: a series of short and non-lethal episodes of induced ischaemia is used to render that specific tissue resistant against subsequent ischaemia-reperfusion injury.[4] This powerful protective effect can reduce the amount of infarcted tissue up to 75%. It should be noted that preconditioning by itself is not enough to protect against ischaemia: reperfusion is still needed and when the period of ischaemia is too long, all ischaemic tissue will be lost. Implementing preconditioning requires both advanced knowledge of an ischaemic event as well as direct access to the target organ. Interestingly, reducing or interrupting blood flow during reperfusion also reduces the amount of tissue

damage in a process called postconditioning.[5] In contrast to preconditioning, postconditioning could also be used to treat an acute and unexpected ischaemic event such as a myocardial infarction.

Other forms of conditioning

Other methods exist that mimic the protective effect of short periods of induced ischaemia. Some examples include the use of opioids, statins and adenosine.[6-9] Volatile anaesthetic agents such as isoflurane and sevoflurane were also found to provide protection similar to ischaemic preconditioning when administered in a repetitive fashion.[10] These methods of conditioning have the benefit of not requiring direct access to a blood vessel or organ. However, volatile anaesthetics are still not the ideal conditioning agents since administration does require specialist equipment and can cause anaesthesia and impaired cardiovascular function.

Other forms of conditioning are likely more practical for clinical application and therefore more promising for use in patients: conditioning by remote ischaemia and helium inhalation.

Remote ischaemic conditioning

Ischaemic conditioning not only confers protection to that specific organ, but also to other parts of the body. This phenomenon is called remote ischaemic conditioning and was first discovered within one organ, when preconditioning one area of the heart was found to provide protection to the rest of the heart as well.[11] This phenomenon has been expanded and it has been demonstrated that a different organ or even skeletal muscle can be stimulated to achieve a protective effect in a distant organ.[12] Indeed, arms and legs are readily accessible and more resistant against ischaemia than most organs, making the idea of inducing ischaemia in the extremities to protect a more vulnerable target organ a very attractive construct. How the protection is being transferred through the body is not fully understood, but early clinical trials have already shown that such a remote protocol can reduce tissue injury after cardiac surgery.[13, 14]

Helium conditioning

In animal models of myocardial infarction, the gas helium also reduced myocardial injury, both when applied as a preconditioning as well as a postconditioning stimulus.[15, 16]

Helium is administered by inhalation in a mixture with oxygen, known as heliox, and is clinically used for its physical properties to treat patients with airway or pulmonary disorders. Whether helium can provide protection against ischaemia-reperfusion injury in humans as well was still unknown. However, in contrast to volatile anaesthetics helium has no known cardiovascular side-effects, is rather cheap and can be administered with only minor modifications to the existing ventilation equipment, making inhalation of helium potentially an attractive strategy to protect tissues against ischaemic injury.[17]

Mechanisms of conditioning

Protective intracellular signalling pathways, such as the reperfusion injury salvage kinase pathway (RISK pathway), play a central role in protection from ischaemia reperfusion injury. Key components of this pathway include extracellular regulated kinase 1 and 2 (ERK1/2), glycogen synthase kinase-3ß and protein kinase A. The common downstream target seems to be the mitochondria, where these pathways converge to prevent opening of the mitochondrial permeability transition pore, stabilising the mitochondrial membrane and inhibiting cell death. As an example, figure 1 shows an updated overview of different cellular mediators of helium-induced cardioprotection. Although there are some differences, significant overlap exists with ischaemic and remote conditioning.

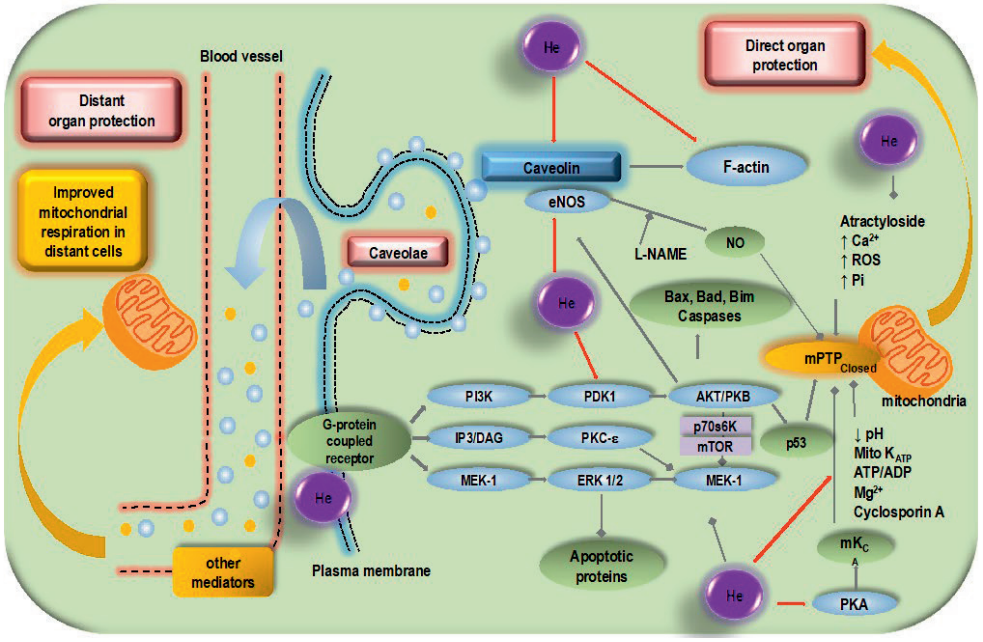

Figure 1 Overview of cellular mechanisms of helium-induced conditioning. Reproduced under Creative Commons License 4.0 from[18]

Helium is represented by the purple circles. The red arrows represent an activating effect; the squares an inhibiting effect. MEK-1 = mitogen-activated protein kinase-extracellular regulated kinase-1; ERK 1/2 = extracellular signal-regulated kinase 1 or 2; IP3 = inositol triphosphate-3; DAG = diacylglycerol; PKC-ε = protein kinase C epsilon; GSK3β = glycogen synthase kinase 3β; PI3K = phosphatidylinositol-3-kinase; PDK-1 = phophoinositide-dependent protein kinase-1; PKB = protein kinase B; mTOR = mammalian target of rapamycin; p53 = tumour protein p53; mPTP = mitochondrial permeability transition pore; eNOS = endothelial nitric oxide synthase; NO = nitric oxide; L-NAME = L-NG-nitroarginine methyl esther; PKA = protein kinase A; mKCa = mitochondrial calcium-sensitive potassium channel; ROS = reactive oxygen species; Pi = inorganic phosphate; ATP = adenosine triphosphate; ADP = adenosine diphosphate

OUTLINE OF THIS THESIS

Part I of this thesis is focussed on remote ischaemic conditioning. In **chapter 2** we performed a systematic review, collecting and assessing the available randomised clinical trials (as of July 2011) of remote ischaemic conditioning. We then performed a meta-analysis to investigate whether remote ischaemic conditioning leads to a reduction in biomarkers of cardiac tissue injury and an improvement in clinical outcome.

In **chapter 3** we investigated in a randomised controlled trial the effects of remote ischemic conditioning on molecular changes in the myocardium. We induced remote ischaemic

preconditioning by occluding forearm blood flow of patients undergoing coronary artery bypass grafting surgery. Since co-morbidities such as diabetes mellitus have a significant effect on organ protection by conditioning, two subpopulations were included; patients with or patients without diabetes mellitus.

Part II of the thesis explores the use of helium as a conditioning agent. First, in **chapter 4** we describe the effects of helium inhalation on endothelial function after forearm ischaemia-reperfusion in human volunteers.

We than investigated in a randomised controlled trial in coronary artery bypass grafting surgery whether helium pre- or post-conditioning could reduce myocardial injury, and whether known protective signal molecules are involved. These results are described in **chapter 5**.

Chapter 6 describes the use of helium in post cardiac arrest patients. Since there was no prior experience in this population we performed an open label safety and feasibility study to evaluate whether treating patients after cardiac arrest with helium inhalation would be an option.

Chapter 7 presents a secondary analysis of these same post cardiac arrest patients, focussing on pulmonary gas exchange. We hypothesized that due to the physical properties of the gas, heliox ventilation would improve carbondioxide (CO_2) removal from the body.

In **chapter 8** the results of these studies intended to reduces ischaemia-reperfusion injury are summarised and discussed in the light of the current literature.

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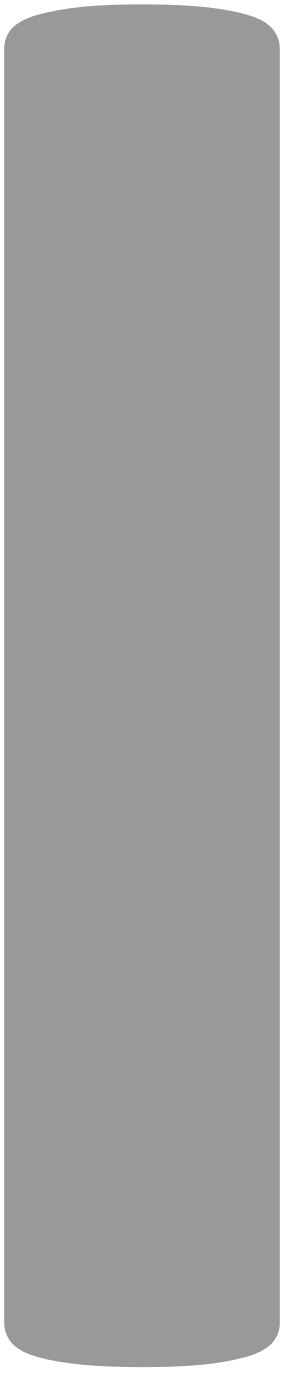

Part I

Remote ischaemia

2

Chapter 2

Remote ischaemic conditioning to protect against ischaemia-reperfusion injury: a systematic review and meta-analysis

Daniel Brevoord, Peter Kranke, Marijn Kuijpers, Nina C. Weber, Markus W. Hollmann, Benedikt Preckel

PLOS One 2012

ABSTRACT

Background: Remote ischaemic conditioning is gaining interest as potential method to induce resistance against ischemia-reperfusion injury in a variety of clinical settings. We performed a systematic review and meta-analysis to investigate whether remote ischaemic conditioning reduces mortality, major adverse cardiovascular events, length of stay in-hospital and in the intensive care unit and biomarker release in patients who suffer from or are at risk for ischaemia-reperfusion injury.

Methods: Medline, EMBASE and Cochrane databases were searched for randomised clinical trials comparing remote ischaemic conditioning, regardless of timing, with no conditioning. Two investigators independently selected suitable trials, assessed trial quality and extracted data.

Results: 23 studies in patients undergoing cardiac surgery (15 studies), percutaneous coronary intervention (four studies) and vascular surgery (four studies), comprising in total 1878 patients, were included in this review. Compared to no conditioning, remote ischaemic conditioning did not reduce mortality (odds ratio 1.22 [95% confidence interval 0.48, 3.07]) or major adverse cardiovascular events (0.65 [0.38, 1.14]). However, the incidence of myocardial infarction was reduced with remote ischemic conditioning (0.50 [0.31, 0.82]), as was peak troponin release (standardised mean difference -0.28 [-0.47, -0.09]).

Conclusion: There is no evidence that remote ischemic conditioning reduces mortality associated with ischaemic events; nor does it reduce major adverse cardiovascular events. However, remote ischaemic conditioning did reduce the incidence of peri-procedural myocardial infarctions, as well as the release of troponin.

INTRODUCTION

Organ ischaemia causes tissue damage in a variety of clinical settings, such as acute infarctions and/or hypoperfusion during surgery and organ transplantations. Restoration of adequate blood flow is the main treatment. However, even after restoration of blood flow tissue damage continues, partly induced by reperfusion itself. Research into the reduction of ischaemia-reperfusion injury has gained an impulse after the discovery of ischaemic preconditioning in 1986 by Murry et al.[1] In ischaemic preconditioning, a series of short non-lethal periods of ischemia interspersed with reperfusion periods, renders a target organ more resistant against a subsequent ischaemic event. In addition, ischaemic postconditioning, e.g. interrupted reperfusion or low-flow reperfusion, results in a reduction of damage compared to full-flow reperfusion.[2]

Remote ischaemic conditioning is an approach of conditioning in which not the target organ, such as the heart, but instead a more accessible tissue is submitted to a conditioning stimulus. First discovered to offer protection within one organ,[3] remote preconditioning has been found to offer protection against ischaemia-reperfusion injury also when a different organ, or even skeletal muscle tissue is used for conditioning.[4] Postconditioning is also possible with remote conditioning, offering the possibility of protecting patients suffering from unpredictable ischaemic events, such as acute infarctions.[5]

There is an increasing number of clinical studies investigating possible protection by remote ischaemic conditioning, mostly in cardiac and major vascular surgery. These studies show a reduction of the release of biomarkers like serum troponin values. The release of troponin is related not only to myocardial infarction, but also to other cardiovascular events in the post-operative period.

In 2008 Takagi et al. conducted a first meta-analysis of the four available studies, with a total of 184 patients undergoing cardiovascular surgery.[6] They found that remote ischaemic preconditioning reduced the release of biomarkers of myocardial injury, however, no analysis on adverse events was done. We performed a systematic review of an increased number of randomised trials investigating remote ischaemic conditioning to answer the question whether remote ischaemic conditioning, either before or during ischemia, improves the outcome of patients suffering from acute ischaemia or who are at risk of developing ischaemia during surgery.

METHODS

Search strategy and selection of papers

Medline, EMBASE and Cochrane databases were searched independently by two investigators (DB, MK) using the following terms: "remote preconditioning", "remote postconditioning", "remote perconditioning", "remote conditioning", "remote ischemic preconditioning", "remote ischemic postconditioning", "remote ischemic perconditioning", "remote ischemic conditioning", "remote ischaemic preconditioning", "remote ischaemic postconditioning", "remote ischaemic perconditioning" and "remote ischaemic conditioning." Literature was searched until July 2011, no search limitations were used and all found abstracts were screened for eligibility. Abstracts of papers identified through other sources were screened as well. Eligible trials were those in which patients suffering from, or being at risk for, ischaemia were randomly assigned to receive either remote ischaemic conditioning, regardless of the timing or the way the stimulus was induced, or no ischaemic conditioning. Full paper manuscripts were obtained of all selected articles based on the assessment of abstracts. Only fully published trials were included, abstracts and congress presentations were not included. Primary outcome to be assessed was mortality; secondary outcome parameter was the combined endpoint of major adverse cardiovascular events, which was defined as stroke, myocardial infarction, atrial fibrillation, or kidney injury. Additional secondary endpoints were length of stay in the hospital and in the intensive care unit, biomarkers of cardiac and kidney injury, and kidney function. Trials not reporting any of these parameters were excluded from the review. When the independent search led to papers included by only one author, this was discussed to reach consensus. Two authors independently reviewed all full reports that could possibly meet inclusion criteria, guided by the Cochrane Handbook for Systematic Reviews and the PRISMA statement.[7, 8] In case of disagreement, consensus was sought by discussion with a third author.

Reviewing and data extraction

All selected papers were reviewed by two investigators (DB, MK), who were not blinded for the origin of the paper, nor the authors. Both investigators independently extracted data to a data extraction sheet. Data on the study population, in- and exclusion criteria, control and intervention protocol, randomisation, blinding and follow-up were collected, as well as the outcome parameters mentioned above. Outcome parameters that were reported in the paper but were not the focus of this review were not extracted. Concerning the timing of events such as mortality, we used peri-operative or in-hospital event rate, if reported. For clinical parameters such as the occurrence of myocardial infarction or kidney injury, the

criteria of the respective paper were used. We did not extract data on myocardial infarction from studies that were done in patients suffering from myocardial infarction.

With respect to biomarker data, we used the peak values as reported in the paper. We renamed both troponin-I and troponin-T "troponin", and to compensate for the different types of troponin as well as the variation induced by different methods of analysis we used the standardized mean difference for analysis. If the data was presented in a graph, but not in text, we requested the corresponding author of the paper to provide these data. If these data were not provided we extrapolated it from the graph if the scale allowed a sufficiently precise estimation, i.e. if the scale and resolution of the graph allows the extraction of the graph without ambiguity. Inconsistency of the extracted data was discussed among the investigators, clarified and accounted for.

After extraction of relevant data to the data sheets by the two investigators, these were checked for inconsistencies, which were then resolved by discussion and joint reviewing of the paper. In case consensus couldn't be reached, a third investigator would be consulted (BP).

Papers were assessed for risk of bias by DB and MK on the following areas: randomisation sequence generation, concealment of randomisation sequence, blinding of intervention, blinding of outcome assessment, incomplete outcome reporting, and were classified as having low risk, high risk or unclear risk of bias for each item, as suggested in the Cochrane Handbook.[7]

Data analysis

The verified data were analysed using Review Manager (Version 5.1).[9] DB entered the data, MK verified data entry. The odds ratio (Mantel-Haenszel), mean difference (inverse variance) and their corresponding 95% confidence intervals (95% CI) were calculated for dichotomous or continuous outcome data, respectively. A fixed-effect model was used in case of no relevant statistical heterogeneity. Statistical heterogeneity was assessed with the I^2-test and assumed when I^2 was >25 %. A random-effect model was used in all other cases. A significant effect of an intervention was assumed if the 95% CI did not include the value 1.0 for odds ratio or 0 for mean difference. When the standard error was reported but not the standard deviation, the standard deviation was calculated. If continuous data was reported as median and range, we calculated an estimation of the mean and standard deviation as described by Hozo et al.[10] When not the range, but the interquartile range was reported, we assumed this to be 1.35 standard deviations.

Studies containing zero events in both arms are excluded when calculating an odds-ratio. To avoid over-estimating a possible effect due to the exclusion of patients in studies with no events, we recalculated the odds-ratio of dichotomous outcomes after adding one event in each arm, allowing us to include the patients in those studies.

Forrest plots were used for graphical presentation, as well as an l'Abbé plot for dichotomous endpoints. We intended to perform a sub-group analysis for different patient populations if more than four studies were available: cardiac surgery, coronary artery bypass grafting surgery, valve-surgery, paediatric cardiac surgery and percutaneous coronary intervention (acute and elective).

RESULTS

Description of studies

Search results

Searching resulted in 501 hits in Medline using PubMed, 226 hits in EMBASE and 42 hits in the Cochrane data base of clinical trials (see Figure 1), as well as one additional abstract identified through other sources. After checking for duplicates, 594 unique references remained. All abstracts were screened and 28 papers were identified for further evaluation, which were obtained in full print. Reading the full prints resulted in the direct exclusion of two papers,[11, 12] and after discussion between the three investigators of another two,[13, 14] that did not report any of the outcome parameters, which left 24 papers to be included for review and analysis (Table 1). One paper[15] provided additional data on kidney injury in patients of two previously published studies,[16, 17] thus, 23 clinical studies were finally analysed.

Included studies

Most studies were done in patients undergoing cardiac surgery, including 10 studies in coronary artery bypass grafting surgery surgery,[16-24] and five in other cardiac surgery.[25-29] Four studies were done in patients undergoing percutaneous coronary intervention, of which two were elective[30, 31] and two were acute studies.[32, 33] Another four studies were done in patients undergoing vascular surgery.[34-37] Median trial size was 66 patients (range 8, 333 patients). Due to the small number of trials we only performed a sub-group analysis for the patients undergoing coronary artery bypass grafting surgery.

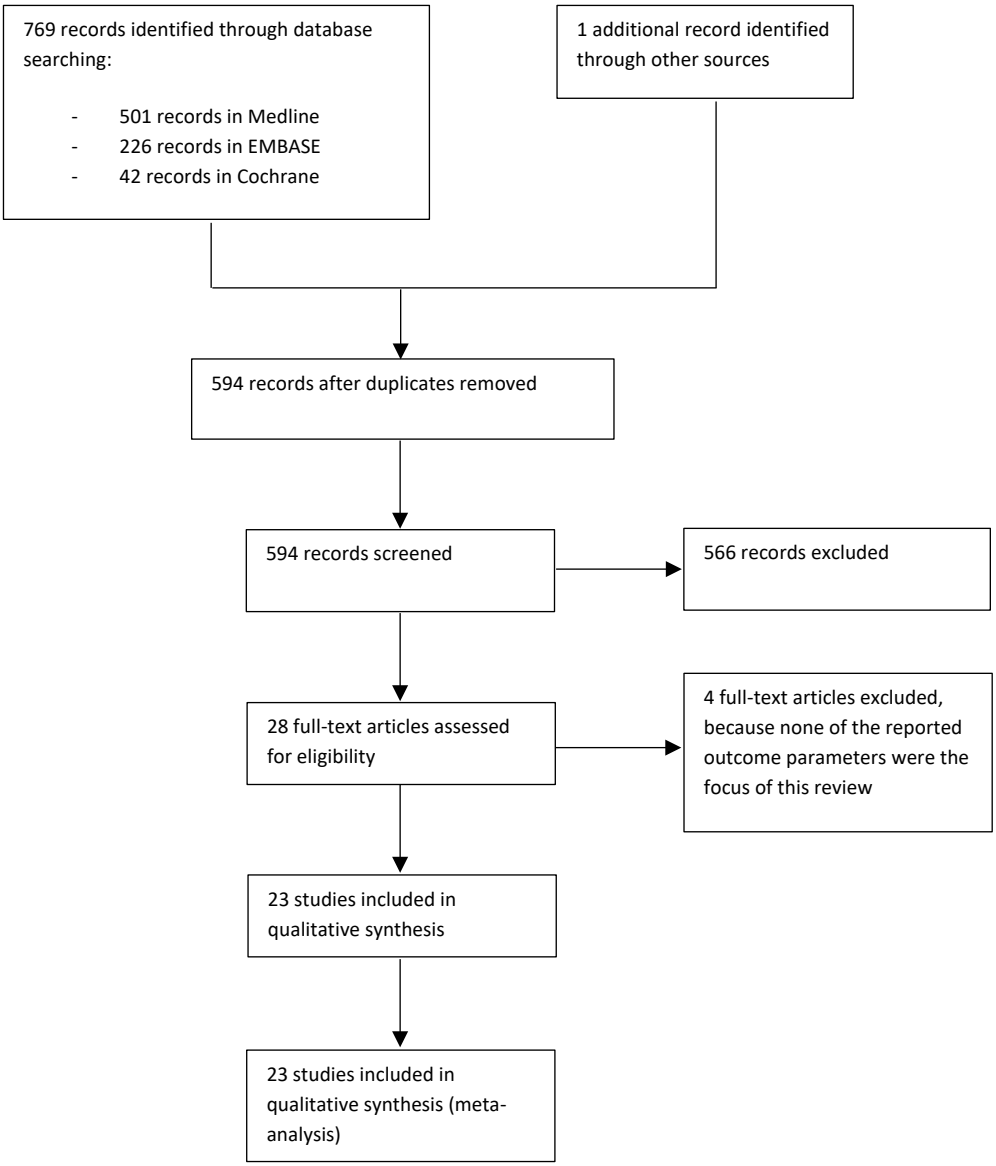

Figure 1. Flow-schedule of search and selection of studies.

Table 1. Overview of included studies.

Study	Patient population	*N*	Groups	Form of remote conditioning	Endpoints	Results
Ali 2007[34]	Open AAA[a] repair	82	Remote ischaemic preconditioning VS control	Leg ischaemia by clamping of the common iliac artery, 10 minutes left and 10 minutes right	Myocardial injury, as defined by an increase in serum cardiac troponin > 0,40 ng/ml Sec: myocardial infarction, kidney injury, death	Preconditioning reduced the risk of myocardial injury
Ali 2010[21]	On-pump CABG[b]	100	Remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at the arm, for 3 x 5 minutes	CK-MB release	Remote ischaemic conditioning reduced release of CK-MB postoperatively
Bøtker 2010[33]	Primary PCI[c]	333	Remote ischaemic perconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at the arm, for 4 x 5 minutes	Salvage index at 30 days measured by scintigraphy Sec.: Infarct size, troponin levels, death, reinfarction, hospitalization for heart-failure, LVEF[d]	Remote ischaemic conditioning increased salvage index, when corrected for ventricle size
Cheung 2006[25]	Heart surgery for congenital defects	37	Remote ischaemic preconditioning VS control	Leg ischaemia and reperfusion, by inflation of a cuff at systolic pressure + 15 mmHg placed at the leg, for 4 x 5 minutes	Troponin levels, mixed venous saturation, urine output, inotropic demand, lung function, systemic inflammatory response	Remote ischaemic conditioning reduced myocardial injury
Choi 2011[29]	Complex valve surgery	76	Remote ischaemic preconditioning VS control	Leg ischaemia and reperfusion, by inflation of a cuff at 250 mmHg placed at the leg, for 3 x 10 minutes	Kidney injury, as defined by in increase in serum creatinine by more than 50% or 0.3 mg/dl (26.5 umol/L). Sec: CK, CK-MB levels	Remote ischaemic preconditioning did not reduce kidney or myocardial injury
Günyadin 2000[18]	On-pump CABG	8	Remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 300 mmHg placed at the upper arm, for 2 cylci of 3 minutes ischaemia and 2 minutes reperfusion	CK-MB and LDH release	Remote ischaemic conditioning increased biomarker release

Hausenloy 2007[16]	On-pump CABG	66	Remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at the arm, for 3 x 5 minutes	Troponin release	Remote ischaemic conditioning reduced troponin release
Hong 2010[38]	Off-pump CABG	133	Remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at the arm, for 4 x 5 minutes	Troponin release	Remote ischaemic preconditioning did not significantly reduce troponin release
Hoole 2009[31]	Elective PCI	242	Remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at the arm, for 3 x 5 minutes	Primary: Troponin levels at 24 hours Secondary: serum creatinine, CRP, estimated GFR. MACE[e] rate at 6 months	Remote ischaemic preconditioning reduced troponin levels
Iliodromitis 2006[30]	Elective PCI	41	Remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at both arms, for 3 x 5 minutes	CRP, troponin, CK and CK-MB levels	Remote ischaemic preconditioning increased the release of cardiac enzymes
Karuppasamy 2011[24]	On-pump CABG surgery	54	Remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at both arms, for 3 x 5 minutes	Troponin and CK-MB	Remote ischaemic preconditioning did not significantly reduce troponin release
Li 2010[27]	Valve surgery	81	Remote ischaemic preconditioning VS remote ischaemic perconditioning VS control	Leg ischaemia and reperfusion, by inflation of a cuff at 600 mmHg placed at the leg, for 3 x 4 minutes	Clinical data and troponin levels	Remote perconditioning, but not preconditioning, reduced troponin levels
Luo 2011[28]	VSD[f] correction.	60	Remote ischaemic preconditioning VS direct postconditioning VS control	Leg ischaemia and reperfusion, by inflation of a cuff at 200-300 mmHg placed at the leg, for 3 x 5 minutes	CK-MB and troponin levels, clinical data	Remote ischaemic conditioning reduced biomarker release
Rahman 2010[22]	On-pump CABG	162	Remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at the arm, for 3 x 5 minutes	Primary: troponin release	Remote ischaemic conditioning did not reduce troponin levels
Rentoukas 2010[32]	Primary PCI	96	Remote ischaemic perconditioning VS remote	Arm ischaemia and reperfusion, by inflation of a	Primary endpoint: achievement of full ST-	Remote ischaemic conditioning increased the

			ischaemic perconditioning plus morphine VS control	cuff at 200 mmHg placed at the arm, for 3 x 5 minutes	segment resolution Secondary: peak troponin	achievement of ST-segment resolution
Thielmann 2010[20]	On-pump CABG	53	Remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at the arm, for 3 x 5 minutes	Primary: Troponin levels. Secondary: Mortality, major adverse cardiovascular events and renal function	Remote ischaemic conditioning reduced troponin release
Venugopal 2009[17]	On-pump CABG, +/- AVR[8]	45	Remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at the arm, for 3 x 5 minutes	Troponin levels	Remote ischaemic conditioning reduced troponin release
Venugopal 2010[15]	On-pump CABG, +/- AVR (retrospective analysis of 2 prior studies)	78	Remote ischaemic conditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 200 mmHg placed at the arm, for 3 x 5 minutes	Kidney injury, defined as an increase of 25 umol/L	Remote ischaemic conditioning reduced kidney injury
Wagner 2010[19]	On-pump CABG, +/- valve surgery	101	Late remote ischaemic preconditioning (18 hours before surgery) VS control VS tramadol	Arm ischaemia and reperfusion, by inflation of a cuff at systolic pressure plus 40 mmHg placed at the arm, for 3 x 5 minutes	Troponin release	Remote ischaemic conditioning reduced troponin release
Walsh 2009[35]	Endovascular AAA repair	40	Remote ischaemic preconditioning VS control	Leg ischaemia and reperfusion, by inflation of a cuff placed at the thigh to a pressure that ensured absence of flow by echo-Doppler, 10 minutes of 1 leg, then 10 minutes of the other leg	Primary: kidney injury, measured by albumin:creatinin ratio and retinol binding protein in urine Secondary: serum creatinin and GFR	Remote ischaemic conditioning did not significantly reduce kidney injury

Walsh 2010[36]	Open AAA repair	40	Remote ischaemic preconditioning VS control	Leg ischaemia by clamping of the common iliac artery, 10 minutes left and 10 minutes right	Primary: kidney injury, measured by albumin:creatinin ratio and retinol binding protein in urine Secondary: serum creatinin and GFR	Remote ischaemic conditioning did not reduce kidney injury
Walsh 2010[37]	Carotid endarteriectomy	70	Remote ischaemic conditioning VS control	Leg ischaemia and reperfusion, by inflation of a cuff placed at the thigh to a pressure that ensured absence of flow by echo-Doppler, 10 minutes of 1 leg, then 10 minutes of the other leg	Primary: saccadic latency, troponin release Secondary: major adverse cardiovascular events	Remote ischaemic conditioning did not significantly improve saccadic latency or troponin release
Zhou 2010[26]	VSD repair	60	Combined late and early remote ischaemic preconditioning VS control	Arm ischaemia and reperfusion, by inflation of a cuff at 240 mmHg placed at the arm, for 3 x 5 minutes	Heart and lung function, inflammatory markers	Remote ischaemic preconditioning had mixed effects on heart and lung function and inflammatory markers
Zimmerman 2011[23]	On-pump cardiac surgery	60	Preconditioning VS no intervention	Leg ischaemia and reperfusion, by inflation of surgical tourniquet to 200 mmHg placed at the thigh for 3 x 5 minutes	Kidney injury (increase of serum creatinine by >26.5 mmol/L)	Reduction in incidence of post-operative kidney injury by preconditioning

a=abdominal aortic aneurysm; b=coronary artery bypass grafting; c=percutaneous coronary intervention; d=left ventricular ejection fraction; e=major adverse cardiovascular event; f=ventricular septal defect; g=aortic valve replacement

Conditioning stimulus

In most studies, remote ischaemic conditioning was induced shortly before an expected period of ischaemia (preconditioning). In two studies preconditioning was induced 18-24 hours before ischaemia (late preconditioning), either by itself[19] or combined with early preconditioning.[26] One study[27] included a remote ischaemic preconditioning group and a remote ischaemic postconditioning group, as well as one control group. We treated these two groups as if they were from two separate studies, thereby including the control patients twice. In both primary PCI studies conditioning was induced during ischaemia.[32, 33]

Remote ischaemic conditioning was almost always induced by inflating a blood-pressure cuff or surgical tourniquet placed at one of the extremities. Only in two studies ischaemia was induced by invasively clamping an artery.[34, 36] In 14 studies the arm was used to induce conditioning by three or four series of five minutes of ischaemia and five minutes of reperfusion.[16-22, 24, 26, 30-33, 38] In nine studies the leg was used for conditioning by two or three series of five or ten minutes of ischaemia.[23, 25, 27-29, 34-37]

Quality of studies

In one third of the studies the process of sequence generation and concealment was done properly, in the other studies the methods were not described in detail (see Figure 2). Adequate blinding was done in nine out of 23 studies. Six studies are at risk for performance bias, since the treating surgeon was not blinded for group allocation. This is in particular the case for the two studies in which the surgeon performed ischaemic conditioning invasively. Nine studies were described as being blinded, but it was not specified how blinding of treating physicians was achieved. These studies were marked as having an unclear risk of bias. In one study, patients undergoing remote conditioning were sedated, while control patients were not.[26] Outcome assessment was blinded in two thirds of the studies, for the other studies it was not well described. Two studies excluded patients after randomisation and might be at risk for attrition bias.[19, 20] One study recruited extra patients after the planned number of patients did not provide enough power, although the numbers in the article do not match the numbers in the flow-schedule.[38] In another study the patient numbers provided in text do not match those in the corresponding tables.[33] Finally, one study did not specify a primary outcome parameter and lacked a power calculation.[25]

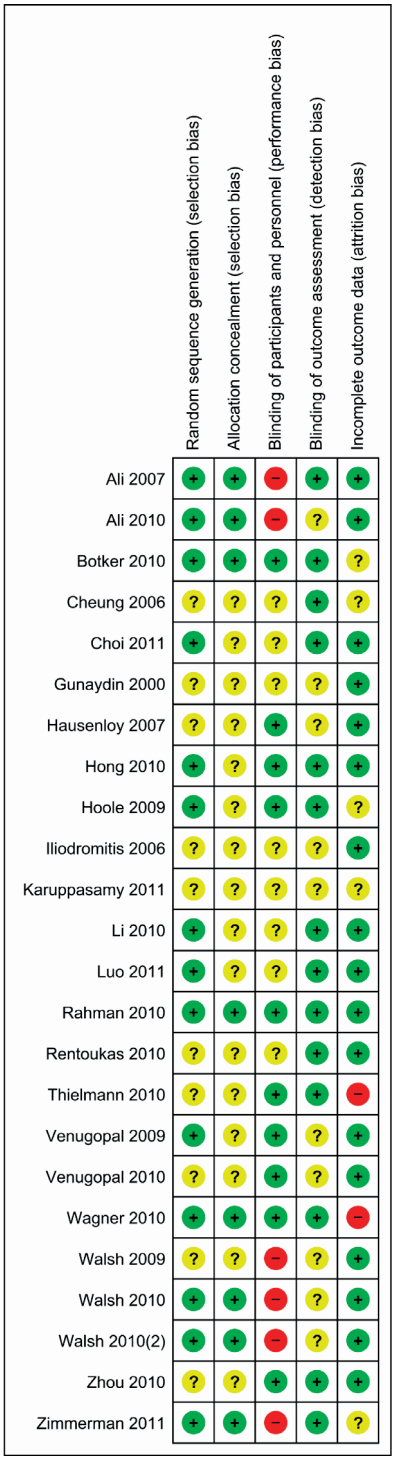

Figure 2. Risk of bias table: green= low risk of bias; yellow= unclear risk of bias; red= high risk of bias.

Effects of remote conditioning

Mortality and major adverse cardiovascular events

In total, 954 patients were treated with remote ischaemic conditioning and were compared with 924 control patients. Remote ischaemic conditioning did not reduce the primary endpoint of mortality (Figure 3), which was reported in 16 studies and occurred in nine (1.3%) patients of 709 treated with remote ischaemic conditioning, compared to seven (1.0%) out of 680 control patients (odds ratio 1.22 [95% confidence interval: 0.48, 3.07]). There was no evidence for statistical heterogeneity (I^2=0%). The incidence of major adverse cardiovascular events was not reduced by remote conditioning (odds ratio 0.65 [0.38, 1.14], Figure 4), nor was the occurrence of atrial fibrillation (odds ratio 1.11 [0.63, 1.95]), or kidney injury (odds ratio 0.63 [0.28, 1.45]). Stroke was reported in five studies, but no events occurred. Remote ischaemic conditioning reduced the risk of myocardial infarction (0.50 [0.31, 0.82]), with no statistical heterogeneity (I^2=0%) (Figure 5). Including the studies with no events by adding one event in each study arm results in a recalculated odds ratio of 0.53 (0.34, 0.85). The l'Abbé plot shows two outliers: the studies Ali '07 and Hoole '09, a mid-size and large trial, both with a rather high incidence of infarctions. As a sensitivity analysis we recalculated the odds ratio without these studies, resulting in an odds ratio of 0.68 (0.28, 1.65). In the subgroup of patients undergoing coronary artery bypass grafting surgery there were four events in the intervention group compared to seven in the control group (odds ratio 0.63 [0.21, 1.89]).

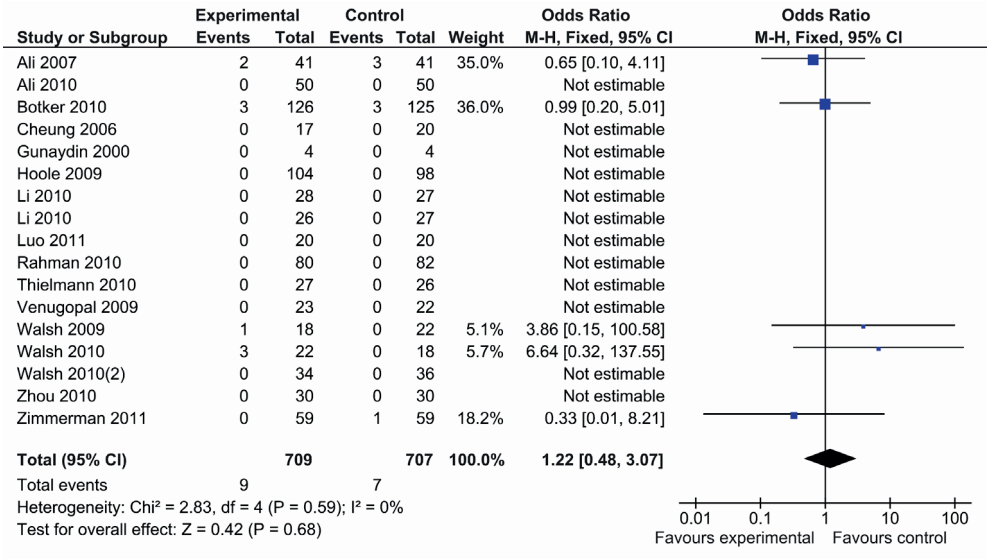

Figure 3. Mortality with remote ischaemic conditioning and without remote ischaemic conditioning.

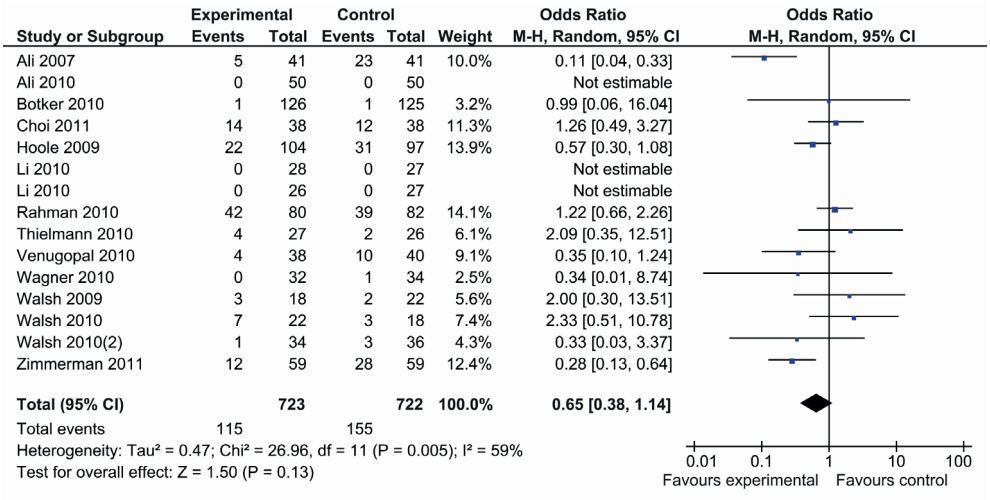

Figure 4. Major adverse cardiovascular events with remote ischaemic conditioning and without remote ischaemic conditioning.

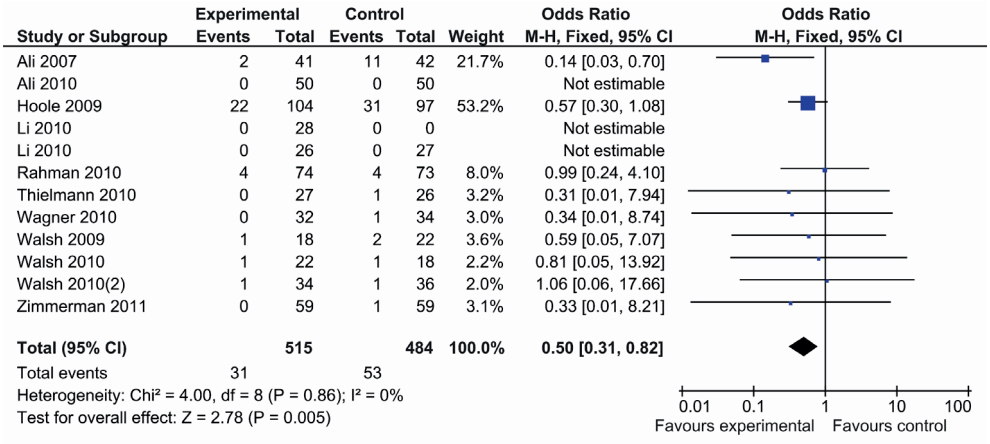

Figure 5. Myocardial infarction with remote ischaemic conditioning and without remote ischaemic conditioning.

Length of stay in hospital and in the intensive care unit

Twelve and ten studies reported the length of stay in the hospital and in the intensive care unit, respectively, but both outcome parameters were not affected by remote ischaemic conditioning (mean difference length-of-stay 0.04 days (95% confidence interval: -0.21, 0.29); mean difference intensive care unit stay -0.16 days (-0.38, 0.06), respectively).

Biomarkers

Troponin was the most reported biomarker of myocardial injury and was significantly reduced by remote ischaemic conditioning (standardized mean difference -0.28 [95% confidence interval: -0.47, -0.09], Figure 6), although there was significant statistical heterogeneity (I^2=66%). Troponin release was also reduced in the coronary artery bypass grafting subgroup by remote ischaemic conditioning as compared to control (standardized mean difference -0.29 [95% confidence interval -0.55, -0.03], Figure 7). Levels of creatine kinase were reported only in three studies (mean difference 19.54 U/L [-5.60, 44.69]), and the levels of creatine kinase muscle-brain in seven studies (mean difference -0.45 ug/L [-6.14, 5.24]), showing no difference between groups.

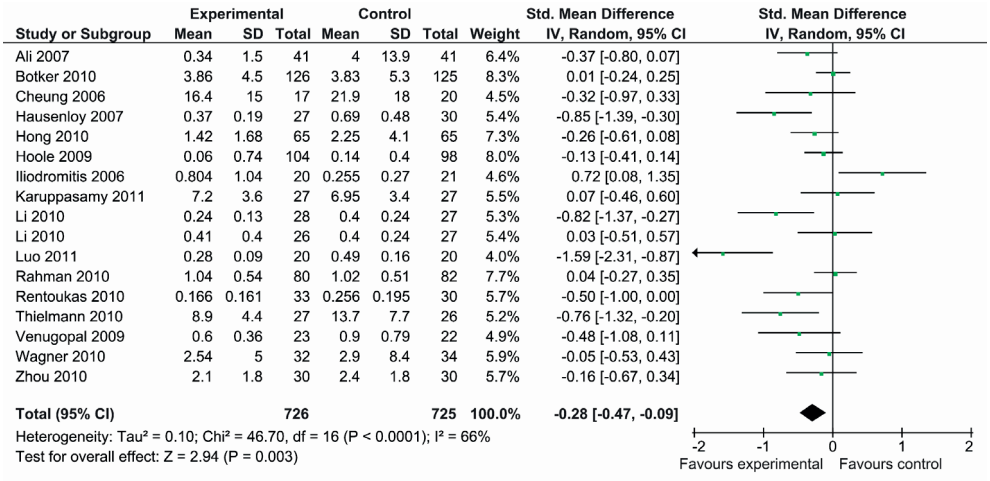

Figure 6. Peak troponin release with remote ischaemic conditioning and without remote ischaemic conditioning.

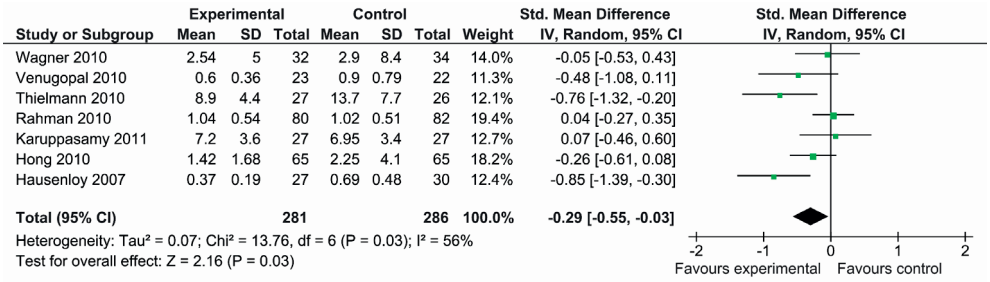

Figure 7. Peak troponin release with remote ischaemic conditioning and without remote ischaemic conditioning in the CABG-surgery sub-group.

Regarding kidney function, creatinine clearance was reported in five studies and plasma creatinine levels in seven, which were both not reduced by remote ischaemic conditioning (mean difference creatinine clearance 1.88 ml/min [-5.10, 8.87]; mean difference creatinine levels -6.50 mmol/L [-17.52, 4.53], respectively).

DISCUSSION

Despite including 23 studies the available evidence on the effect of remote ischaemic conditioning on the clinical outcome of patients after ischaemic events is limited, and strong conclusions cannot be drawn. The present analysis does not support the hypothesis that remote ischaemic conditioning reduces mortality associated with ischemic events, nor did remote conditioning reduce the combined endpoint of major adverse cardiovascular events. However, the secondary endpoint of periprocedural myocardial infarction was reduced by remote ischaemic conditioning, as was the post-procedural peak release of troponin, although there are some limitations to these conclusions.

Most included clinical studies were rather small, and focused on the effect of remote conditioning on biomarker release and other surrogate parameters of organ injury. Short or long-term outcome was not the primary outcome variable in any study. Some studies did not report on the occurrence of adverse events, such as death or myocardial infarction. The overall mortality in studies that reported on this outcome was 1.2 %, and the total number of events was 14, meaning that the incidence of the relevant event was quite low. It is debatable whether a reduction in mortality is possible in these patients. But to demonstrate an effect on mortality, if there is any, thousands of patients have to be included. For mortality the required group-size cannot be calculated using our data, since we found no trend towards a reduction in mortality by remote conditioning. However, based on our data, with an incidence in the control group of 21%, 760 patients per group will be needed to detect a relative risk reduction of 27% in major adverse cardiovascular events, with an alpha of 0.05 and a beta of 0.80. Providing statistical significance would be reached, inevitably the question of the clinical impact of such findings would appear. The intervention of remote conditioning might prove to be more beneficial in high-risk patients; however, these high-risk patients were generally excluded from the clinical trials that were available for inclusion in the current meta-analysis.

Our analysis shows that remote conditioning reduces the incidence of myocardial infarction. When examining the endpoint of myocardial infarction, we used the definition applied by the investigators of the respective studies. This means that different events

could have been classified as myocardial infarctions, and we could have wrongly pooled these events in the meta-analysis. Additionally, the results of the meta-analysis are largely driven by the study of Ali et al.[34] In this study the control group had a rather high event rate of approximately 26% and might not represent the population as a whole. Also, the study intervention (invasive crossclamping of the iliac artery) was administered by the operating surgeon, which could have induced bias. In addition to a reduction in myocardial infarction, we also found that the release of biomarkers of myocardial injury was reduced after remote conditioning. In contrast, however, we found no reduction in mortality, or length of stay in hospital or in the intensive care unit. As mentioned in the meta-analysis of myocardial infarctions the studies by Hoole and Ali were assigned a high weight, of 53% and 22% respectively. The Hoole study was performed in low risk patients undergoing elective percutaneous coronary intervention, no mortalities occurred and could therefore not have been improved; length of stay was not reported. Ali et al did find a significant reduction in length of stay on the intensive care unit, as well as a non-significant reduction in length of stay in hospital. However, in the meta-analysis of these parameters their study was assigned a low weight and the effect was lost. This could explain why the reduction in myocardial infarctions does not translate into a reduction in length of stay, or mortality.

Troponin was commonly used as biomarker for myocardial injury. Quantities reported varied; some studies reported peak values, some area under the curve, while again other studies mentioned values at several time-points (which also differed between studies). We used peak values when reported, and otherwise approximated them as good as possible. However, this inevitably leads to variance since the reported time-points differed between studies and the extracted peak values varied greatly between studies, even in comparable settings. Therefore, it is uncertain whether our meta-analysis estimates the true effect of remote ischaemic conditioning by missing the true peak values in some studies. In view of a resulting research agenda and as with other preconditioning studies, it would be advisable that the international research community establishes standards to measure organ injury in clinical studies.

An issue that troubles most studies is adequate blinding, which is probably more difficult to achieve than in trials investigating a pharmacological agent. Only in a minority of the trials all care providers were blinded, leaving the others at risk for bias.

In the present review we included studies with significant clinical heterogeneity. Studies differed in setting, patient population and the extent of ischaemia the patients were at risk for. Patients undergoing cardiac surgery, vascular surgery, elective or acute percutaneous coronary intervention were all pooled for the meta-analysis on the chosen outcome parameters. We intended to perform sub-group analysis on special patient populations, but while analysing the data it became obvious that only the group of studies in patients

undergoing coronary artery bypass grafting was large enough to permit a sub-group analysis.

There is no uniform protocol to induce remote ischaemic conditioning in the included studies. Different limbs are made ischaemic and the duration and number of the ischaemic periods differ. In a prior study Loukogeorgakis et al.[39] compared different regimes of remote postconditioning, comparing both arms and legs, and two and three cycles of five minutes ischaemia and reperfusion. Their findings suggest that a threshold stimulus must be reached in order to achieve protection. Therefore, it might be that some studies used a form of conditioning which is not sufficient to achieve the maximal protective effect. Different kinds of anaesthesia have been used in different clinical studies. From experimental and clinical studies, it is known that pre- and postconditioning are influenced by different anaesthetics. It is most likely that there is an effect of aesthetic regime on conditioning, and it would be interesting to perform an analysis based on different anaesthetics used within our meta-analysis. However, most studies do not report the anaesthetics used, and unless the aesthetic regime is specified in the protocol, it is impossible to identify this in retrospect. Therefore, we chose not to perform such an analysis.

We used a random-effect model when statistical heterogeneity was high, and a fixed-effect model when it was low. Since random-effect models tend to provide a broader confidence interval than fixed-effect models, and can therefore be seen as more conservative, one may argue to use them for all analysis. This is not necessarily true, however, since random-effect models also put greater emphasis on small trials, and can be less conservative in those instances.[40] A comparison of the results of this approach and those of the respective random-effect model was performed, which did not alter the results (data not shown).

Recently Takagi and Umemoto published an update of their previous meta-analysis on remote ischaemic preconditioning,[41] which confirmed their previously determined reduction in biomarker release. However, they found no reduction in mortality or peri-operative myocardial infarctions. Our findings are in line with their study, as we can show an effect on biomarker release, but no reduction in mortality. However, we did see a reduction in myocardial infarctions. There are a several differences between the study by Takagi and the present analysis, which might explain these different results. We included 23 studies compared to 9 studies with a total of 1878 patients compared to 482 included by Takagi. Next to a broader timeframe (until July 2011 versus April 2010) we also included a broader population of studies, encompassing four studies in patients undergoing a PCI. Concerning biomarker release, Takagi extracted different entities such as area-under-the-curve, peak values and single time-points, and subsequently pooled the data using the standardized mean differences, whereas we tried to extract peak values from all studies.

In summary, remote ischemic conditioning is an experimental technique and until today not part of accepted treatment protocols. Although studies on biomarker release show promising results, there is until today not enough evidence to recommend the routine use of remote ischaemic conditioning to treat ischaemic injury. Large trials are needed to investigate whether remote conditioning actually improves clinical outcomes. Research should also include high-risk patients, who might benefit most from protection by remote ischaemic conditioning.

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Chapter 3

A randomised controlled trial on the effect of remote ischaemic preconditioning on ERK1, ERK2, STAT3 and STAT 5 in patients with and without diabetes mellitus undergoing coronary artery bypass grafting surgery

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Submitted

ABSTRACT

Background: Remote ischaemic preconditioning (RIPC) is less effective in patients with diabetes, and the molecular mechanisms behind this lack of protection are not fully understood. In this study we investigated the effects of remote ischaemic preconditioning on the intracellular signal cascade (known as the RISK pathway), e.g. ERK1/2, STAT3 and STAT5 in patients with and without diabetes undergoing coronary artery bypass graft (CABG) surgery.

Methods: Twenty-six patients with and 24 patients without diabetes mellitus undergoing CABG surgery under a total-intravenous, propofol-based anaesthetic regime, were randomised to either remote ischaemic preconditioning induced by 3x 5 minutes of forearm ischaemia or untreated controls. In each patient, two atrial tissue samples were collected: the first before, and the second after preconditioning, but before initiation of cardiopulmonary bypass. Tissue samples were homogenised and the ratio of total-to-phosphorylated ERK1 and 2, STAT 3 and STAT5 were determined by Western Blotting. Myocardial injury after CABG was assessed by analysing postoperative troponin release.

Results: In the patients without diabetes, no effect of RIPC on any of the molecular markers was observed. In addition, we found no reduction in postoperative peak troponin release after RIPC. In patients with diabetes, ERK1 and ERK2 phosphorylation was increased in the control group, while in the RIPC treated patients only ERK2 was significantly increased. STAT3 phosphorylation was increased in both the control and RIPC group, while there was no effect on STAT5 phosphorylation. Again, there was no effect on postoperative troponin release.

Discussion: We found no effect of RIPC on ERK 1, ERK2, STAT 3 or STAT 5 in either patients with or without diabetes. Additionally, there was no reduction in troponin T release by RIPC. The major limitation of our trial is the small sample size.

BACKGROUND

Remote ischaemic preconditioning (RIPC) has become a promising method of providing protection against ischaemia-reperfusion injury, most notably in cardiac surgery.[1, 2] Even after recent large trials showed negative results, [3, 4] RIPC remains an intriguing concept.

Preconditioning is less effective in patients with diabetes mellitus,[5, 6] while simultaneously these patients have an increased risk for adverse cardiovascular events.[7-9] Not only is the risk of sustaining an acute myocardial infarction or requiring coronary revascularization increased for patients with diabetes; they also suffer from increased myocardial injury and poorer outcomes when these events occur.[10, 11]

How diabetes interferes with remote conditioning is not fully understood. RIPC activates intracellular pro-survival kinases, commonly referred to as RISK and SAFE pathways.[12, 13] Key components include extracellular signal-regulated kinase 1/2 (ERK-1/2), protein kinase B (Akt)[14], protein kinase C (PKC),[15] signal transducer and activation of transcription 3 (STAT3)[16] and/or signal transducer and activation of transcription 5 (STAT5)[17, 18]. The common target for these pathways seems to be the mitochondria, where opening of mitochondrial potassium channels (K_{ATP}) helps to stabilize the membrane, and helps to prevent opening of the mitochondrial permeability transition pore (mPTP), thereby preventing apoptosis.[19] Animal studies indicated that most of the important intracellular kinases involved in ischaemic preconditioning are downregulated in diabetic subjects.[20] However, clinical data on RIPC in patients with diabetes is scarce since most clinical trials excluded these patients. Nevertheless, subgroup analysis of larger trials that did include patients with diabetes suggest that cardioprotection by remote ischaemic preconditioning is abolished by diabetes.[14, 21]

We present a randomised controlled trial investigating the effects of RIPC on known mediators of cardioprotection in diabetic and non-diabetic patients. We aimed to confirm that remote conditioning activates STAT 3 or STAT 5 and we hypothesize that the activation of these kinases is diminished in diabetic patients.

METHODS

Subjects and setting

This study was a randomised controlled single-centre trial, performed at an academic centre. The study was approved by the local medical ethics committee and conducted in accordance to the principles of the Declaration of Helsinki. The trial was registered in the Dutch trial register as NTR1931. All participants provided written informed consent. The study adhered to the CONSORT statement on reporting on clinical trials.[22]

We recruited patients scheduled for elective coronary artery bypass grafting (CABG) surgery, with or without concurrent aortic valve repair. Exclusion criteria were an age < 18 years, severe organ dysfunction, including left ventricular ejection fraction <30%, severe chronic obstructive pulmonary disorder or SpO_2 <90% at room air, renal clearance <30 ml/min, liver failure, or the use of the antidiabetic drug glibenclamide which is known to block protection by preconditioning.[21, 23]

Study procedures

Patients were randomised 1:1 to either a control or intervention group, using a web-based application (ALEA; NKI, Amsterdam, The Netherlands) with a random block method and stratified for diabetes. Patients were blinded to treatment allocation, as was the investigator performing the laboratory analyses. The anaesthesist was not blinded, and although the surgeons were unaware of group allocation, we didn't take special precautions to prevent them from becoming aware whether a cuff was inflated on the patients arm or not.

Standard monitoring was used, including an arterial line, central venous catheter and trans-oesophageal echocardiography. Anaesthesia was induced and maintained using a total intravenous anaesthesia regimen consisting of midazolam, propofol, sufentanil and rocuronium.

In all patients a researcher placed a pneumatic tourniquet on the upper arm on the side without arterial line or intravenous cannula. In the control group, no further intervention was done and tissue samples were collected as described later. In the intervention group, after obtaining the first tissue sample, the tourniquet was inflated to 200 mmHg during 3 cycles of 5 minutes, with 5 minutes deflating the cuff between the cycles.

Two samples of right atrial tissue were taken during the procedure. After opening of the sternum but before harvesting of the left internal mammary artery, the surgeon took the first sample at the site where the venous cannula for bypass would later be inserted and closed the resection point with a suture. Later, when the right atrium was cannulated, the second biopsy was taken. Tissue samples were immediately frozen in liquid nitrogen before storage at -80° C.

Outcome

The study was designed to compare the effects of RIPC on biochemical markers ERK 1 and 2, STAT3 and STAT5 between patients with and without diabetes. We analysed the data as if there were two separate studies, one with only patients without diabetes, and the other one with only patients with diabetes. Additionally, we pooled the data of patients with and without diabetes.

Primary outcome parameter

Initially (see Dutch trial register NTR1931), the study planned to analyze differences in phosphorylation of the signalling enzymes ERK1, ERK2, HSP27 and PKC.[24, 25] However, during the inclusion period, new, most likely more relevant markers of RIPC in humans had been published.[17] We therefore adjusted the biochemical analyses and investigated the effect of RIPC on ERK 1and 2, STAT3 and STAT5.

Secondary outcome parameters

Myocardial injury was estimated by comparing the peak postoperative troponin values. We used the data collected for postoperative monitoring of troponin on the ICU, and samples were generally taken at arrival on the ICU, 4 hours later and at 6.00 AM the next day. In patients with significantly increased troponin values, samples were drawn more frequently to monitor possible myocardial injury.

We assessed clinical outcome by examining the charts and discharge letter from the ICU.

Laboratory analyses

Tissue samples were homogenised and separated into cytosolic, membrane and particulate fraction before analysing. The samples were crushed and dissolved in a lysis buffer consisting of Sigma 7-9, EGTA, NaF, Na3VO4 with the freshly added proteases aprotinin, lepetin and pepsatin, and DTT and okadaic acid. They were kept on ice until they were centrifuged at 530 G at 4° C for 10 minutes. The supernatant, containing the cytosolic

fraction, was centrifuged again at 13.000 G at 4° C for 15 minutes to clean up reaming debris. The pellet from the first step was dissolved in lysis buffer with 1% Triton and incubated for 60 minutes on ice. Then, this solution was centrifuged at 13.000 G at 4° C for 15 minutes. The supernatant containing the membrane fraction was transferred and the pellet containing the particulate fraction was dissolved in lyses buffer.

Western Blotting was performed as described previously.[26] Protein content was quantified using the Lowry method,[27] and the proteins were separated by electrophoresis using Criterion™ XT precast gels (Biorad, Hercules, CA) and transferred to a polyvinyliden fluorid membrane (Immobilin-FL, Licor, Bad Homburg, Germany). These membranes were incubated with the primary antibodies: pERK 1:1000, ERK 1:5000, pSTAT3 1:500, STAT3 1:000, pSTAT5 1:500 and STAT5 1:1000. They were then washed with phosphate buffered saline containing tween, incubated with a suitable secondary antibody and washed again. Thereafter, the membranes were scanned using the Odyssey Infrared Imaging System (Licor) and the signal intensity quantified using Odyssey Imaging Studio Software (Licor).

Sample size calculation

Sample size was based on previous work on the cardioprotective effect and molecular changes by sevoflurane.[28] We therefore assumed a sample size of 12 patients would be sufficient to detect a difference in patients without diabetes. We expected the molecular changes in patients with diabetic to be less profound and therefore increased the sample size in these patients to 16 subjects per group. This led to a total sample size of 56 patients.

Statistics

SPSS v24 (IBM, Armonk, New York, USA) was used for statistical analysis. Continuous data are presented as mean with standard deviation when normally distributed, or otherwise as median and interquartile range. Categorical data are presented as numbers with proportions. Student's T-test was used for normally distributed data, or skewed data after log transformation when appropriate, and the Mann-Whitney U-test or Wilcoxon signed rank test for not normally distributed data. Statistical significance was defined as $p \leq 0.05$. Figures were created using Graphpad Prism v7.

RESULTS

Baseline characteristics and study procedures

From august 2009 until October 2011, 50 patients (24 patients without diabetes and 26 with diabetes) were included. Due to concurrent trials the inclusion of the last patients proved to be difficult. We felt that storing the samples longer would not be beneficial, and decided to terminate the trial.

Patients without diabetes

Of 24 patients without diabetes, 13 were randomised to the control group and 11 to the intervention group. Groups were well matched for known risk factors and medication usage (Table 1). One patient in the intervention group accidentally received sevoflurane during the procedure; otherwise the study intervention was executed according to protocol. In the control group, 1 patient developed postoperative atrial fibrillation, 1 patient underwent a percutaneous coronary intervention and 1 patient had a re-thoracotomy for excessive bleeding. In the intervention group, 2 patients suffered from postoperative atrial fibrillation.

Patients with diabetes

Of 26 patients with diabetes, 13 were randomised to the control group and 13 to the intervention group. In the control group there were more smokers (10 vs 3), but groups were well matched otherwise. Cardiac tissue samples were not collected in 1 patient in the intervention group. In the control group, 1 patient had postoperative atrial fibrillation; in the intervention group, 3 patients suffered from atrial fibrillation, 1 patient had a delirium and 1 patient had a compartment syndrome postoperatively.

Table 1. Baseline characteristics and demographics

	Patients without diabetes (24)		p-value	Patients with diabetes (26)		p-value
	Control (11)	Intervention (13)		Control (13)	Intervention (13)	
Age (mean±SD)	67±8	66±6	0.59	63±7	69±11	0.65
Male sex n(%)	12 (92)	10 (91)	1	9(69)	11 (85)	0.65
Body mass index	26±3	28±4	0.20	30±4	30±5	0.20
Euroscore	3.5±2	2.8±2	0.35	2.5±1	3.2±2	0.35
NYHA class angina			0.58			0.42
1	0	0		1 (8)	0 (0)	
2	4 (31)	6 (55)		6 (46)	2 (17)	
3	6 (46)	4 (36)		3 (23)	6 (50)	
4	1 (8)	10 (42)		2 (15)	2 (17)	
Prior myocardial infarction	3 (23)	1 (9)	0.60	6 (46)	5 (39)	1
Ejection Fraction >50	1			10 (77)		
Ejection Fraction 30-50				2 (15)		
Left main stenosis	8 (62)	3 (27)	0.12	7 (54)	3 (23)	0.23
Percutaneous coronary intervention	0			0 (0)	1 (8)	0.045
Smoker	9 (69)	5 (46)	0.41	10 (77)	3 (23)	0.017
Hypertension	6 (46)	6 (54)	1	9 (69)	11 (85)	0.65
Dyslipidemia	4 (31)	5 (46)	0.68	6 (46)	9 (69)	0.43
Family history	6 (46)	3 (27)	0.42	5 (39)	5 (39)	1

NYHA class heartfailure			0.10			0.53
1	7 (54)	7 (64)		8 (62)	10 (77)	
2	2 (15)	4 (36)		2 (15)	2 (15)	
3	4 (31)	0		2 (15)	2 (15)	
4	0	0		0	0	
Medication						
Statin	12 (92)	11 (100)	1	13 (100)	12 (92)	1
Beta blocker	8 (62)	10 (91)	0.17	11 (85)	12 (93)	1
Angiotensin converting enzyme inhibiters	4 (31)	1 (9)	0.33	7 (54)	3 (23)	0.23
Angiotensin receptor blocker	3 (23)	4 (36)	0.66	2 (15)	5 (39)	0.38
Calcium	3 (23)	3 (27)	1	4 (31)	6 (46)	0.67
Diuretics	1 (8)	3 (27)	0.30	5 (39)	5 (39)	1
Nitrates	5 (39)	3 (27)	0.68	3 (23)	6 (46)	0.41
Aspirin	11 (85)	10 (91)	1	11 (85)	10 (77)	1
Clopidogrel	4 (31)	3 (27)	1	6 (46)	5 (39)	1
Dual anti platelet therapy	4 (31)	3 (27)	1	5 (39)	5 (39)	1
Insulin	0	0		5 (39)	5 (39)	1
Biguanide	0	0		12 (92)	9 (69)	0.32
Sulfonylurea	0	0		3 (23)	3 (23)	1

Western Blotting

Part of the tissue samples processed during the trial, and the rest at the conclusion of the trial. However, due to the significant time delay we felt that the stored samples would probably produce different results compared to freshly processed samples, especially regarding the phosphorylated fraction of proteins. Therefore, we finally only analysed the recently processed samples. Additionally, the protein content of 2 samples of 2 different

patients in the non-diabetic intervention group was too low for Western Blotting, leaving a sample size of 4 non-diabetic patients treated with RIPC and 6 subjects in all other groups.

Patients without diabetes

We observed an increase in the ratio of phosphorylated versus total ERK1 in both the control group as the intervention group, but this was not statistically significant at p=0.06 and p=0.23. No difference in p/t ratio of ERK2 was seen in either group (figure1). In the control group there was no difference in p/t ratio of STAT3. In the RIPC group we observed an increase in the p/t ratio after preconditioning, but this did not reach statistical significance (p= 0.14). There was no difference in the p/t ratio of STAT5 in either of the groups.

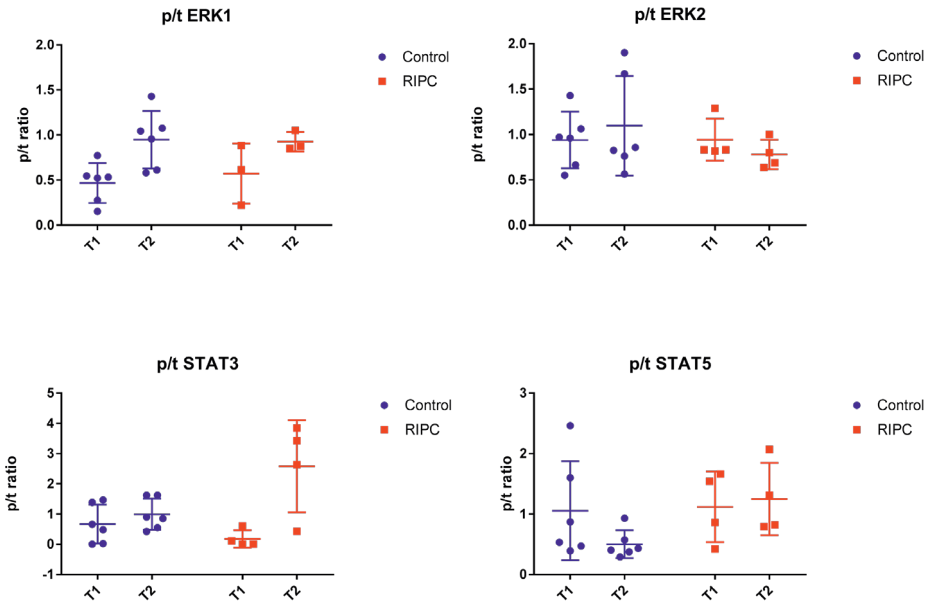

Figure 1. p/t ratio of ERK1, ERK2, STAT3 and STAT5 in patients without diabetes

Patients with diabetes

We observed a significant increase in the p/t ratio of ERK1 in the control group (p<0.01), but not in the RIPC group (p=0.07). For ERK2, we found a significant increase in both groups (figure 2). For STAT3, the p/t ratio increased significantly in both the control group, and the

RIPC group (p=0.03 for both groups). No difference on STAT5 p/t ratio was seen in either group.

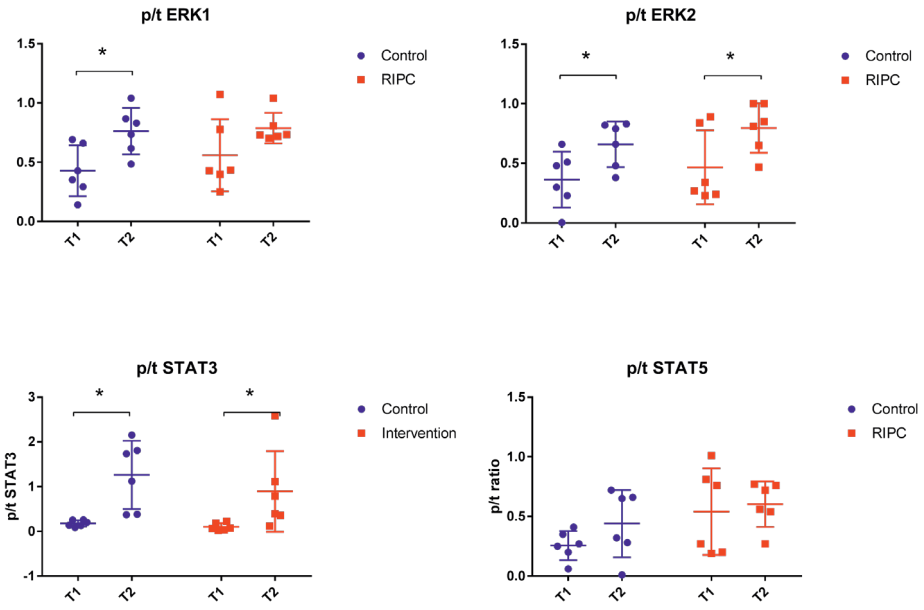

Figure 2. p/t ratio of ERK1, ERK2, STAT3 and STAT5 in patients with diabetes

All patients

There was an increase in the p/t ratio of ERK1 in both the control and the intervention group (figure 3). For ERK2, only the control group showed a significant increase in p/t ratio. We observed in both groups a significant increase in the p/t ratio of STAT3. There was no effect on the p/t/ ratio of STAT5.

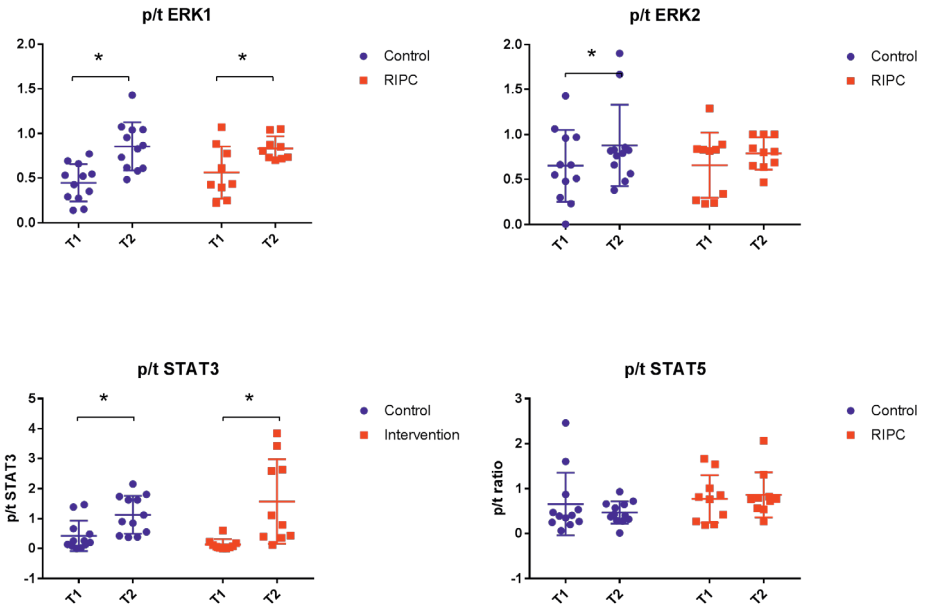

Figure 3. p/t ratio of ERK1, ERK2, STAT3 and STAT5 in all patients combined

Myocardial injury

In the patients without diabetes there was no difference in peak troponin value between groups (0.85±0.8 in the control group versus 1.1±1.2 in the intervention group, p=0.91) (figure 4). Neither was there a difference in peak troponin value between groups in the patients with diabetes (0.91±1.5 in the control group vs 0.6±0.39 in the intervention group, p=0.34). Also, when combining the data on patients with or without diabetes, we found no difference between patients subjected to remote conditioning or control treatment (0.88±1.2 in control patients vs 0.83±0.89 in conditioned patients, p=0.31) (figure 5).

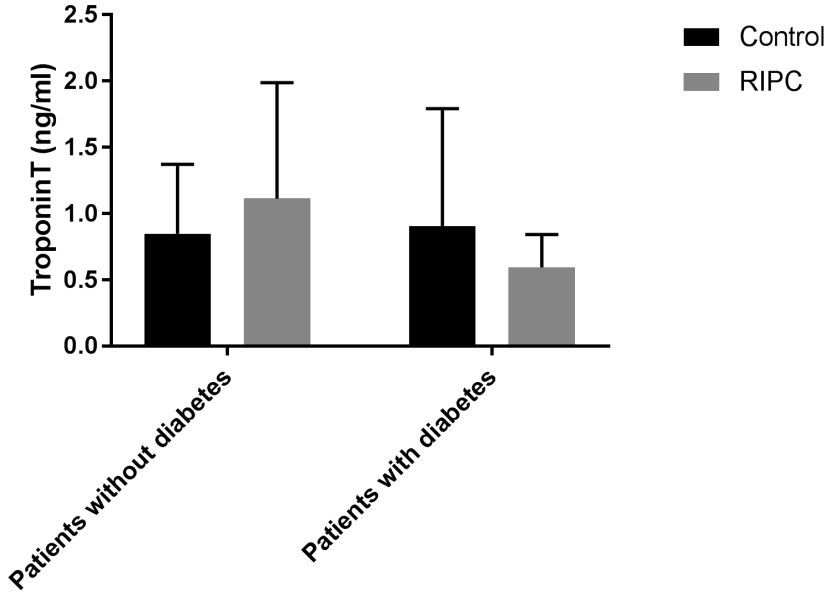

Figure 4. Peak troponin T levels (ng/mmol) in control (black) versus RIPC (grey) treated patients, in patients with and without diabetes

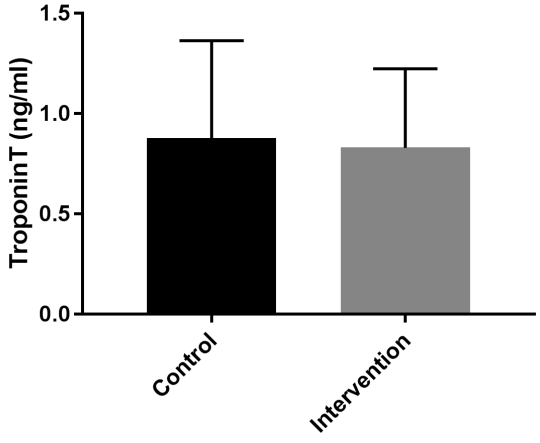

Figure 5. Peak troponin T levels (ng/mmol) in control (black) versus RIPC (grey) treated patients, in all patients combined

DISCUSSION

This is the first study specifically looking at the molecular effects of RIPC in diabetic patients undergoing cardiac surgery. In this randomised controlled trial we observed no effect of RIPC on ERK1, ERK2, STAT3 or STAT5. We did find an increase in phosphorylation of ERK1, ERK2 and STAT3 over time in patients with diabetes, with no differences between control and intervention groups. And in the patients without diabetes we observed a non-significant increase in STAT3 phosphorylation by RIPC.

We did not observe a reduction in troponin release with RIPC in either patient population. Although this study was not powered to detect a difference in troponin release, similar sized trials did find a reduction in biomarker release.[1] Also, using peak troponin levels derived from the clinically collected samples might be less sensitive than sequential sampling and calculating the area under the curve. However, in STEMI patients treated with primary PCI, peak values of troponin were similarly predictive of infarct size as area-under-the-curve of troponin release was.[29]

The main limitation for these findings is that the number of usable cardiac samples was smaller than anticipated at the design of the trial, thereby reducing sample size and power. This is especially true for the non-diabetic patients. This unfortunately makes it difficult to draw strong conclusions from our data.

Another possible limitation of this study is the choice of anaesthetics. A great number of factors can affect preconditioning-like cardioprotection during cardiac surgery, including drugs taken by the patient preoperatively as well as those administered during surgery; Beta-blockers, statins, antidiabetic drugs and opioids are a just a few, although the effects are not always well established.[23, 30-32] Of special interest are the aesthetic drugs that are being used. Volatile anaesthetics are known to induce preconditioning and it would be expected that this could interfere with remote ischaemic preconditioning, for example, by making it more difficult to demonstrate an additional benefit.[28, 33-35] Indeed, in a meta-analysis of randomised trials remote ischemic preconditioning seemed less effective in patients undergoing cardiac surgery using a volatile anaesthetics.[32] This reasoning led us to use a total intravenous anaesthesia with propofol. Contrary, other studies suggest that it is a propofol-based anaesthesia that inhibits remote ischemic preconditioning and reduces its protective effects.[36, 37] In a subset of patients from a larger randomised trial Kottenenberg et all found that remote ischaemic preconditioning could reduce the area under the curve of troponin I release by 50% in isoflurane anaesthetised patients, but with only 30% in patients under propofol anaesthesia, which was lower than the expected 40% used in their power calculation and therefore not significant.[36] Then again, another

recent randomised trial strictly avoiding propofol did not see a reduction in troponin T release by remote ischaemic preconditioning.[38] Overall, it is likely that the choice of anaesthetics could have influenced the results of our trial, although the exact way is less clear.

This study contrasts with previous studies. Heusch et al. investigated multiple molecular targets and found specifically STAT5 activated by RIPC,[17] while Gedik et al. showed an increased activation of STAT3 by RIPC in non-diabetic patients.[16] In children with congenital heart disease, both STAT3 and STAT5 were activated by RIPC.[39] On the other hand, Kottenberg et al. found no effect of RIPC on either STAT3 or STAT5, which might have been due to the effects of propofol anaesthesia.[40] However, in these studies no patients with diabetes were included.

Indeed, there are very few studies investigating RIPC in diabetic patients. In a small subgroup from a larger trial, Kottenberg et al. reported no effect of RIPC on troponin I release after CABG surgery in patients with diabetes treated with sulfonylurea derivatives.[21] In a trial following elective PCI in diabetic patients, RIPC failed to reduce myocardial injury.[41]

The signal cascade involved in conditioning seems less suited for preconditioning in diabetic patients.[20] In type 2 diabetics undergoing CABG surgery, atrial tissue contains increased levels of phosphatase and tensin homolog, which inhibits preconditioning, and reduced levels of phosphorylated AKT, Bcl and endothelial nitric oxide synthetase.[42] The mitochondria of patients with diabetes appear more susceptible to ischaemic injury, due to an increased sensitivity to intracellular Ca^{2+} and thereby increased tendency for the mPTP to open.[43]

CONCLUSIONS

In conclusion, in this trial specifically designed to examine remote ischaemic preconditioning in patients with diabetes and those without, we found no effect of remote ischaemic preconditioning on ERK1, ERK2, STAT3 or STAT5 or troponin release.

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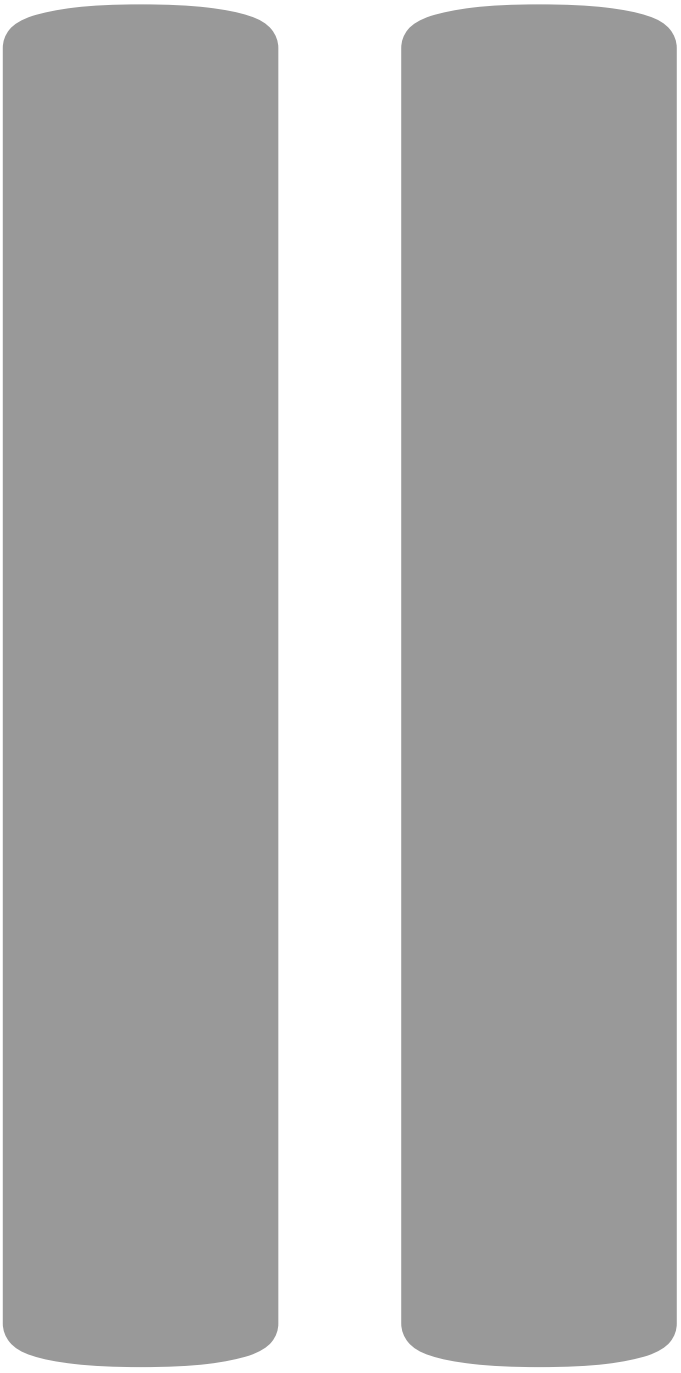

Part II

Helium inhalation

Chapter 4

Helium induces preconditioning in human endothelium *in vivo*

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ABSTRACT

Aims: Helium protects myocardium by inducing preconditioning in animals. We investigated whether human endothelium is preconditioned by helium inhalation in vivo.

Methods: Forearm ischaemia-reperfusion (I/R) in healthy volunteers (each group n=10) was performed by inflating a blood pressure cuff for 20 minutes. Endothelium-dependent and –independent responses were measured following cumulative dose-response infusion of acetylcholine and sodium nitroprusside, respectively, at baseline and after 15 minutes of reperfusion using strain-gauge, venous occlusion plethysmography. Helium preconditioning was applied by inhalation of helium (79% helium, 21% oxygen) either 15 min (helium early preconditioning, He-EPC), or 24 hours before I/R (helium late preconditioning). Additional measurements of He-EPC were done after blockade of endothelial nitric oxide synthase. Plasma levels of cytokines, adhesion molecules and cell-derived microparticles were determined.

Results: Forearm I/R attenuated endothelium dependent vasodilation (acetylcholine) with unaltered endothelium-independent response (sodium nitroprusside). Both He-EPC and helium late preconditioning attenuated I/R-induced endothelial dysfunction (max increase in forearm bloodflow in response to acetylcholine after I/R was 180±24% (mean±SEM) without preconditioning, 573±140% after He-EPC, and 290±32% after helium late preconditioning). Protection of helium was comparable to ischaemic preconditioning (max forearm bloodflow 436±38%) and was not abolished after endothelial nitric oxide synthase blockade. He-EPC did not affect plasma levels of cytokines, adhesion molecules or microparticles.

Conclusion Helium is a non-anesthetic, non-toxic gas without haemodynamic side effects that induces early and late preconditioning of human endothelium in vivo. Further studies have to investigate whether helium may be an instrument to induce endothelial preconditioning in patients with cardiovascular risk factors.

INTRODUCTION

Ischaemic preconditioning (IPC) results in protection of organs against ischaemia-reperfusion (I/R) injury by short, non-lethal periods of ischaemia.[1] Two phases are distinguished, an early phase of protection induced by a stimulus directly before I/R, known as classical or early preconditioning, and a second window of protection that arises 12-72 hours after administration of the stimulus, which is known as late preconditioning. Next to ischaemia, pharmacological agents (i.e.volatile anaesthetics[2]) can also induce preconditioning. The endothelium circumvents all blood vessels and serves as a first-line defence mechanism against organ and tissue injury. The protective functions of the endothelium include anti-coagulation, anti-inflammation, prevention of platelet activation, regulation of permeability and regulation of vascular tone. I/R elicits profound changes in the endothelial homeostasis, as attested by a significant suppression of endothelium-dependent vasodilation.[3] Endothelial dysfunction is regarded as an independent risk factor for cardiovascular events[4] and is a surrogate marker for monitoring the efficacy of therapeutic strategies.[5, 6] Postischaemic endothelial dysfunction can be attenuated by IPC, as was shown in a human forearm model using venous occlusion plethysmography. In this study, 20 minutes of forearm I/R resulted in a blunted vasodilatory response to acetylcholine, which could be restored by IPC.[3]

Animal studies demonstrated that the noble gas helium induces early[7] and late[8] preconditioning of the heart. Because helium is readily available, easy to administer and has no known side effects, it has the potential to become a perfect preconditioning agent.

Forearm I/R induces endothelial dysfunction by reducing vasodilation induced by increasing dosages of acetylcholine.[3] Our primary hypothesis is that helium preserves postischaemic endothelial function.

There are markers of endothelial function present in plasma. Activated endothelial cells release nitric oxide products, infammatory cytokines, adhesion molecules, regulators of hemostasis and microparticles.[9] Microparticles are vesicles circulating in plasma, which are derived from various cells in response to cell activation, injury or apoptosis. Endothelial microparticles have been used as a clinical and quantitative marker of endothelial cell dysfunction,[10] and their presence is inversely associated with acetylcholine-induced vasodilation in coronary arteries.[11] Our secondary hypothesis is that the underlying mechanism of helium preconditioning might be related to endothelial nitric oxide synthase production, circulating cytokines and adhesion molecules, or cell-derived microparticles released after I/R.

MATERIALS & METHODS

The Institutional review board of the Academic Medical Centre, Amsterdam, The Netherlands approved the trial (www.trialregister.nl, NTR1124), which was conducted in accordance with the International Conference on Harmonization on Good Clinical Practice Guidelines and the Declaration of Helsinki. All subjects gave written informed consent.

Subjects

A total of 58 healthy volunteers were included (baseline characteristics and demographics are shown in Table 1). Volunteers abstained from caffeine, alcohol and smoking 12 hours before onset of the experiment. All experiments were performed in a quiet, temperature-controlled (20-24°C) room. Fifty volunteers were randomised to one of five groups using randomisation software (ALEA; NKI; Amsterdam, The Netherlands) provided by Clinical Research Unit of the Academic Medical Centre. An additional group of 8 volunteers received the endothelial nitric oxide synthase (eNOS) blocker N^G-monomethyl-L-arginine (L-NMMA).

Study design

The study protocol is outlined in figure 1. Forearm ischaemia was induced by inflating a 12-cm wide blood pressure cuff placed on the non-dominant upper arm to a pressure of 200 mmHg for 20 minutes. Helium preconditioning was induced by administration of a helium mixture (Heliox: Helium 79%, Oxygen 21%, BOC, Mordon, United Kingdom) using a non-invasive delivery system (Helontix™vent, Linde Therapeutics, Eindhoven, The Netherlands) via a normal facemask with pressure support of 3 cm H_2O. Volunteers were given three cycles of helium for 5 minutes, followed by 5 minutes of normal air breathing either directly (helium preconditioning, He-EPC) or 24 hours (helium late preconditioning, He-LPC) before I/R (see figure 1).

The group receiving L-NMMA received a dosage of 0.4 mg min^{-1} dL^{-1} forearm tissue volume, and this dosage effectively blocked nitric oxide production in previous studies.[12]

L–NMMA was started 5 min before He-EPC and was continued during helium preconditioning (35 min in total). Ninety min after termination of this preconditioning protocol, FBF was restored to baseline values, indicative of normal eNOS activity before forearm I/R.

Inflating the blood pressure cuff around the non-dominant upper arm to 200 mmHg for 3 times 5 minutes interspersed with time 5 minutes reperfusion directly before I/R induced ischaemic preconditioning.

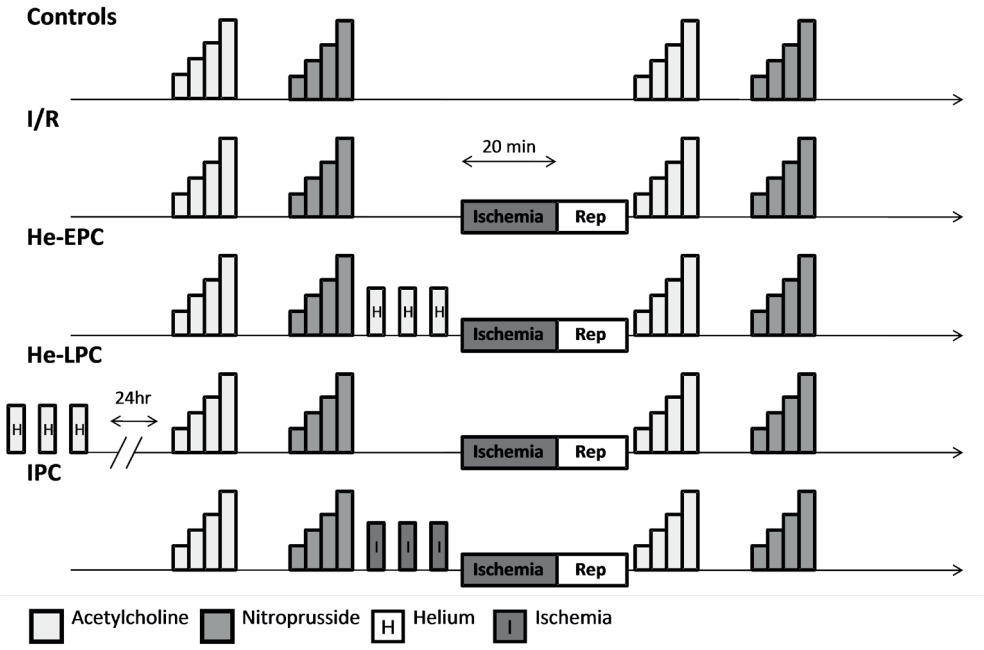

Figure 1. Study protocol

Controls, control group without ischaemia; He-EPC, helium early preconditioning group; He-LPC, helium late preconditioning group; I/R, ischaemia-reperfusion group; IPC, Ischaemic preconditioning group.

Endothelial function was established at baseline and was repeated after 20 min of forearm I/R **(I/R)** except for controls **(controls).** Treatment with helium preconditioning was administered directly before forearm I/R **(HE-EPC)** or 24 hours before **(He-LPC).** Treatment of ischaemic preconditioning **(IPC)** was administered directly before forearm I/R.

Assessment of endothelial function

Assessment of vascular function was performed using venous occlusion plethysmography (EC4; Hokanson, Inc. Bellevue, WA). After local anaesthesia with lidocaine 2%, the non-dominant brachial artery was cannulated under aseptic conditions using a 22-gauge needle. Bilateral forearm bloodflow (FBF) was measured with mercury-in-silastic strain gauges and expressed in ml/min/100ml forearm tissue volume. Forearm tissue was measured by water displacement. Endothelial function was assessed as described before[13] and measured in response to intra-arterial infusion of the endothelium-dependent vasodilator acetylcholine (0,1; 0,5; 1,5; 5,0 μg/100ml forearm tissue volume/min, Novartis AG, Stein, Switzerland) and the endothelium-independent vasodilator sodium nitroprusside (6; 60; 180; 600ng/100ml/forearm tissue volume/min, AZH, The Hague, The Netherlands). Each dose was given for 5 minutes, and intrabrachial infusion was kept constant at a rate of 90 ml/hour. To reconfirm that our postischaemic measurement was consistent, we repeated our postischaemic baseline measurement twice within 10 minutes, after which we continued the measurement with infusion of acetylcholine and sodium nitroprusside.

Blood Samples

A venous cannula was inserted in the non-ischaemic arm to collect blood samples at baseline, after 10 minutes of reperfusion[14] and after 3 hours of reperfusion (at the end of the protocol) to allow activation of interleukins. Samples were centrifugated (1550*g*, 20 min, 20°C) within 15 minutes and aliquots were snap frozen in liquid nitrogen and stored at -80°C.

Flowcytometry of microparticles

Samples of frozen citrate plasma of subjects from control, I/R- and He-EPC group were analysed for circulating cell-derived microparticles.[15] Samples were thawed and microparticles were isolated and incubated with annexinV and the cell-specific monoclonal antibody or isotype-matched control antibodies, and were analysed for 1 min in an automated cell sorter (FACSCalibur flow cytometer with CELLQuest 3.1 software (BD Immunocytometry Systems; San José, CA, for details see supplemental digital content 1).

Enzyme-Linked Immuno Sorbent Assay (ELISA)

We used serum samples to determine levels of circulating interleukin (IL)-1ß, E-selectin, soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1. Citrate plasma was used to determine levels of IL-6 and IL-8 (all kits from R&D systems, Minneapolis, MN). Samples were analysed by ELISA according to recommendations of the manufacturer.

Calculation & Statistics

All plethysmography results are presented as mean ± standard error of the mean (mean±SEM). Demographic and cytokine data are presented as mean ± standard deviation (mean±SD), microparticle data are presented as median and 25-75 percentiles.

As in previous studies (3) we planned to only perform a within group analysis. Therefore, we performed a repeated measure ANOVA in each group, and compared the first measurements (responses to acetylcholine and sodium nitroprusside) with the respective postischaemic measurements after I/R. We focussed on a group main effect and did not perform post hoc tests for each dosage. A probability value of $P<0.05$ was considered significant. FBF measurements were analysed using SPSS (version 16.0, Chicago, IL). The mean ratio of flow in the infused (measurement) arm/non-infused (control) arm was calculated (FBF measurement/control arm ratio). Baseline FBF measurement/control arm ratios were normally distributed, and comparison within groups of first and second measurement was performed by two-sided paired student's *t*-test (one outlier (>2SD) in the IPC group was excluded before analysis). ELISA data were analysed by one-way ANOVA using Dunnett's multiple comparison as posthoc test. Microparticle data were unequally distributed and Friedman's test was used for within group analysis.

RESULTS

Demographic data of randomised volunteers are presented in table 1, no statistical difference was observed between groups. All subjects tolerated the procedures without complications. Data of one patient were excluded because of violation of the preconditioning protocol, (IPC group n=9, all other groups n=10). Helium administration was well tolerated, and no effects on blood pressure and heart rate were observed.

Table 1. Demographics and baseline data of randomised volunteers

	Controls	I/R	He-EPC	He-LPC	IPC
Male/Female	5/5	6/4	6/4	6/4	5/4
Age (yrs)	24±6	22±3	27±8	23±5	23±5
BMI	23.7±4.2	22.2±2.6	22.5±3.0	21.6±1.3	23.8±4.2
SBP (mmHg)	126±14	124±17	121±9	118±14	122±7
DBP (mmHg)	75±7	73±8	73±9	70±6	72±8
ESR (mm/hr)	3.7±2.2	4.3±4.9	4.3±5.0	3.8±2.4	3.6±1.5
Heart Rate (beats/min)	58±8	60±9	62±8	63±10	67±12
Hemoglobin (mg/dL)	13.4±2.2	13.5±1.3	13.7±1.3	14.3±1.1	13.8±1.1
Thrombocytes (10^9/L)	228±24	230±48	234±61	220±31	261±56
Leukocytes (10^9/L)	5.2±0.8	6.4±3.2	5.8±1.5	5.7±1.0	6.3±1.9

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ESR, erythrocyte sedimentation rate. Controls, control group without ischaemia; He-EPC, helium early preconditioning group; He-LPC, helium late preconditioning group; I/R, ischaemia-reperfusion group; IPC, Ischaemic preconditioning group. All data are expressed as mean±SD. No statistical differences were observed between groups.

Effect of forearm I/R on endothelial function

Forearm I/R resulted in a persistent postischaemic hyperaemia resulting in a significant increased baseline FBF at the start of the second measurement. The prolonged postischaemic hyperaemia was completely abolished after IPC and HE-EPC, resulting in a similar baseline FBF at the start of the second measurement. However, after helium late preconditioning a non-significantly increased postischaemic baseline FBF was observed (Figure 2). Acetylcholine caused dose-dependent increases in FBF in all groups. In the control group, both the baseline and the second measurement showed similar responses to acetylcholine, illustrating the reproducibility of our methodology (Figure 3A). Forearm

I/R significantly blunted the dose-dependent response to acetylcholine, reflecting postischaemic endothelial dysfunction (*P*= 0.001, Figure 3B). The response to sodium nitroprusside remained unaffected after forearm I/R, and maximal increase of FBF was in the same range as maximal increase in response to acetylcholine (supplemental digital content 2 figure 1).

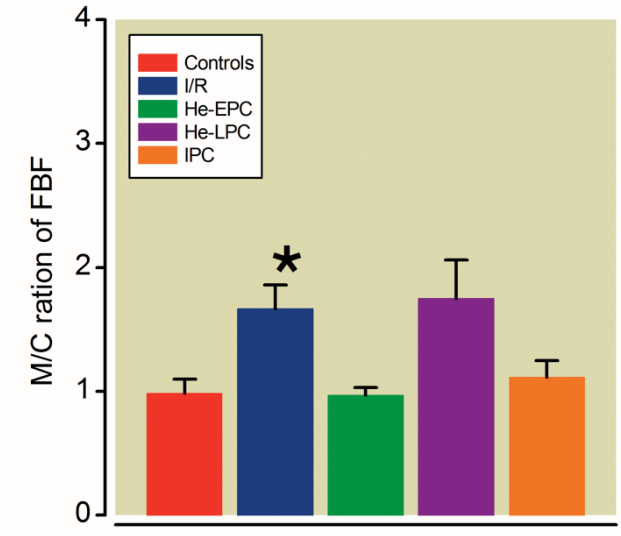

Figure 2. Baseline forearm blood flow (FBF) measurement/control arm ratio of second measurement

Controls, control group without ischaemia; He-EPC, helium early preconditioning group; He-LPC, helium late preconditioning group; I/R, ischaemia-reperfusion group; IPC, Ischaemic preconditioning group. Bar plot showing Baseline FBF measurement/control arm ratio (mean±SD) calculated by dividing FBF from the infused (measurement) arm/non-infused (control) arm. I/R caused significant postischaemic hyperaemia (*, P<0.05) compared to the first measurement, which was prevented by He-EPC, IPC and He-LPC.

Effect of helium preconditioning on endothelial function

He-EPC prevented postischaemic endothelial dysfunction by preserving the response to acetylcholine (*P*= 0.581 first versus second measurement, Figure 3C). Even when administered 24 hours before forearm I/R, helium late preconditioning preserved postischaemic endothelial function (*P*= 0.165, Figure 3D). The protection by helium preconditioning was comparable to protection elicited by IPC, (*P*=0.657, Figure 3E).

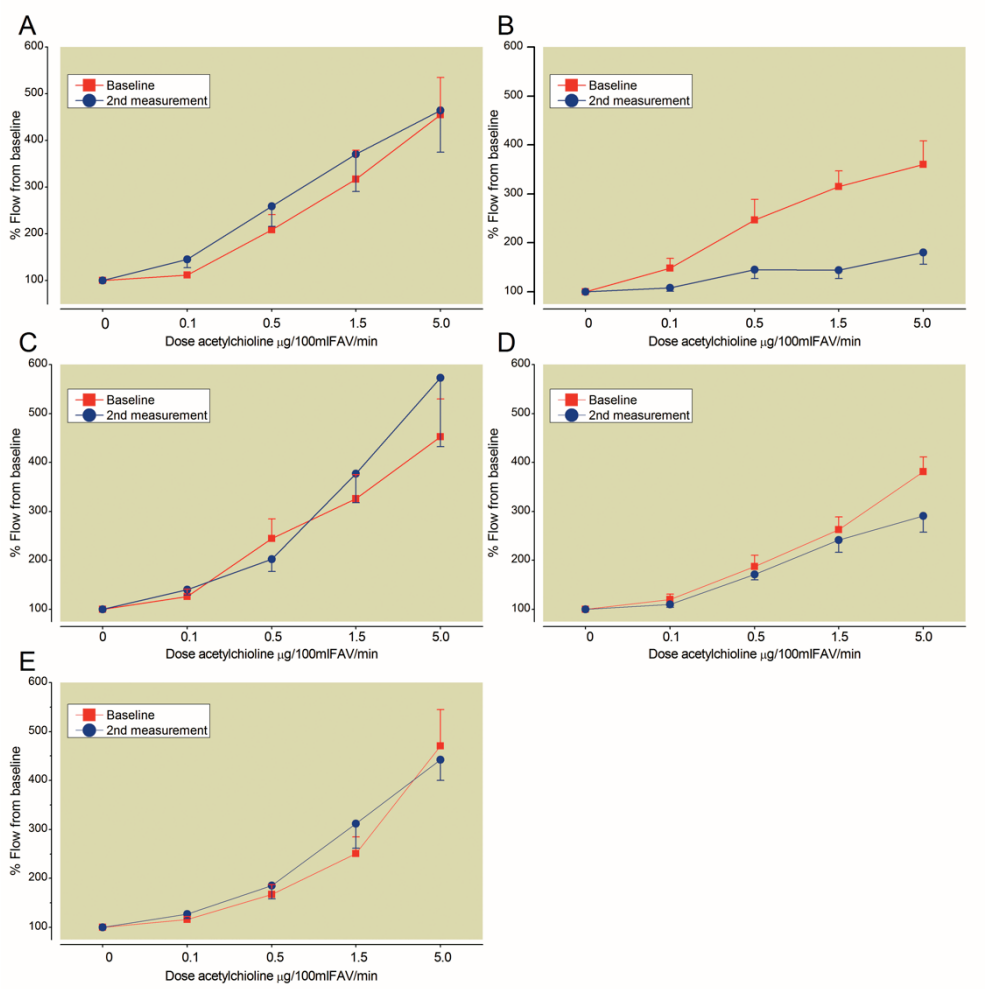

Figure 3. Acetylcholine dose response curves

Controls, control group without ischaemia; He-EPC, helium early preconditioning group; He-LPC, helium late preconditioning group; I/R, ischaemia-reperfusion group; IPC, Ischaemic preconditioning group. All data are represented as mean±SEM. Figure 3A shows similar dose response curves to acetylcholine for the first and the second measurement in controls. (*P*=0.59). Figure 3B shows that I/R of the forearm significantly blunted dose-dependent response to acetylcholine (*P*= 0.001) indicating postischaemic endothelial dysfunction. Helium early (figure 3C, *P*= 0.581) and late preconditioning (figure 3D, *P*= 0.165) prevented postischaemic endothelial dysfunction, as did ischaemic preconditioning (figure 3E, *P*=0.657).

Effect of helium preconditioning on endothelial function

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In the additional L-NMMA group (male/female 1/7, age 23±5 years, body mass index 21.7±2.1, systolic and diastolic blood pressure 122±19 mmHg and 69±9 mmHg, respectively), L-NMMA significantly reduced FBF by 61±8 %, indicating eNOS blockade during application of He-EPC. This blockade however did not block the protective effect of He-EPC as the response to acetylcholine was preserved (*P*=0.720, Figure 4).

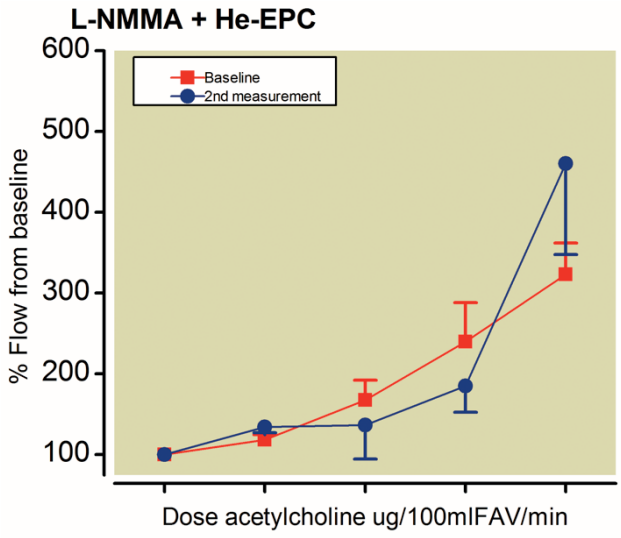

Figure 4. Acetylcholine dose response curves after infusion of L-NMMA during helium preconditioning

FAV; forearm volume. Infusion of N^G-monomethyl-L-arginine (L-NMMA) during helium preconditioning (He-EPC) did not block helium preconditioning, as postischaemic endothelial function was still preserved.

Effect of I/R on circulating pro-inflammatory cytokines and adhesion molecules

We measured the levels of soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1 and E-selectin. All baseline values were within the normal limits reported for healthy subjects. To investigate whether forearm I/R would affect these adhesion molecules, we measured the levels after 10 minutes of reperfusion and after 3 hours of reperfusion. We observed no significant effects on the plasma levels of soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1 and E-selectin.

The baseline levels of the pro-inflammatory cytokines IL-1β and IL-8 were all below the lowest standard provided by the manufacturer. The levels of IL-1β after 10 minutes and 3 hours of reperfusion remained below the detection limit of the assay and no increases were observed. Forearm I/R did not affect systemic levels of IL-8 after 10 minutes or 3 hours of reperfusion. In contrast, IL-6 increased in all groups in time without significant differences between controls, I/R or preconditioning groups (Table 2).

Table 2. Adhesion molecule expression and cytokine levels at 3 hours of reperfusion

Target	Control	I/R	He- EPC	He-LPC	IPC
			3 hours of reperfusion		
sVCAM-1, ng/ml	730±83	716±130	674±246	604±136	690±128
sICAM-1, ng/ml	235±77	213±48	211±77	203±23	208±57
E-selectin, ng/ml	37.7±19.1	28.2±12.1	30.4±8.6	23.2±7.6	32.0±12.5
IL-1β, pg/ml	2.0±0.7	1.3±0.8	1.0±0.9	0.9±1.1	0.9±1.0
IL-6, pg/ml	3.46±2.3	4.96±3.4	4.80±4.1	4.43±2.5	3.76±2.4
IL-8, pg/ml	3.85±1.7	5.07±3.4	5.79±2.4	3.70±1.5	3.33±2.5

Abbreviations: Controls, control group without ischaemia; He-EPC, helium early preconditioning group; He-LPC, helium late preconditioning group; I/R, ischaemia-reperfusion group; IPC, Ischaemic preconditioning group. IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8, sICAM-1, soluble intercellular adhesion molecule-1, sVCAM-1, soluble vascular cell adhesion molecule-1; Data are expressed as means ±SD. No statistical differences were observed between groups.

Effect of I/R on circulating cell-derived microparticles

To investigate whether helium preconditioning or forearm I/R affected the release of endothelial microparticles, plasma samples from the control, He-EPC, and I/R group were analysed for the presence of microparticles (all groups n=8). An example of the microparticles results is given in supplemental digital content 2 figure 2) Neither forearm I/R nor He-EPC affected the total levels of circulating microparticles in blood.

In line with earlier studies we found that the levels of microparticles originating from endothelial cells, i.e. microparticles binding antibodies directed against E-selectin (CD62e), VE-cadherin (CD144) or melanoma cell adhesion molecule (CD146), were very low and almost below the detection limit, except for samples from one volunteer in the I/R group who showed a strong increase of endothelial microparticles after I/R.

We further investigated the cellular origin of microparticles from platelets (CD61, CD62p, CD63), lymphocytes (CD4, CD8, CD20), monocytes (CD14), granulocytes (CD66b) or erythrocytes (CD235a). We found a large variation in the baseline levels of erythrocyte-derived microparticles in all groups, reflecting large heterogeneity in our volunteers, which is possibly caused by mild haemolysis (Table 3). Forearm I/R resulted in a non-significant increase of systemic circulating erythrocytes derived microparticles (Table 3). Furthermore, we observed no effect of He-EPC or forearm I/R on microparticles exposing tissue factor, or microparticles derived from leukocytes. Because we focused on a group main effect of endothelial function and did not perform a power analysis for the effect of forearm I/R or He-EPC on microparticles, we cannot conclude that forearm I/R affects levels of systemic circulating microparticles, and that microparticles do not play a role in He-EPC on a systemic level. Since we did not investigate the levels of microparticles in the ischaemic arm, we cannot exclude a possible effect on a local level.

Table 3. Analysis of circulating microparticles at different timepoints

	Controls			I/R			He-EPC		
Marker	**Baseline**	**Rep 10 min**	**Rep 3 hours**	**Baseline**	**Rep 10 min**	**Rep 3 hours**	**Baseline**	**Rep 10 min**	**Rep 3 hours**
CD235a	6.3 (4.2-20.5)	3.5 (1.96-10.0)	2.6 (2.0-9.3)	6.0 (1.0-10.0)	3.0 (2.0-20.0)	20 (4.0-50)	9.0 (4.0-36.7)	10.5 (5.1-34.4)	7.1 (3.6-3.4)
CD61	36.6 (15.7-13.8)	40.0 (10.0-50.0)	38.9 (13.4-192)	26.7 (10.0-76.2)	20.6 (9.0-44.2)	18.8 (7.2-38.5)	44.5 (33.7-52.5)	30.6 (5.1-58.9)	18.6 (5.3-35.7)
CD62p	3.0 (1.0-8.0)	2.0 (0.8-3.0)	2.4 (1.3-8.3)	1.0 (0.4-3.0)	0.7 (0.4-1.0)	2.0 (0.7-3.0)	0.9 (0.3-4.0)	0.4 (0.3-3.9)	0.7 (0.1-1.0)
Tissue Factor	1.0 (0.5-3.0)	0.4 (0.2-1.0)	1.0 (0.3-2.0)	0.3 (0.1-0.5)	0.2 (0.1-0.7)	0.4 (0.3-1.0)	0.4 (0.2-0.9)	0.3 (0.2-0.5)	0.2 (0.09-0.4)
% CD62p pos. platelets	9.8 (2.9-14.6)	3.7 (2.2-13.8)	4.8 (3.8-13.0)	3.9 (2.0-5.7)	3.4 (2.3-6.7)	5.3 (2.5-6.9)	2.2 (1.2-6.9)	3.5 (1.8-4.7)	2.8 (1.1-9.6)

Controls, control group without ischaemia; He-EPC, helium early preconditioning group; I/R, ischaemia-reperfusion group; All data are expressed as mean±SD. All values are corrected for isotope controls and represent $*10^5$ microparticles/ml. The percentage of positive CD62p particles was calculated by dividing particles positive for both CD62p and CD61 by the total amount of CD61 positive particles. Data are presented as median with 25-75 quartiles. No significant differences were observed between groups.

DISCUSSION

In this study, we show for the first time that inhalation of helium in humans prevents impairment of acetylcholine-induced vasodilation following I/R. A similar protection was observed 24hrs after helium administration. Therefore, our data show that helium induces not only early but also late endothelial preconditioning in humans in vivo. This conclusion is supported by the improved postischaemic FBF upon infusion of the endothelial-dependent vasodilator acetylcholine, while the response to the endothelial-independent vasodilator sodium nitroprusside was unaffected.

Forearm blood flow model

A time-control group was included in our study to demonstrate that the results were reproducible. In accordance with previous studies using the same model,[3, 16] ischaemic preconditioning protected against endothelial dysfunction. Although an increased baseline FBF after ischaemia (hyperaemia) in the I/R group might interfere with postischaemic response curve to acetylcholine, it is unlikely that this effect is responsible for the absent response to acetylcholine. The postischaemic response to sodium nitroprusside (Supplemental Digital Content 2, figure 1) demonstrates that vasodilation can still be achieved after I/R, indicating postischaemic vasodilation was not maximal.

Acetylcholine results in calcium-mediated activation of eNOS via the endothelial muscarinic receptor.[17] Under physiological conditions, nitric oxide diffuses to the vascular smooth muscle cell layer and activates soluble guanylate cyclase eventually leading to cyclic guanosine monophosphate-mediated vasodilation and flow increase.[18] Following I/R, acetylcholine-induced vasodilation is reduced probably by a decrease in the release of nitric oxide which can be due to eNOS uncoupling, i.e. endothelial depletion of essential cofactors of eNOS like tetrahydrobiopterin and L-arginine.[19] As a result of this uncoupling, scavenging of endothelial nitric oxide by increased reactive oxygen species can lead to production of peroxynitrite, which in turn can induce cellular injury and vasoconstriction.[20] There could be a contribution of vascular smooth muscle cell dysfunction, however this is unlikely since sodium nitroprusside-induced vasodilation was unaltered by I/R.

Preconditioning by inhalational substances

Volatile anaesthetics as well as the anaesthetic noble gas xenon induce organ protection by preconditioning,[21, 22] as was shown in several in vitro[23] and in vivo[8] models. This organ protective effect cannot be attributed to analgesic actions of these gases as the analgesic gas nitrous oxide did not precondition the rat heart in vivo.[24] Noble gases without anaesthetic properties induced preconditioning in rabbits in vivo,[25] and for the noble gas helium both the early and late phase of preconditioning have been demonstrated.[8, 26, 27]

For volatile anaesthetics, the translation to clinical I/R situations was made by showing the preconditioning effect of sevoflurane in patients undergoing coronary artery bypass graft surgery.[28, 29] Although previous studies demonstrated a late phase of preconditioning by gases,[8, 22] the present study shows for the first time that inhalation of helium induces early and also late endothelial preconditioning in humans.

In contrast to our data showing helium preconditioning in the human endothelium, another study investigating helium preconditioning in human endothelium found that helium provided modest anti-inflammatory effects, but did not protect against I/R.[14] There are several differences between the two studies. First, postischaemic reactive hyperaemia was used to assess endothelial function, which is less reliable to measure endothelial function compared to infusion of a vasodilator like acetylcholine. Second, the lack of a distinct preconditioning protocol could be the reason why helium failed to induce preconditioning, since the noble gas was applied continuously before, during and after ischaemia. Previous clinical studies demonstrated that the preconditioning protocol plays a major role in volatile-anaesthetic induced organ protection in humans: protection could only be evoked by a distinct and repetitive stimulus.[28, 29] Another difference between our study and the study from Luchinetti et al. is the helium concentration used to induce endothelial protection (50% compared to 79%). The minimal required concentration of helium to induce preconditioning in humans is unknown, and could be above 50%, although in experimental studies in rats a concentration of 30%-70% helium was sufficient to induce late preconditioning whereas 10% was not.[8]

Possible mechanisms of protection

Although the mechanisms of helium induced preconditioning are not fully clarified, some mediators have been identified and are discussed in a recent review about the possible effects of helium in different organs.[30] One experimental study showed that administration of the non-selective nitric oxide synthase inhibitor *N*-nitro-*L*-arginine-methyl-esther during helium preconditioning abolished cardioprotection in rabbits.[31]

Data from this study suggest that helium preconditioning is mediated by nitric oxide generated by eNOS in vivo. In order to investigate the possible role of eNOS in He-EPC, we administered L-NMMA during helium breathing. Our data (see figure 4 and supplemental digital content 2 figure 3) demonstrate that in our experiments eNOS blockade administered during helium preconditioning does not abolish endothelial protection. There are some limitations to our L-NMMA administration protocol: we only administered L-NMMA during helium preconditioning, and stopped infusion before the start of I/R. We cannot rule out from the current data that a prolonged infusion of L-NMMA might be able to block the helium preconditioning effect. We carefully considered our administration protocol for L-NMMA, and administration during forearm ischaemia and reperfusion could have altered the postischaemic damage. This is in line with another study in which infusion of L-NMMA was continued during acetylcholine infusion, resulting in a decreased response to acetylcholine even in the control group.[32] Experimental data have shown that L-NMMA, given during ischemia and reperfusion, attenuated postischaemic endothelial dysfunction in the Langendorff perfused heart[33] making it impossible to compare results of these groups with groups not receiving L-NMMA.

Although it is very unlikely, L-NMMA might have induced preconditioning by itself, thereby overcoming a blockade of the helium preconditioning effect. However, this preconditioning effect of L-NMMA has never been demonstrated in animal studies before. Although blood flow was significantly reduced by L-NMMA, we did not observe any aspects of forearm ischaemia caused by L-NMMA in the physically non-active study situation.

Endothelial injury may lead to the increased expression of inflammatory cytokines and adhesion molecules resulting in increased adhesion and migration of leukocytes. One of the cytokines that mediates endothelial dysfunction is tumour necrosis factor-α,[34] which stimulates the production of IL-6. IL-6 is of vital importance to induce ischemic late preconditioning[35] and increased levels of IL-6 are associated with poor prognosis in patients with heart failure.[36]

Although we did not perform a power analysis of the effect of forearm I/R on cytokines, the current data suggest that forearm I/R does not affect the systemic levels of IL-1ß, IL-6, IL-8, soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1 and E-selectin after 15 minutes or 3 hours of reperfusion. However, we cannot exclude a local contribution of these cytokines to the endothelial dysfunction after I/R.

Another mechanism of endothelial dysfunction is the presence of circulating endothelial microparticles, which proved to be an independent risk factor for impaired endothelial vasodilation.[37, 38] Microparticles from patients with acute myocardial infarction selectively impaired nitric oxide production and caused severe endothelial dysfunction shown by impairment of acetylcholine-induced vasodilator response in isolated vessels.[11]

We showed that forearm I/R did not significantly affect the amount of circulating microparticles derived from platelets and erythrocytes. Microparticles derived from endothelial cells and leukocytes were at or below the detection limit, also after I/R. It is unknown whether forearm I/R causes microparticles release, and it is possible that a local increase of microparticles, possibly endothelial microparticles, in the venous outflow tract was missed because of systemic dilution.

Study limitations

Since we collected our blood samples from the control arm, only systemic effects of cytokines and microparticles could be investigated. There are no previous studies in healthy volunteers that investigated cytokine release or its time course after forearm I/R. We therefore cannot exclude the possibility that there is cytokine involvement at other time points than those investigated in our present study.

In this study we focused on group main effects of forearm I/R and helium inhalation on endothelial function, statistical analyses between groups were not performed. We also cannot exclude effects of helium on cytokines and microparticles in a larger study population. However, previous studies found significant differences in similar sized study populations.[38]

Inhalation of helium has been shown to affect ventilation parameters in patients with chronic obstructive airway disease.[39] Volunteers inhaled helium via a non-invasive ventilation machine and changes in ventilation parameters (e.g. breathing frequency) were not observed. We did not measure arterial partial pressure of oxygen during helium inhalation. However, significant changes in oxygen tension after inhalation of Heliox containing 21% oxygen is not to be expected in healthy volunteers. In addition, helium inhalation was stopped at least 5 min before forearm I/R, and because helium rapidly diffuses, a significant effect on oxygen tension during forearm I/R is most unlikely. We did not investigate the direct effect of helium on forearm bloodflow without I/R.

Helium is a non-anaesthetic, non-toxic gas without any haemodynamic side effects that can easily be applied to patients. This inhalational gas could be a perfect instrument to induce preconditioning in patients subjected to clinical I/R situations, i.e. coronary artery bypass graft surgery. However, whether helium preconditioning can protect patients with co-morbidities like atherosclerosis, hypertension or diabetes mellitus still has to be investigated.

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Supplemental Content

Isolation of microparticles

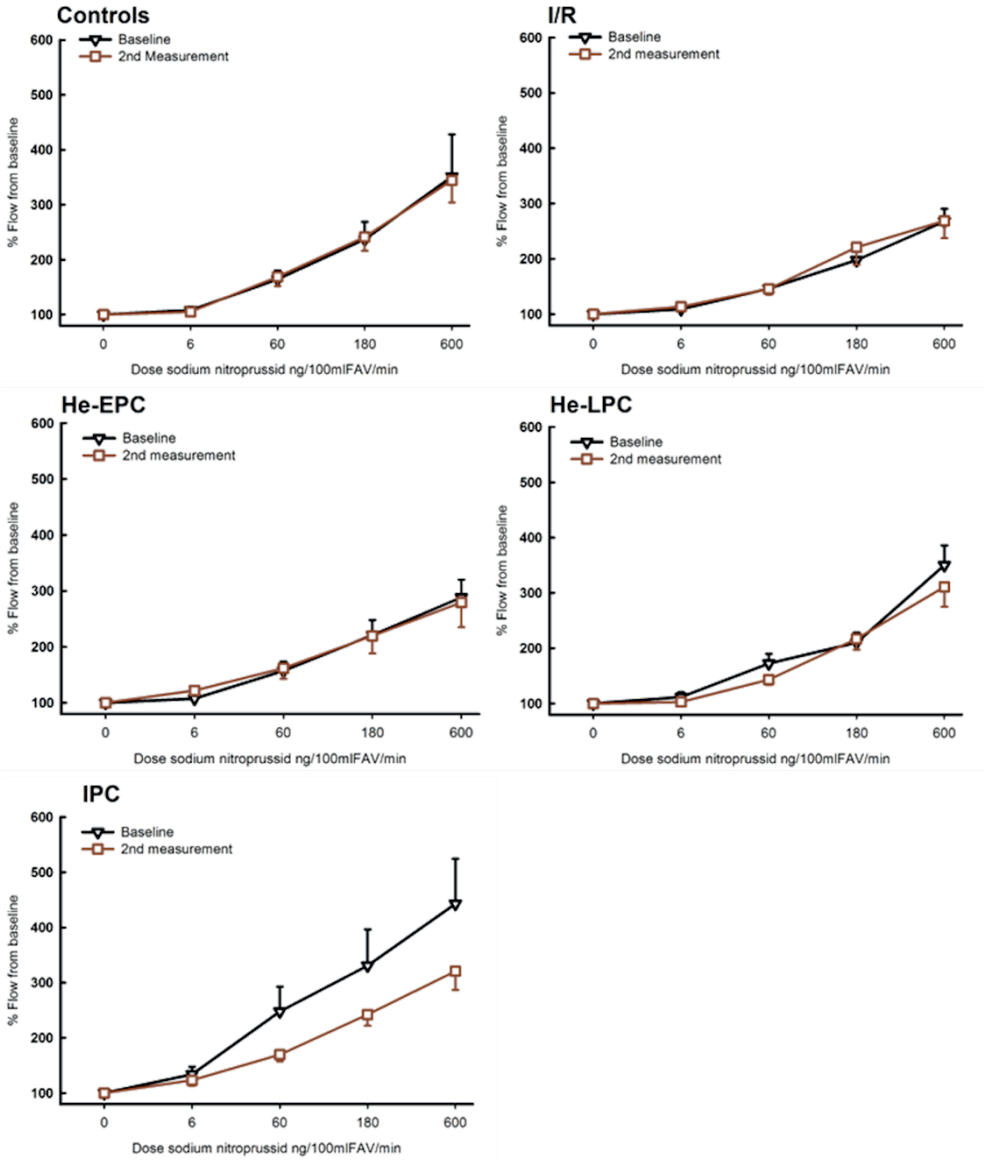
A sample of 250 µL of frozen plasma was thawed on melting ice for 1 h and centrifuged for 30 min at 18,890*g* at 20°C to pellet the microparticles. After centrifugation, 225 µL of the supernatant were removed. The pellet was resuspended in 225 µL phosphate-buffered saline (PBS) containing citrate, after which samples were centrifuged again and supernatants were removed again. The pellet containing microparticles was resuspended in 75 µL PBS-citrate for the final concentration.

Flowcytometry of microparticles

Five microliters of the microparticles suspension was diluted in 30 µL $CaCl_2$ (2.5 mmol/L)-containing PBS. Than 5 µL allophycocyanin-labelled annexin V were added to all tubes plus 5 µL of the cell-specific monoclonal antibody or isotype-matched control antibodies (total volume: 50 µL). The samples were incubated in the dark for 15 min at room temperature. After incubation, 900 µL of calcium-containing PBS were added to all tubes (except to the annexin V control, to which 900 µL of citrate-containing PBS were added). Samples were analysed for 1 min in a fluorescence automated cell sorter FACS Calibur with CellQuest software (Becton Dickinson, San Jose, CA). Both forward scatter and sideward scatter were set at logarithmic gain. To establish the origin of the microparticles, we performed a triple labeling on each sample using different fluorochromes (Fluorescein isothiocyanate (FITC)-labeled IgG_1, phycoerythrin (PE)-labeled IgG_1, and allophycocyanin conjugated annexin V). These antibodies were used to analyse the origin of the circulating microparticles: CD61-PE (exposed on thrombocytes), CD63 (expressed by activated platelets), CD14 (mostly found on macrophages), CD4 (from T-cells), CD8 (also from T-cells), CD20 (from B-cells), glycophorin A-FITC (CD235a) from erythrocytes, CD144-FITC exposed on endothelial cells, CD62e-PE (E-selectin exposed on activated endothelial cells). Microparticles were identified based on their size and density and on their ability to bind cell-type specific CD antibodies and annexin V. Microparticles positive for annexin V, CD62e-PE and Cd144-FITC were considered to be derived from activated endothelial cells.

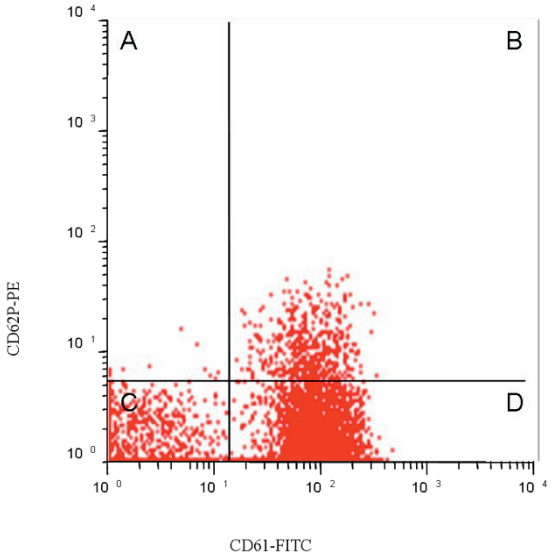
Antibodies

FITC-labeled IgG_1, PE-labeled IgG_1, CD4-PE, CD8-PE, CD14-PE, CD20-FITC, CD61-PE, CD146-PE, Tissue Factor-PE were obtained from Becton Dickinson, Ig2b-PE and IgGpoly-FITC from Immuno Quality Products (Groningen, The Netherlands), CD24-PE (Serotec, Oxford, United Kingdom), CD61-FITC, CD235a from DAKO (Glostrup, Denmark), CD62e-PE from Ancell corporation(Bayport, MN), CD63-PE from Beckman Coulter Inc. (Fullerton, CA), CD144-FITC from Alexis Biochemicals (San Diego, CA), Allophycocyanin-conjugated annexinV was obtained from Caltag (Burlington, CA).



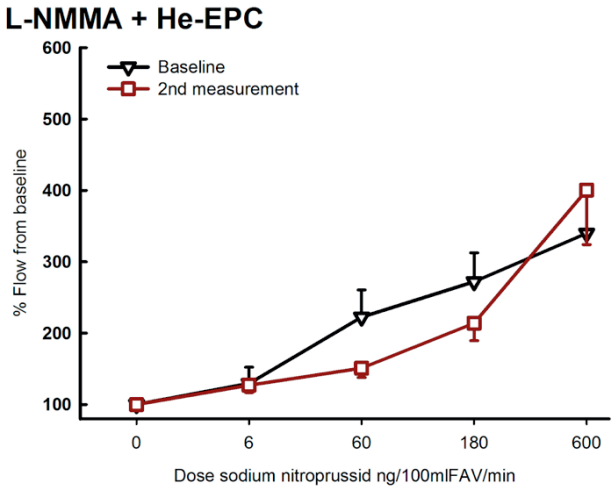
Supplemental figure 1. Results of Nitroprusside infusion

Controls, control group without ischaemia; He-EPC, helium early preconditioning group; He-LPC, helium late preconditioning group; I/R, ischaemia-reperfusion group; IPC, Ischaemic preconditioning group. FAV; Forearm volume; FBF, Forearm blood flow. All data are represented as mean±SEM, no significant statistical differences were observed between first and second FBF measurement.



Supplemental figure 2. Example of microparticle results

These are the results of the microparticle analysis of a volunteer from the helium early preconditioning group, at timepoint 10 minutes of reperfusion. Microparticles positive for CD 61-FITC are shown in panels B and D, and microparticles positive for CD62p-PE are shown in panels A and C. Microparticles positive for CD61-FITC that are also positive for CD62p-PE (which indicates that these particles are derived from activated platelets) are shown in panel B, and are 4,37%. (FITC; Fluorescein isothiocyanate, PE, phycoerythrin.)



Supplemental figure 3. L-NMMA+He EPC

Results of Helium early preconditioning group with N^G-monomethyl-L-arginine (L-NMMA) infusion (L-NMMA + He-EPC). FAV; Forearm volume; FBF, Forearm blood flow. All data are represented as mean±SEM, no significant statistical differences were observed between first and second FBF measurement.

5

Chapter 5

Effect of helium pre- or postconditioning on signal transduction kinases in patients undergoing coronary artery bypass grafting surgery

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ABSTRACT

Background: The noble gas helium induces pre- and postconditioning in animals and humans. Volatile anaesthetics induce cardioprotection in humans undergoing coronary artery bypass graft (CABG) surgery. We hypothesized that helium induces pre- and postconditioning in CABG-patients, affecting signalling molecules protein kinase C-epsilon (PKC-ε), p38 mitogen activated protein kinase (p38 MAPK), extracellular signal-regulated kinase 1/2 (ERK-1/2) and heat shock protein 27 (HSP-27) within cardiac tissue, and reducing postoperative troponin levels.

Methods: After ethical approval and informed consent, 125 elective patients undergoing CABG surgery were randomised into this prospective, placebo controlled, investigator blinded, parallel arm single-centre study. Helium preconditioning (3x5 min of 70% helium and 30% oxygen) was applied before aortic cross clamping; postconditioning (15 min of helium) was applied before release of the aortic cross clamp. Signalling molecules were measured in right atrial appendix specimens. Troponin-T was measured at 4, 12, 24, and 48 hours postoperatively.

Results: Baseline characteristics of all groups were similar. Helium preconditioning did not significantly alter the primary outcome (molecular levels of kinases PKC-ε and HSP-27, ratio of activated p38 MAPK or ERK 1/2). Postoperative Troponin T was 11 arbitrary units (5, 31; area-under-the-curve (interquartile range)) for controls, and no statistically significant changes were observed after helium preconditioning [He-Pre: 11 (6, 18)], helium postconditioning [He-Post: 11 (8, 15)], helium pre- and postconditioning [He-PP: 14 (6, 20)] and after sevoflurane preconditioning [APC: 12 (8, 24), p=0.13. No adverse effects related to study treatment were observed in this study.

Conclusions: No effect was observed of helium preconditioning, postconditioning or the combination thereof on activation of p38 MAPK, ERK 1/2 or levels of HSP27 and PKC-ε in the human heart. Helium pre- and postconditioning did not affect postoperative Troponin release in patients undergoing CABG surgery.

INTRODUCTION

Noble gases like xenon can induce cardioprotection via preconditioning.[1-6] The signal transduction cascade mediating this effect has partly been described and shares similarities with transduction cascade mediating ischaemic preconditioning.[7, 8] This noble gas induced cardioprotective effect was abolished on a cellular level by blockers of protein kinase C (PKC) and p38 mitogen activated protein kinase (p38 MAPK).[3] Xenon preconditioning also involves extracellular-signal-regulated kinases-1 and -2 (ERK1/2),[6] leading to intracellular translocation of heat shock protein 27 (HSP-27).[4]

The non-anaesthetic noble gas helium has no relevant cardiopulmonary side effects and is already clinically used in patients with airway diseases.[9, 10] It can easily and safely be administered using readily available ventilators and in critical care patients.[11, 12] Experimental data from different laboratories in different animal species have demonstrated profound protective effects of helium against ischaemia-reperfusion damage of the heart.[1, 13, 14] In a previous study in healthy volunteers, we demonstrated that 3 times 5 minutes of 79% helium inhalation prevented post-ischaemic endothelial dysfunction.[15] Experimental data indicated involvement of similar signal cascades during helium conditioning as were shown before for xenon- and anaesthetic-induced conditioning.[1] However, the exact underlying mechanism of helium protection in humans remains unclear.

Different preconditioning protocols are currently used to induce anaesthetic preconditioning, either via continuous administration throughout surgery,[16] during ischaemia-reperfusion,[17] or before aortic cross clamping.[18] It is known that both timing and repetition of the preconditioning stimulus, are central for producing the respective protection.

Based on the experimental and first clinical data on helium conditioning we hypothesised that helium induces pre- and/or postconditioning in human myocardium of patients undergoing coronary artery bypass grafting (CABG) surgery, involving regulation of PKC, p38 MAPK, ERK 1/2 and HSP-27, and reducing postoperative troponin T release.

METHODS

The institutional review board of the Academic Medical Centre, Amsterdam, The Netherlands, approved the trial registered in the Dutch trial register (number NTR1226). Inclusion into this prospective, placebo controlled, investigator blinded, parallel arm single-centre study took place from 8-7-2008 to 3-7-2011 at the Academic Medical Centre, Amsterdam, The Netherlands, in accordance with the International Conference on Harmonization on Good Clinical Practice Guidelines and the Declaration of Helsinki. Patients were randomised to one of five parallel groups in a 1:1:1:1:1 allocation ratio using web-based randomisation software (ALEA; NKI; Amsterdam, The Netherlands) with a fixed block scheme, a block size of 5 patients and stratification on sex (see figure 1). While the anaesthetist and the investigator in the operating room were not blinded, the patients as well as the investigators performing laboratory data analysis (Troponin T values, Western Blot experiments) were blinded to the randomisation result. Patients were recruited by self-selecting, and all subjects gave written informed consent. Exclusion criteria were age < 18 years, legal incapacity, emergency operations, combined coronary artery and heart valve procedures, off-pump procedures, diabetes mellitus, severe chronic obstructive pulmonary disease (COPD), and left ventricular ejection fraction < 30%. The last two criteria were added after publication on www.trialregister.nl but before start of the study.

Study protocol

At least two cycles inhalation of sevoflurane were necessary to induce preconditioning in humans.[18, 19] We wanted to extend the preconditioning stimulus and decided to use three cycles of conditioning, as this protocol was also used in most experimental studies.[20] The first group received helium preconditioning (He-Pre) by inhalation of three cycles of helium for five min, followed by five min inhalation of oxygen-enriched air (30% oxygen). Helium was obtained as a mixture with oxygen (Heliox: 79% helium and 21% oxygen, BOC, Mordon, United Kingdom) and administered using a non-invasive helium delivery system (Helontix Vent, Linde Therapeutics, Eindhoven, The Netherlands) modified to allow manual ventilation in a Maplesons A configuration. All patients were ventilated the same way by the same investigator. Extra oxygen was added and the final concentration of the gas-mixture was 70% helium and 30% oxygen. He-Pre was administered shortly before start of cardiopulmonary bypass (CPB). A graphical presentation of our study protocol is represented in figure 1. The postconditioning group (He-Post) received at least 15 minutes of helium at the end of aortic cross-clamping, lasting up to five minutes after release of the clamp. The third group received helium as pre- and postconditioning stimulus (He-PP). Patients receiving helium pre- and postconditioning thus received two conditioning stimuli

of helium with double time of helium ventilation. To compare the effects of helium with the known effects of anesthetic preconditioning (APC), the fourth group received three cycles of five minutes sevoflurane inhalation with a minimal alveolar concentration (MAC) of 1.0 MAC, and the auto-flow function of the anaesthetic machine (Zeus, Dräger Medical, Lübeck, Germany) was used to ensure rapid wash in and wash out of sevoflurane. The fifth group was an untreated control group.

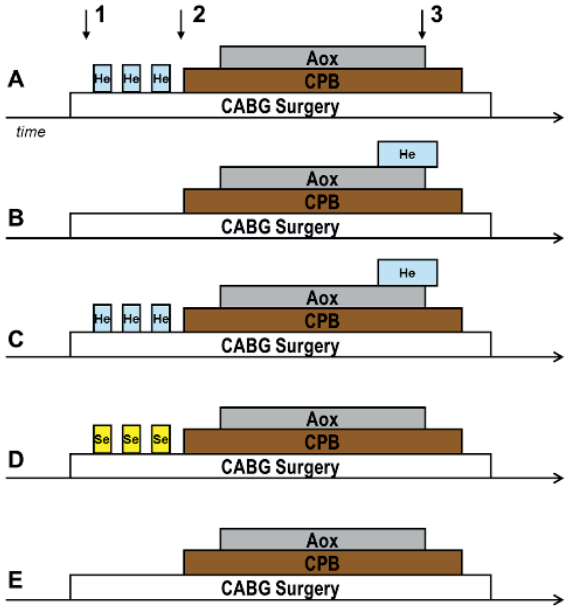

Figure 1. Protocol outline

Schematic timeline of the study protocol. The black arrows represent the time points at which atrial myocardial biopsies were taken. Aox: aortic cross clamp. CPB: cardiopulmonary bypass**. A:** Helium preconditioning group. Helium was administered in three cycles for 5 minutes, followed by 5 minutes inhalation of oxygen enriched air (30% oxygen). **B:** Helium postconditioning group, helium administration started at the end of aortic cross clamping for 15 minutes and was continued for 5 minutes after begin of reperfusion. **C:** Helium pre-and postconditioning group. Helium was administered both as preconditioning stimulus before cardiopulmonary bypass and as postconditioning stimulus at the end of aortic cross clamping. **D:** Anaesthetic preconditioning group in which sevoflurane was administered in three cycles of 5 minutes. **E:** untreated controls.

Anaesthesia

Patients received premedication with temazepam 10 mg per os. Induction of anaesthesia was performed with intravenous administration of midazolam 0.1-0.2 mg kg^{-1} and target

controlled infusion of propofol (dosage was 1-2 mg/kg for induction), sufentanil 1.0-1.5 µg kg^{-1}, and rocuronium 0.6 mg kg^{-1} for muscle relaxation. Target controlled infusion of propofol was continued to maintain anaesthesia in combination with either continuously or intermittently sufentanil.

Surgery

All patients received routine monitoring during operation and routine surgical techniques were used. A pulmonary artery catheter was used for cardiac output monitoring. The left internal mammary artery was used to graft the left anterior descending artery. As additional grafts, harvested veins from the leg, the right internal mammary artery or one of the radial arteries were used. Both cold crystalloid and cold blood cardioplegia were administered antegrade via the aortic root, and management of the cardiopulmonary bypass (CPB) was according to standard procedure.

Median sternotomy was performed, followed by pericardiotomy after which the first sample of the right atrial appendage was obtained. Then the left internal thoracic artery was prepared, during which time systemic heparinization was started (300 IU/kg goal: coagulation time > 450 s). After venous and arterial cannulas for CPB were inserted and secured, the second sample of the right atrium was obtained which was directly after preconditioning in the applicable groups. Then CPB was started, and the aorta was cross-clamped and cardioplegia solution was infused. All distal anastomoses were performed during aortic cross-clamping. Additional cardioplegic solution was administered at intervals to maintain a flat electrocardiogram. Fifteen minutes before expected release of the aortic cross clamp, we started helium postconditioning in the designated groups, and continued helium ventilation until 5 minutes after the start of reperfusion. After completion of coronary artery bypass grafting, CPB was discontinued and the third sample of the right atrium was obtained. After surgery, patients were transferred to the intensive care unit (ICU), received routine therapy and were weaned from the ventilator. ICU and ward staffs were blinded to the treatment allocation.

Blood sampling and tissue preparation

Blood samples were taken before cardiopulmonary bypass, 10 min after cardiopulmonary bypass and at the end of operation, as well as at 4, 12, 24, and 48 hours after cardiopulmonary bypass. We measured troponin-T, creatinine kinase and its myocardial specific isoform creatine kinase-muscle/brain as markers of cellular injury. All samples were

analysed in the Laboratory of Clinical Chemistry of the Academic Medical Centre, Amsterdam, The Netherlands.

Atrial samples were immediately flash frozen in liquid nitrogen and stored at -80°C until further processing. Tissue fractionation was performed as described by Weber et al.[6] Cytosolic, membrane, and the particulate fraction were immunoblotted using the Criterion Western Blotting system (Biorad, Hercules, CA).

After protein determination by the Lowry method, samples were thawed and diluted 1:5 with Sample Buffer 5 times containing Tris–HCl, glycerol and bromophenol blue. Samples were vortexed and boiled at 95°C before being subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using Criterion™ XT precast gels (Biorad, Hercules, CA). The proteins were separated by electrophoresis and transferred to a polyvinylidenfluorid membrane by tank blotting (Voltage 200V for 50-55 minutes). Non-specific binding of the antibody was blocked by incubation with 5% fat dry milk powder or bovine serum albumin solution in Tris-buffered saline containing Tween (TBS-T) for 2 h. Subsequently, the membrane was incubated overnight at 4°C with the respective primary antibody at indicated concentrations. After washing in fresh, cold TBS-T, the blot was subjected to the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Immunoreactive bands were seen by chemiluminescence detected on X-ray film (Hyperfilm ECL, Amersham) using the enhanced chemiluminescence system Santa Cruz. The blots were quantified using a Kodak Image station® (Eastman Kodak Co., Rochester, NY, USA) and the results are presented as the ratio of phosphorylated to total protein. Values are expressed as ***x***-fold average light intensity (AVI) compared with control. Equal loading of protein on the gel was additionally confirmed by detection of actin/α-Tubulin and Coomassie staining of the gels.

Antibodies

We used anti-phospho PKC-ε, antibody (1:10.000) and total PKC-ε, both from Upstate (Lake Placid, NY). Phospho-ERK1/2 (1:10.000), total ERK 1/2 (1:10.000), phospho p38 MAPK (1:5.000) and total p38 MAPK (1:5.000) were obtained from Cell Signalling (Danvers, MA), HSP 27 (1:5.000) from Abcam (Cambridge, UK). Both Actin (1:10.000) and α-Tubulin (1:40.000) were obtained from Sigma (St. Louis, MO). Peroxidase-conjugated goat anti-rabbit and donkey anti-mouse antibodies were from Jackson Immunoresearch (Suffolk, UK). The enhanced chemiluminescence protein detection kit was purchased from Santa Cruz (Heidelberg, Germany).

Endpoints and data collection

Primary endpoints of this study are phosphorylation of ERK1/2, p38MAPK and expression of HSP27 and PKC-ε in the particulate fraction. Secondary endpoints include post-operative troponin T release.

Data were collected on age, sex, race, length and weight, co-morbidities and risk factors for cardiovascular disease, EuroSCORE, medication usage, duration of bypass, and aortic clamping, number and type of grafts. Because of technical difficulties establishing reproducible results for the western blot we lost n=5 patients per group for these targets (p38 MAPK, ERK1/2, HSP27 and PKC- ε).

Sample size calculation and statistics

Regarding our primary endpoint, no data on the effect of noble gas preconditioning on protein expression in human myocardial tissue was available while setting up the study. A proper sample size calculation was therefore not possible at start of the study. However, based on previous experimental research and a similar clinical study,[19] we expected to find any - also clinically relevant differences - with a sample size of 25 patients per group.

Numerical data are presented as mean ± SD or median with interquartile range, as appropriate. Categorical data are presented as numbers and percentages. Statistical analyses were done using SPSS version 22 (IBM, Armonk, New York, USA).

We considered a p-value of < 0.05 to be statistically significant. Statistical testing of the Western Blot data was done using Shapiro Wilk test for normality and Friedmann test for non-parametric data followed by Bonferroni correction for multiple testing (GraphPad Prism version 5.0, GraphPad, La Jolla, CA). We chose to graphically represent our data as mean+SD, however detailed information regarding the mean differences of the timepoints from our primary endpoints is available in the online data supplement 1.

To compare post-operative troponin T release the area-under-the-curve was calculated (mentioned as arbitrary units) and compared in a one-way-ANOVA.

RESULTS

Baseline characteristics

Four patients were excluded from the study [no helium available (1), no investigational team available (1), unplanned additional valve surgery (1), previously unknown decreased left ventricular ejection fraction <30% (1)], leaving data from 121 patients available for statistical analysis (CONSORT diagram see figure 2). Baseline characteristics of all groups were similar with regard to age (66±9) and sex (83% male, table 1). Preoperative demographic data showed that in controls, less patients with hypercholesterolaemia, or who used statins or beta-blockers were present compared to the other groups. The predictive additive risk determined by EuroSCORE was similar in all groups. The number of bypass grafts and duration of aortic cross clamping and CPB duration were also similar in all groups (table 2).

Pre- and postconditioning protocols

Helium and sevoflurane pre- and/or postconditioning were administered without any problems during surgery. Neither the preconditioning nor the postconditioning stimulus altered haemodynamics, and no differences in heart rate, mean arterial pressure, cardiac index or pulmonary artery pressure were observed between groups (table 3). We did not measure myocardial oxygen supply directly, but no differences were observed between groups regarding arterial oxygen tension and haemoglobin levels (see supplement 1).

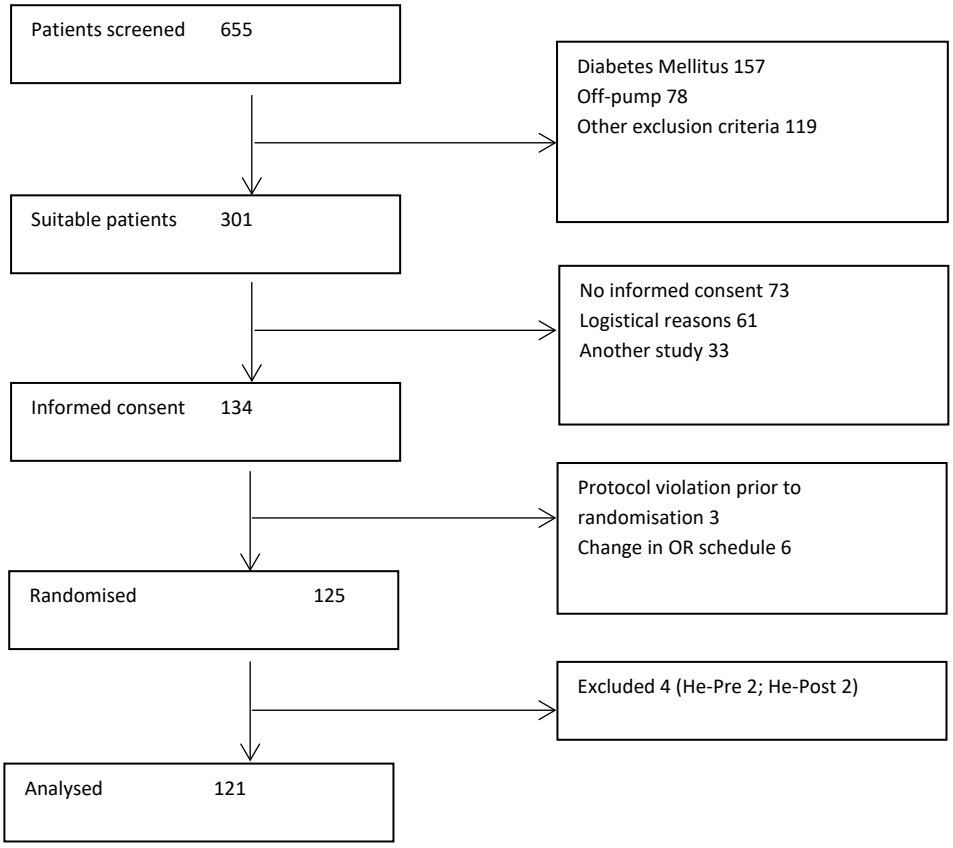

Figure 2. CONSORT diagram

Table 1. Demographic data

	Con	He-Pre	He-Post	He-PP	APC	*p value*
N	28	23	22	24	24	
Age (years)	66,7 (±7,0)	62,8 (±11,7)	66,2 (±7,8)	66,2 (±8,3)	66,9 (±7,6)	0.47
Male (%)	22 (82)	21 (91)	20 (83)	20 (83)	21 (88)	0.88
BMI	28,4 (3,7)	27,3 (±2,7)	26,7 (±3,8)	27,4 (±3,7)	27,7 (±3,2)	0.55
Risk factors						
Hypertension	16 (59)	9 (39)	13 (57)	10 (42)	13 (54)	0.52
Hypercholesterolaemia[a]	6 (22)	11 (48)	10 (44)	5 (21)	14 (58)	0.023[a]
Smoking (past or present)	13 (48)	10 (44)	8 (34)	11 (46)	10 (42)	0.89
Diabetes	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Family	12 (44)	9 (39)	7 (30)	9 (38)	13 (54)	0.56
Myocardial Infarction	7 (26)	10 (44)	8 (36)	10 (42)	8 (33)	0.71
Cerebrovascular Accident	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	0.40
Heartfailure						
NYHA I	20 (74)	17 (74)	19 (86)	17 (71)	16 (70)	0.97
NYHA II	2 (7)	2 (9)	1 (5)	2 (8)	3 (13)	0.97
NYHA III	5 (19)	4 (17)	2 (9)	4 (17)	4 (17)	0.97
Ejection Fraction						
>50%	21 (81)	16 (73)	12 (57)	16 (67)	19 (90)	0.15
30-50%	5 (19)	6 (27)	7 (33)	4 (17)	1 (5)	0.15
Medication						
Salicylate	24 (86)	19 (83)	22 (92)	23 (96)	23 (92)	0.57
Clopidogrel	9 (32)	9 (39)	12 (50)	9 (38)	4 (16)	0.15
Statin[b]	27 (96)	23 (100)	24 (100)	20 (83)	21 (84)	0.04[b]

Beta-blocker[c]	19 (68)	22 (96)	24 (100)	21 (88)	21 (84)	0.008[c]
ACE-inhibitor	10 (36)	8 (35)	10 (42)	4 (17)	11 (44)	0.30
AT2-receptor-blocker	6 (21)	1 (4)	5 (21)	5 (21)	3 (12)	0.40
Calciumchannel-blocker	10 (36)	7 (30)	4 (17)	10 (42)	6 (24)	0.35
Diuretics	6 (21)	4 (17)	4 (17)	7 (29)	4 (16)	0.77
Nitrates	10 (36)	9 (39)	7 (29)	12 (50)	13 (52)	0.43
EuroSCORE	3,0 (±1,6)	2,4 (±2,0)	2,7 (±1,7)	3,3 (±1,8)	2,4 (±2,0)	0.36

Age and Body Mass Index are presented as mean ± SD; Euroscore is presented as mean (±IQR); other data are numbers and percentage, n (%). He-Pre= Helium preconditioning; He-Post=Helium Postconditioning; He-PP= Helium Pre- and Postconditioning; APC=Anaesthetic preconditioning. Age and Body Mass Index are presented as mean ± SD; EuroSCORE is presented as mean (±IQR); other data are numbers and percentage, n (%). [a] In the control group significantly less patients with hypercholesterolemia were present. [b] Use of statins was significantly lower in our control group compared to other groups. [c] Use of beta-blockers was significantly lower in our control group compared to other groups.

Table 2. Surgical Specifications

	Controls	He-Pre	He-Post	He-PP	APC	*p-value*
ECC time (min)	88 ±7	84 ±6	91 ±5	100 ±7	95 ±6	*0.53*
Cross-clamp time (min)	59 ±4	54 ±4	58 ±5	67 ±5	62 ±4	*0.51*
Cardioplegia						
Blood (%)	18 (82)	17 (85)	18 (82)	14 (67)	18 (78)	*0.36*
Saline (%)	4 (18)	3 (15)	4 (18)	7 (33)	5 (22)	*0.36*
Number of coronary arteries						
1	1 (4)	1 (5)	0 (0)	2 (8)	1 (4)	*0.19*
2	9 (33)	6 (27)	4 (17)	8 (33)	7 (29)	*0.19*
3	17 (63)	15 (68)	17 (81)	14 (58)	16 (67)	*0.19*
Left Main	9 (32)	8 (36)	3 (13)	20 (42)	10 (40)	*0.88*

Data are presented as mean ± SD; no significant differences were observed between groups. He-Pre= Helium preconditioning; He-Post=Helium Postconditioning; He-PP= Helium Pre- and Postconditioning; APC=Anaesthetic preconditioning.

Table 3. Haemodynamic data

	Controls	He-Pre	He-Post	He-PP	APC
Heart Rate (beats min^{-1})					
After sternotomy	56±7	59±8	62±12	60±10	60±13
After preconditioning	60±11	59±8	59±9	61±11	61±13
After CPB	72±7	73±10	73±14	71±12	80±15
Mean arterial pressure (mmHg)					
After sternotomy	76±12	74±13	76±14	77±13	80±16
After preconditioning	66±12	69±13	64±12	67±11	68±13
After CPB	63±12	64±7	66±11	70±11	70±14
Cardiac Index ($L/min/m^2$)					
After sternotomy	2.1±0.6	2.1±0.6	2.0±0.4	2.1±0.4	2.2±0.5
After preconditioning	2.0±0.7	2.1±0.5	2.0±0.4	2.3±0.4	2.1±0.5
After CPB	2.4±0.7	2.7±0.8	2.4±0.4	2.5±0.7	2.1±0.8
Pulmonary artery pressure (mmHg)					
After sternotomy	18±6	16±5	16±5	17±4	20±3
After preconditioning	18±6	18±6	14±5	17±4	18±5
After CPB	20±6	18±4	18±4	20±5	20±4
PCWP (mmHg)					
After sternotomy	11±6	10±6	8±6	10±2	8±4
After preconditioning	4±5	9±6	9±7	9±3	9±5
After CPB	15±6	11±3	8±3	13±3	10±1

Data are presented as mean ± SD; no significant differences were observed between groups. He-Pre= Helium preconditioning; He-Post=Helium Postconditioning; He-PP= Helium Pre- and Postconditioning; APC=Anaesthetic preconditioning; CPB=cardiopulmonary bypass; PCWP=pulmonary capillary wedge pressure.

Phosphorylation of p38 MAPK in cytosolic fraction

We determined in the cytosolic fraction of the myocardial atrial tissue the phosphorylated-to-total (p/t) p38 MAPK ratios within three biopsies from each patient taken at time points described in figure 3. In controls, no statistically significant changes were observed at begin of CPB and after CPB compared to baseline (figure 3). In groups receiving a preconditioning stimulus (He-Pre, He-PP, APC), no effect of the preconditioning stimulus on the ratio of p/t p38 MAPK was observed (biopsy 2 vs. biopsy 1). In addition, also postconditioning (He-Post, He-PP) did not affect the ratio p/t p38 MAPK (biopsy 3 vs. biopsy 1).

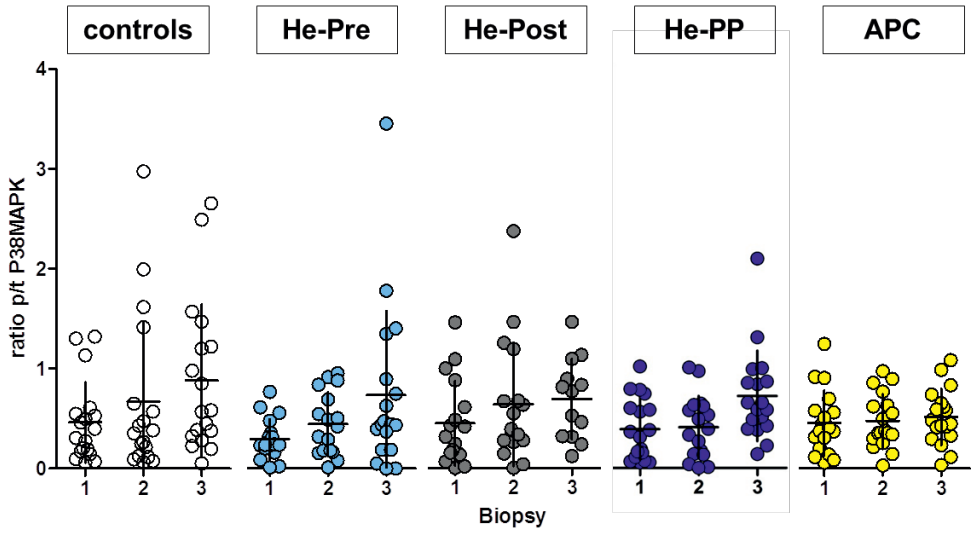

Figure 3. Ratio of activated p38 MAPK

The ratio of activated (phosphorylated to total p/t p38MAPK) at three different time points. The baseline ratio p/t p38MAPK is represented by biopsy 1. Biopsy 2 represents the ratio directly after application of the preconditioning stimulus, and the biopsy 3 is at the end of cardiopulmonary bypass. Values presented in mean ± SD. No significant differences were observed in the ratios of p/t p38MAPK at different time points in the controls (white dots), after preconditioning with helium (He-Pre; light blue dots) or sevoflurane (APC; orange dots), or after postconditioning (He-Post; grey dots) or the combination of helium pre- and postconditioning (He-PP; dark blue dots, Kruskall Wallis rank sum analysis).

Phosphorylation of ERK-1 and ERK-2 in cytosolic fraction

The ratio of activated (phosphorylated to total, p/t) ERK-1 of the cytosolic fraction was determined. In the preconditioning groups (He-Pre, APC), a statistically significant increase

of ratio p/t ERK-1 was observed after the preconditioning stimulus (biopsy 2 vs. biopsy 1; figure 4 upper panel). While these changes were still evident after CPB in the APC group, the changes were no longer significantly different from baseline at the end of CPB in the He-Pre group. Postconditioning with helium had no effect on ratio p/t ERK-1.

Regarding the ratio p/t ERK-2, no statistically significant changes were observed after either pre- or postconditioning with helium or sevoflurane, nor in the control group (figure 4, lower panel).

Protein expression of HSP-27 in particulate fraction

Total levels of HSP-27 were determined in the particulate fraction of myocardial atrial tissue. In controls, we observed a statistically significant increase of HSP-27 immediately before as well as after CPB (biopsy 2 and 3 vs. biopsy 1, respectively; figure 5). The same pattern of changes in HSP-27 as observed in the control group was seen in all other pre- and postconditioning groups, with the only exception that in the He-Pre group no increase of HSP-27 after CPB (biopsy 3) was seen.

Expression of PKC-ε in the particulate fraction

Total PKC-ε was determined in the particulate fraction of the atrial tissue. In control patients, we did not observe any statistically significant difference of PKC-ε levels immediately before or after CPB compared to baseline (biopsy 2 and 3 vs. biopsy 1, respectively; figure 6). Neither preconditioning with helium or sevoflurane (He-Pre, He-PP, APC), nor postconditioning with helium (He-PP, He-Post) had any statistically significant effect on levels of PKC-ε before or after CPB.

Plasma concentrations of postoperative troponin T

Postoperative troponin T was 11 arbitrary units (5, 31; area-under-the-curve (interquartile range)) for the controls, and no statistically significant changes were observed after helium preconditioning [He-Pre: 11 (6, 18)], helium postconditioning [He-Post: 11 (8, 15)], helium pre- and postconditioning [He-PP: 14 (6, 20)] and after sevoflurane preconditioning [APC: 12 (8, 24), p=0.13, one-way ANOVA after log transformation, figure 7].

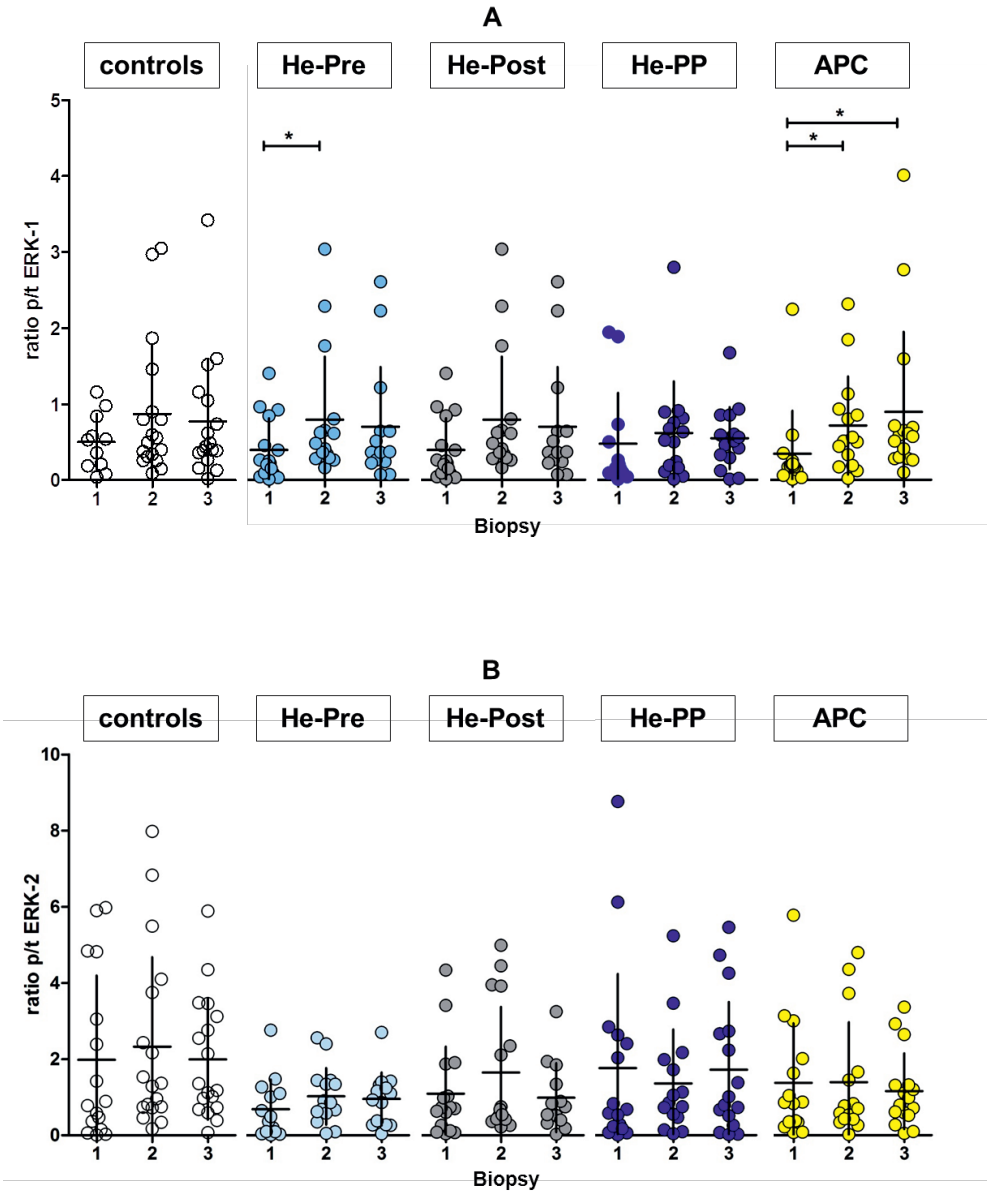

Figure 4. Ratio of activated ERK-1 and ERK-2

Ratio of activated (phosphorylated to total, p/t) Extracellular-signal Regulated Kinases-1 and -2 at different time points. Panel A shows ERK-1 (p44) and Panel B shows ERK-2 (p42). X-axis presents ratio of p/t ERK -1 or -2, Y-axis represents different biopsies (1-3) for each group (see also legend figure 3). Values presented in mean ± SD. For ERK-1, we observed a significant increase in p/t ratio in the helium-preconditioning group in the second biopsy (taken after preconditioning) compared to baseline. In the anaesthetic preconditioning group, we observed a significant increase compared to controls for biopsy 2 and 3. No significant differences in the ratio of p/t ERK-2 were observed at the different time points.

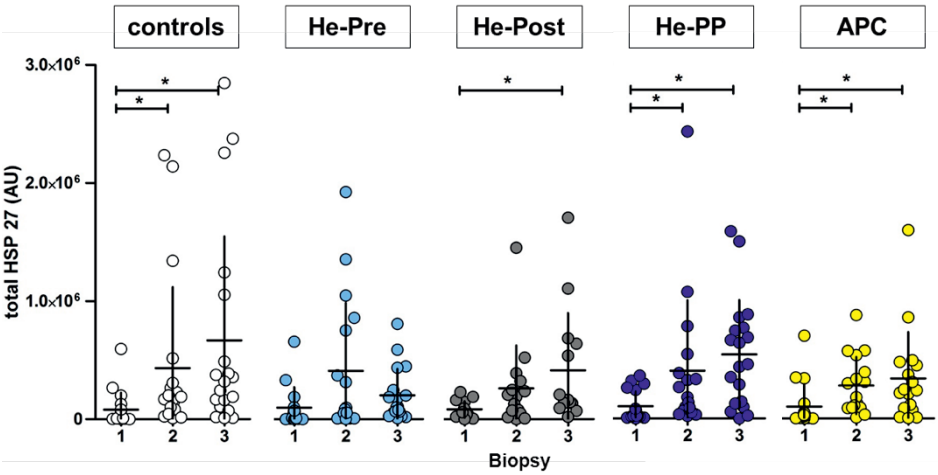

Figure 5. Levels of HSP-27

Amount of heat shock protein (HSP)-27 (mean ± SD) of the particulate fraction at different time points; X-axis is net intensity of the signal of HSP-27. Y-axis represents different biopsies (1-3) for each group (see also legend figure 3). Except for the helium-preconditioning group, a significant increase of total HSP-27 at the end of cardiopulmonary bypass (biopsy 3) compared to the respective baseline values was found. For the controls, helium pre- and postconditioning group and the anaesthetic preconditioning group this increase was also significant directly after preconditioning (biopsy 2) compared to baseline.

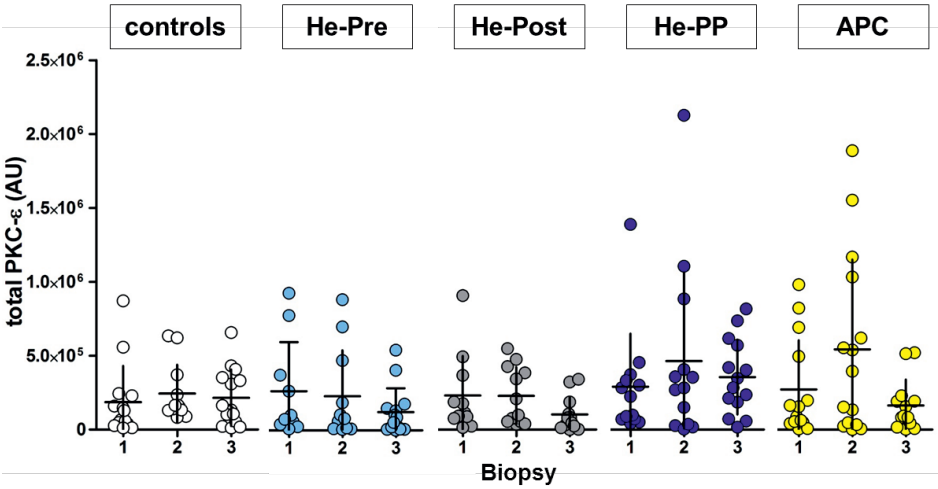

Figure 6. Levels of PKC- *ε* in particulate fraction

Levels of protein kinase C-ε (PKC-ε) in particulate fraction of myocardial atrial tissue at different time points (mean ± SD, see also legend figure 3). No differences of PKC-ε were observed at different time points in controls, after preconditioning with helium (He-Pre) of sevoflurane (APC) or after helium postconditioning (He-Post) or the combination of helium pre-and postconditioning (He-PP).

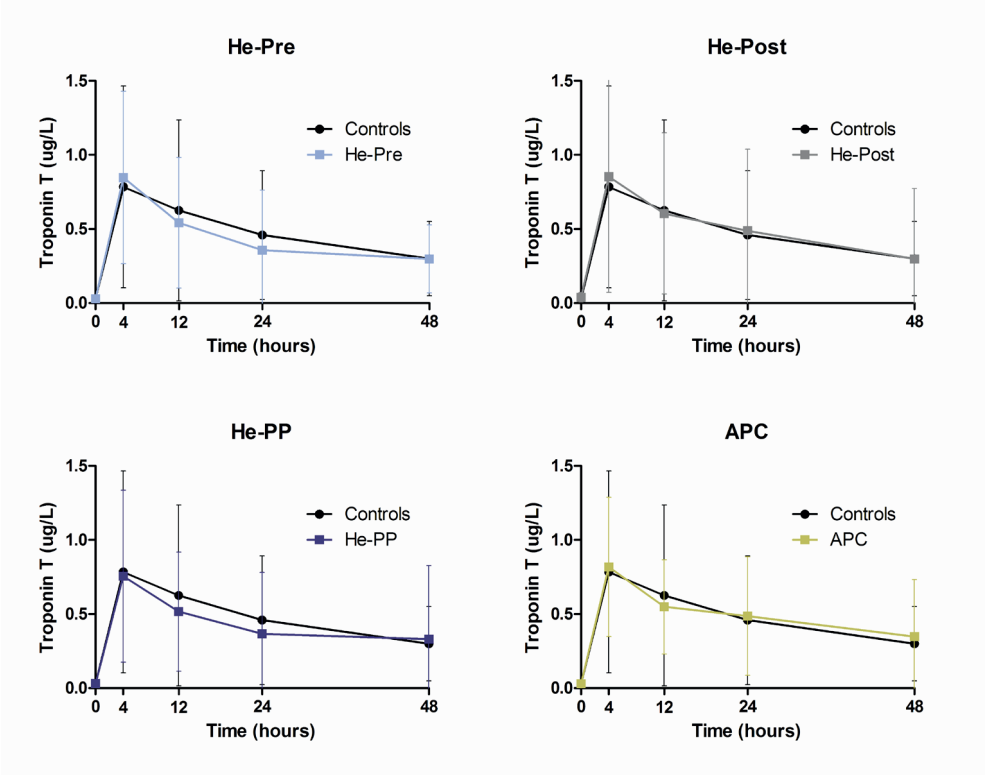

Figure 7. Postoperative Troponin T levels

The course of postoperative Troponin T release; all groups compared to controls (represented in black in all panels). Helium preconditioning (He-Pre) does not affect postoperative Troponin T levels compared to controls (panel A), nor does helium postconditioning (He-Post, panel B) or the combination of helium pre- and postconditioning (He-PP, panel C). Anaesthetic preconditioning (APC) with sevoflurane did also not affect postoperative Troponin T levels compared to controls in the present study (panel D). X-Axis represents Troponin T level in µg/L, values in median ± interquartile range. Y-axis represents time (hours).

DISCUSSION

In the present study, we did not observe any relevant differences on a molecular level in the regulation patterns of the signal transduction kinases p38 MAPK, ERK-1, ERK-2, or PKC-ε after helium treatment as compared to the control group. He preconditioning alone (without postconditioning) prevented HSP-27 increase as observed in all other groups. Helium pre- and postconditioning - alone or in combination - did not affect postoperative Troponin T release in patients undergoing CABG surgery.

Molecular changes by helium

Mechanisms underlying the protection by volatile anaesthetics and noble gasses have been investigated extensively in animal experiments.[8, 21, 22] However, mechanistic data from human studies are scarce. We investigated whether helium has any influence on signal transduction markers known to play a role in noble gas induced cardioprotection we demonstrated before.[3-5, 7, 23] Although p38 MAPK plays a role in inhalational anaesthetic induced preconditioning,[4] we were unable to demonstrate a role for p38 MAPK in preconditioning with either helium or sevoflurane in this clinical study: the ratio of phosphorylated to total p38 MAPK in the cytosolic fraction of the myocardial atrial tissue showed no statistically significant differences at various time-points in all groups. In contrast, Pouzet et al. demonstrated an increase of p38 MAPK after CPB in controls and sevoflurane treated patients undergoing CABG surgery.[24] No statistically significant difference in PKC-ε levels after preconditioning with helium, sevoflurane or in untreated controls was observed, which is in contrast to a previous study showing translocation of PKC-ε to the particulate fraction after sevoflurane preconditioning.[19]

The ratio of phosphorylated-to-total ERK-1 was increased after helium preconditioning compared to the baseline value, but this effect was no longer present after reperfusion at the end of CPB. In contrast, the increased ratio of phosphorylated-to-total ERK-1 after sevoflurane preconditioning was still present at the end of CPB. For ERK-2, no statistically significant effects were found at any time-point in any of the groups. In contrast, Talmor et al. demonstrated in atrial tissue obtained at similar time-points during CABG surgery an increase in ERK-1/2 activity after ischaemia and reperfusion.[25]

Several studies investigated changes of HSP-27 during cardiac surgery, most of them measuring HSP in patient blood.[26-28] Our data demonstrate that in untreated controls the level of HSP-27 in atrial tissue increases significantly after reperfusion compared to baseline levels. Except for the helium-preconditioning group, this effect was seen in all

treatment groups, indicating that aortic cross clamping and subsequent reperfusion increases the levels of HSP-27.

Besides the data on PKC-ε, which were used for the power calculation, there were no data available of ERK1/2, HSP-27 or p38MAPK in human myocardial tissue after preconditioning at start of the current study. Although unlikely, we cannot completely exclude the possibility that our study was underpowered to detect a difference in ERK1/2, HSP-27 or p38MAPK. A clinically relevant outcome parameter to be alternatively used for power calculation would have been troponin T values during the postoperative course. This parameter was used in a previous study with much smaller groups sizes (n=10),[16] showing a significant difference between groups. Therefore, we expected to find any clinically relevant differences of troponin release with the current group size of 25 patients per group.

Lack of sevoflurane preconditioning

In the present study we were unable to show protection in the suggested positive control, namely sevoflurane preconditioning. Demographic data, duration of CPB and aortic cross clamping did not differ compared to recent studies showing cardioprotection by sevoflurane.[18, 19]

In our previous study,[19] all patients received crystalloid cardioplegia, while in the current study crystalloid as well as blood cardioplegia was allowed. However, the distribution of crystalloid and blood cardioplegia was not significantly different between groups. Theoretically, a diminished ischaemic burden could have affected the power needed to obtain protection. Only two cardiac surgeons performed all procedures in the previous study, while in the current study numerous surgeons were involved. Larger than expected variations in biopsies, both in size as in composition (percentage of muscle and fatty tissue), might also have influenced our molecular results. More detailed information regarding difficulties we encountered during protein analysis of these samples are described in online supplement 3. Whether these increased diversities in clinical practices might have blunted potential cardioprotective effects of sevoflurane preconditioning remains unclear.

Opioid-induced cardioprotection might affect additional cardioprotection by inhalational agents,[29] however all patients received opioids in a comparable dosage, which was also performed in our previous study, and it is unlikely that this has significantly influenced the results.

Surprisingly, we did not observe any cardioprotective effect as measured by troponin T release (see figure 7). The volatile anaesthetic sevoflurane is one of the few preconditioning

agents that so far was successfully translated from experimental studies into clinical practice: sevoflurane reduced postoperative troponin release after CABG surgery.[17-19] Several meta-analysis showed that the modern volatile anaesthetics sevoflurane and desflurane were associated with a reduction in mortality after cardiac surgery when compared with total intravenous anaesthesia.[30, 31] The original data from the studies included in these reviews contain small patient groups, and the studies used different conditioning protocols and stimuli. Another review, focussing on the preconditioning protocol used,[32] mentioned that protection could be a side effect of sevoflurane induced alterations in myocardial oxygen demand and supply, not necessarily indicating preconditioning. Despite the initial successful translation of anaesthetic preconditioning into clinical practice, more recent studies show more variable or even contradictory results. For a definitive answer on whether sevoflurane induces preconditioning and which modality of its application is most effective, larger randomised controlled trials are needed to provide more robust evidence.

Lack of helium pre- and postconditioning

While the stimulus for preconditioning is applied before myocardial ischaemia, postconditioning is the protection induced by a stimulus applied during ischemia or at the beginning of reperfusion. Presence of the stimulus during reperfusion seems to be essential for its success to evoke protection. Ischaemic postconditioning decreased postoperative troponin release after cardiac surgery in children,[33] and decreased postoperative CK-MB but not troponin I release in adults.[34]

Helium induces protection by postconditioning,[13] but the current results do not show a beneficial effect of helium postconditioning. We started helium postconditioning at the end of aortic cross clamping by manual ventilation of the lungs; while the patient was still on CPB. We did not measure coronary artery (collateral) flow, nor did we measure helium concentration in coronary blood. We therefore cannot confirm that sufficient helium was present within the coronary artery system at the beginning of cardiac reperfusion, and it is possible the postconditioning stimulus was insufficient. We used 70% helium, allowing an inspiratory concentration of oxygen of 30%. Although experimental data indicate a concentration of 30-70% helium to be enough to induce preconditioning,[14] it cannot be excluded that 70% helium was too low to induce protection in CABG patients with increased age and multiple comorbid conditions. We previously showed helium induced preconditioning was abolished in aged[35] as well as in hypertensive animals.[36] However, in the hypertensive rat, a combination of helium induced pre- and postconditioning was able to overcome the barrier for cardioprotection, leading to reduction of infarct size.[36] In our current study, thirteen patients (57%) from the He-PP group had hypertension.

However, even the combination of helium pre- and postconditioning did not result in reduction of troponin release in this patient group.

It could be that the general trauma for CABG has reduced over time and therefore it will become more and more difficult for a protecting agent to show an additional benefit. Most likely, the “healthiest” CABG patients will not profit from additional protection, and the possible protective effects of helium in high-risk cardiac surgery patients (e.g., valve-plus-CABG surgery, thoracic aortic surgery) are still unknown.

CONCLUSION

In patients subjected to on-pump CABG surgery, we could not observe any statistically significant effect of helium on enzymes of the signal transduction cascade of pre- and postconditioning in human atrial tissue, or on troponin T release. The use of helium as a cardioprotective agent is still a matter of debate between different study groups; however, this is not the case with sevoflurane, which was also without activity in the current study, but brings into question the robustness and true translational value of this type of cardioprotection in CABG surgery.

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Supplemental files

Supplemental table 1. Perioperative arterial oxygen tension and haemoglobin

	Controls	He-Pre	He-Post	He-PP	APC
Haemoglobin (mmol/L)					
before CPB	5,7±0,6	5,7±0,8	5,4±0,7	5,4±0,8	5,9±1,0
after CPB	6,0±0,6	5,9±1,0	5,6±0,8	5,6±0,8	6,2±0,7
Arterial oxygen tension (kPa)					
before CPB	23±7	21±8	21±7	21±8	19±7
after CPB	21±14	23±19	18±7	23±19	20±13

Data are presented as mean ± SD; no significant differences were observed between groups. He-Pre= Helium preconditioning; He-Post=Helium Postconditioning; He-PP= Helium Pre- and Postconditioning; APC=Anaesthetic preconditioning; CPB=cardiopulmonary bypass.

Supplemental table 2. Mean differences of p38 Mitogen Activated Protein Kinase

	Mean difference (95% confidence interval)	
	Time 1-2	**Time 1-3**
Control	0.21 (-0.13 to 0.54)	0.42 (0.07 to 0.77)
He-Pre	0.15 (-0.01 to 0.31)	0.44 (0.02 to 0.86)
He-Post	-0.19 (0.04 to 0.34)	0.34 (0.09 to 0.59)
He-PP	1.35 (-4.33 to 1.62)	-1.91 (-6.63 to 2.80)
APC	0.02 (-0.18 to 0.21)	0.06 (-0.13 to 0.25)

Supplemental table 3. Mean differences extracellular regulated kinase -1

	Mean difference (95% confidence interval)	
	Time 1-2	**Time 1-3**
Control	0.62 (-0.04 to 1.28)	0.56 (0.05 to 1.08)
He-Pre	0.17 (0.02 to 0.31)	0.13 (-0.05 to 0.31)
He-Post	0.40 (-0.01 to 0.80)	0.33 (-0.22 to 0.88)
He-PP	0.35 (-1.98 to 0.90)	0.19 (-0.13 to 0.51)
APC *	0.31 (0.13 to 0.50)	0.35 (-0.13 to 0.51)

Supplemental table 4. Mean differences extracellular regulated kinase-2

	Mean difference (95% confidence interval)	
	Time 1-2	**Time 1-3**
Control	0.57 (-0.46 to 1.59)	0.18 (-0.70 to 1.05)
He-Pre	0.39 (-0.04 to 0.81)	0.26 (-0.23 to 0.75)
He-Post	0.58 (-0.25 to 1.41)	-0.008 (-0.58 to 0.57)
He-PP	0.26 (-0.19 to 0.71)	0.11 (-0.83 to 1.05)
APC	0.09 (-0.27 to 0.45)	0.05 (-0.24 to 0.34)

Supplemental table 5. Mean differences heat shock protein 27

	Mean difference (95% confidence interval)	
	Time 1-2	**Time 1-3**
Control	351923 (54200 to 649648)	587276 (195040 to9795120
He-Pre	303491 (1571 to 605412)	112993 (-13115 to 239101)
He-Post	178773 (-1678 to 359224)	331187 (66224 to 596151)
He-PP	294947 (-15916 to 605810)	420241 (225811 to 614670)
APC	233016 (74261 to 391773)	323358 (97583 to 549133)

He-Pre: Helium preconditioning; He-Post: Helium postconditioning; He-PP: Helium pre- and postconditioning; APC: Anaesthetic preconditioning with sevoflurane

Technical problems using human atrial tissue for protein detection with western blot

Our first problem was the difference in size of the atrial biopsies that were taken by different surgeons. This problem was solved during the time of the study, as both the surgeons and the investigators became more experienced. However, even with the sizes of the biopsies similar, the content and more specifically the amount of fat tissue involved, varied significantly among patients. This resulted in great variations of protein content of the samples after preparation for Western blotting. As all the samples were standardised to protein content for western blotting, standardising was more difficult with varying concentrations. For some of the samples with low protein concentrations, the amount of sample for Western Blot was low, allowing us only to perform a few blots.

The Western Blot method itself was not different with human tissue, but the antibody concentration needed for quantifiable bands was different from experiments in tissues from other species. We needed several blots to establish steady and quantifiable results. This involved switching back from our infrared odyssey imager to chemiluminescence detection on X-ray film.

After establishing a sound method for analysis of these four targets (P38 MAPK, ERK1/2, HSP27 and PKC- ε) we changed to western blot system using precast gels and a larger western blot tank, allowing more samples to be analysed at the same time. However, establishing these methods left us with partly insufficient sample for analysis with our final blotting system.

6

Chapter 6

Helium ventilation for treatment of post-cardiac arrest syndrome: a safety and feasibility study

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ABSTRACT

Aim: Besides supportive care, the only treatment available for comatose patients after cardiac arrest is target temperature management. Helium reduces ischaemic injury in animal models, and might ameliorate neurological injury in patients after cardiac arrest. As no studies exist on the use of helium in patients after cardiac arrest we investigated whether this is safe and feasible.

Methods: The study was an open-label single arm intervention study in a mixed-bed academic intensive care unit. We included 25 patients admitted after circulatory arrest, with a presenting rhythm of ventricular fibrillation or pulseless tachycardia, return of spontaneous circulation within 30 minutes and who were treated with hypothermia. Helium was administrated in a 1:1 mix with oxygen for 3 hours. A safety committee reviewed all ventilation problems, complications and causes of mortality.

Results: Helium ventilation was started 4:59±0:52 (mean±SD) hours after circulatory arrest. In one patient, helium ventilation was discontinued prematurely due to oxygenation problems. This was caused by pre-existing pulmonary oedema, and imposed limitations to PEEP and FiO_2 by the study protocol, rather than the use of helium ventilation. Sixteen (64%) patients had a favourable neurological outcome.

Conclusions: We found that helium ventilation is feasible and can be used safely in patients treated with hypothermia after cardiac arrest. No adverse events related to helium ventilation occurred during the three hours of administration.

INTRODUCTION

Out of hospital cardiac arrest (OHCA) is a major cause of morbidity and mortality, afflicting 335 per million per year in the Netherlands with an overall mortality of 81%.[1] Half of the patients admitted to the intensive care unit (ICU) leave the hospital with an unfavourable outcome.[2, 3] Circulatory arrest and subsequent return of circulation leads to ischaemia-reperfusion injury of the whole body and is particularly injurious to the brain and myocardium.[4, 5] Brain injury is the major cause of mortality and morbidity after cardiac arrest.[6] Therefore, patients admitted after cardiac arrest should receive treatment aimed at reducing brain injury as part of the post-resuscitation care. The only effective treatment currently available is target temperature management.[7-10] Despite this therapy, outcome results are disappointing and therapies to further reduce ischaemia-reperfusion injury after OHCA are needed.

Helium reduces ischaemia-reperfusion injury to the heart and the brain in animal models, suggesting that helium might be capable of reducing neurological and myocardial injury in patients after OHCA.[11-17] However, no clinical studies in this field have been done yet. Recently we demonstrated that helium induces preconditioning in healthy volunteers, thereby protecting against endothelial dysfunction after regional forearm ischaemia.[18] Clinically, helium is used to ventilate both adults and children with severe obstructive pulmonary disease and helium inhalation is generally considered to be safe.[19] Prior to investigating the use of helium as a therapeutic agent in neurological damaged patients, we performed a safety and feasibility study, investigating whether helium ventilation can safely be used in patients admitted to the ICU after OHCA.

METHODS

This was an open-label single arm intervention study, performed in the mixed surgical and medical ICU of the Academic Medical Centre, Amsterdam, the Netherlands. The study was approved by the medical ethics committee of the Academic Medical Centre (protocol number NL 30466.018.09) and was conducted in concordance with the principles of the declaration of Helsinki and good clinical practice. Patients were included after obtaining informed consent from their legal representative.

Inclusion criteria were admission after witnessed OHCA, with the first registered rhythm being ventricular fibrillation (VF) or tachycardia (VT) and treatment with mild hypothermia (target temperature 33° Celsius). Return of spontaneous circulation (ROSC) had to occur

within 30 minutes and helium ventilation had to be started within 6 hours after cardiac arrest. Exclusion criteria were oxygenation problems (necessitating a FiO_2 >50% and >10 cmH_2O positive end expiratory pressure [PEEP]), neurological deficits or severe disability before cardiac arrest, and comorbidities with a life expectancy of less than 6 months. The described ventilation settings were limits during the study-protocol as well.

Study procedures

After inclusion, helium ventilation was initiated as soon as possible. Helium was administered using a heliox compatible Servo-I ventilator (Maquet, Netherlands), which was calibrated to accurately measure tidal volumes when using heliox. Helium was supplied from a pressurised cylinder containing 1780 L heliox (Heliox21, BOC Ltd, UK), as a 79/21% helium/oxygen mixture, and was mixed in the ventilator with oxygen to obtain a final gas mixture of 50% helium and 50% oxygen. Helium ventilation was done in pressure control mode, which was the standard ventilation mode in our ICU; peak pressure was set to achieve a tidal volume of 6 ml/kg ideal body weight, with 5-10 cmH_2O of PEEP and the respiratory frequency was controlled to maintain a pCO_2 of 4.5-5.5 kPa and a pH of 7.35-7.45 (alpha-stat). A pO_2 of ≥10 kPa and a saturation of ≥95% were aimed for. After switching to helium, a setup period with repeated blood gas analyses was used to reach the target values for pCO_2 and pH. When these measurements were within the target values helium ventilation was continued for a 3-hour period. Since the objective of this study was to investigate the safety and feasibility, and not the effectiveness, helium ventilation was stopped if the cylinder was empty before the end of the 3-hour period.

Data collected were age, gender, Body Mass Index (BMI), simplified acute physiology score II (SAPS II), acute physiology and chronic health evaluation score II (APACHE II), pre-existent cardiovascular disease or malignancy, cause of arrest, time until first shock, time to ROSC, the use of coronary angiography and percutaneous coronary interventions and the need for haemodynamic support at admission.

Serum samples for analysis of creatine kinase (CK), creatine kinase muscle-brain (CK-MB) and troponin T were drawn at admission and at 6, 12, 18, 24, and 48 hours. Serum samples for analysis of neuro-specific enolase (NSE) levels were drawn 24 and 48 hours after admission. NSE serum samples were centrifuged and stored at -80° Celsius until analysis by immunoassay (kit for ELECSYS, Roche).

Outcome was assessed by telephone interview of the patient or caregiver 30 days after admission. The Glasgow Outcome Scale (GOS) was used; poor outcome was defined as death or a vegetative state (GOS 1-2).[20] Primary objective of the study was to investigate the safety and feasibility of helium administration in patients after cardiac arrest. Safety

endpoints were the inability to adequately ventilate the patient using helium within the predetermined limits (FiO_2 50% and ≤10 cmH_2O PEEP), and death related to helium. To determine the probability of an adverse event being related to helium treatment all serious adverse events were evaluated by an independent safety committee, consisting of an independent intensive care physician, anaesthetist and neurologist.

Secondary objectives were to investigate the effect of helium ventilation on outcome (GOS), brain injury (NSE) and cardiac injury (CK, CK-MB, and troponin T).

Statistics

There is no data on the effectiveness or the occurrence of adverse events of helium treatment in patients after OHCA. Therefore, a formal sample size calculation could not be performed. We expected a mortality rate of approximately 50%, and therefore chose to include 25 patients, to be able to detect an increase in adverse events related to helium. This is also a sample size that is used in similar studies.[21, 22]

SPSS 19 (IBM, Armonk, New York, USA) was used for statistical analysis unless stated otherwise. Continuous data are presented as mean with standard deviation when normally distributed, and otherwise as median and interquartile range, while categorical data are presented as numbers with proportions.

RESULTS

Between April 2010 and October 2011, 106 patients admitted after OHCA were screened for eligibility, of which 64 patients were not eligible, 13 patients were eligible but were missed by attending physician, in four patients study participation was refused by the legal representative, and finally 25 patients were included (fig. 1). Baseline characteristics of patients are presented in table 1.

Helium ventilation was started 4:59±0:52 (mean±SD) hours after arrest, and 21±13 (mean±SD) minutes was used to reach target values for pCO_2 and pH. After that, helium ventilation was continued for a total of 3:10±0:39 (mean±SD) hours. One patient was ventilated longer than the planned 3-hour period. The effect of helium ventilation on blood gas values and respiratory settings is described in detail elsewhere.[23] In six patients the treatment was stopped prematurely; in five patients the heliox cylinder was empty before completion of the 3-hour treatment protocol, due to high minute volumes needed and the

duration of the adjustment period. In one patient, ventilation with helium was terminated prematurely. This patient had slight hypoxaemia at the time of inclusion due to pulmonary oedema following cardiac arrest, requiring 10 cmH_2O PEEP and a FiO_2 of 50% to maintain an oxygen saturation (sO_2) of >90% and a PaO_2 of 8.4 kPa. Shortly after the initiation of helium ventilation, the sO_2 dropped to 84% and the PaO_2 to 7.1 kPa, and it was decided to discontinue the study protocol and switch back to a normal gas mixture. Only after increasing FiO_2 to 70% and PEEP to 12, oxygenation improved in this patient. These ventilation settings had to be maintained for several days. As the hypoxaemia was pre-existing and persisting, the safety committee concluded that the ventilation disorders were not caused by the short use of helium. Although helium treatment was stopped for safety reasons, this was probably due to the restrictions the study protocol posed on the settings of PEEP and FiO_2, rather than the use of helium itself.

Nine patients died within 30 days (36%); in all patients post-anoxic brain injury was the cause of death. None of these deaths were related to helium ventilation. At 30 days follow-up, the surviving 16 patients (64%) all had a favourable outcome, 13 patients (81%) resided at home, two patients (13%) in a rehabilitation centre and one patient was still hospitalised (6%).

Serum levels of CK, CK-MB, troponin T and NSE are presented in table 2. Helium treated patients had a mean NSE value of 44±51 µg/L at 24 hours, and 54±94 µg/L at 48 hours after arrest.

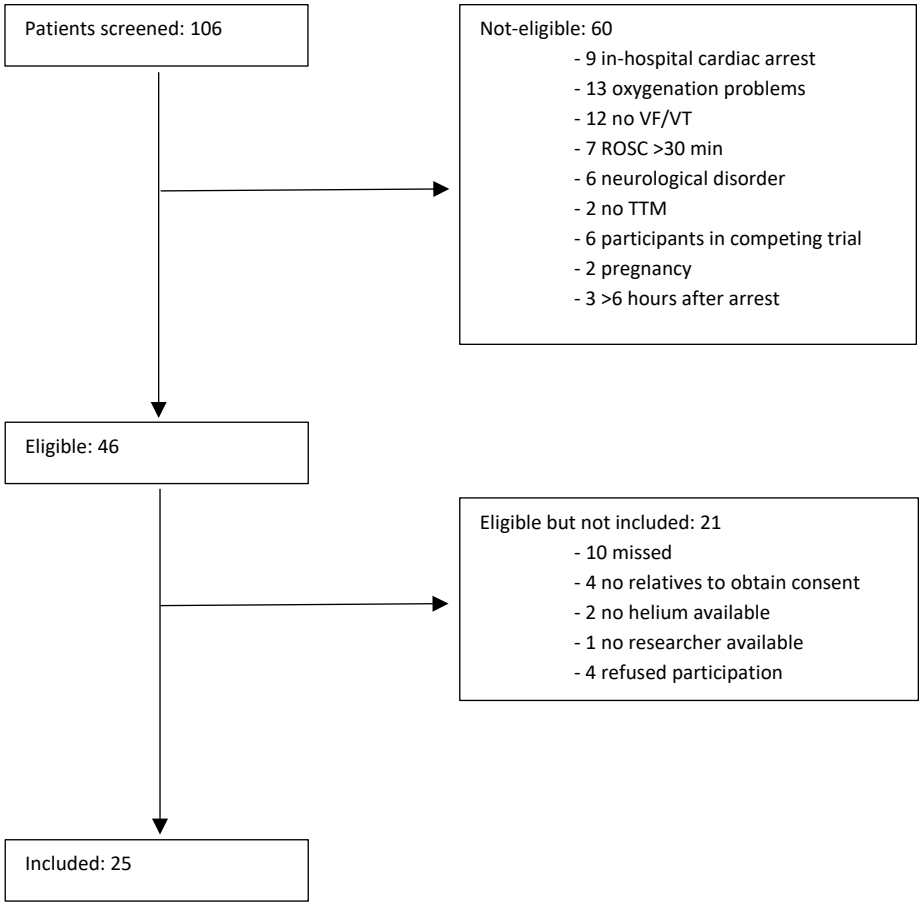

Figure 1. Flow schedule of patients

Table 1. Baseline characteristics

	Patients (25)
Male sex	20 (80%)
Age (years)	64·8±12·1
BMI[a] (kg/m^2)	27·4±4·8
SAPS II score[b]	53·6±18·6
APACHE II score[c]	20·0±8·6
Comorbidity Cardiovascular disease	14 (56%)
Malignancy	4 (16%)
Cause of OHCA[d] Acute Infarction	17 (68%)
Chronic Infarction	4 (16%)
Structural Heart Disease	3 (12%)
Unknown	1 (4%)
Time to 1st shock (min)	8±7
Time to ROSC[e] (min)	16±7
CAG[f]	20 (80%)
PCI[g]	15 (60%)
IABP[h] or Impella	9 (36%)
Inotropics or vasopressors	12 (48%)

A= Body Mass Index, b= Simplified Acute Physiology Score II, c= Acute Physiology and Chronic Health Evaluation II, d= Out-of-Hospital Cardiac Arrest, e= Return of spontaneous circulation, f= Coronary Angiography, g= Percutaneous Coronary Intervention, h= Intra-aortic Balloon Pump

Table 2. Biomarkers of neurological and myocardial injury

	Baseline	6 hrs	12 hrs	18 hrs	24 hrs	48 hrs
CK[a] (U/L)	340±314	1205±961	1627±1263	1963±2115	1828±1775	1556±1749
CK-MB[b] (µg/L)	22±27	128±238	169±241	180±248	154±223	64±74
Troponin T (µg/L)	0·3±0·4	1·0±0·76	2·2±2·7	1·8±2·6	1·6±1·8	1·3±1·4
NSE[c] (µg/L)					44±51	54±94

a= Creatinine Kinase, b= Creatinine Kinase Muscle-Brain, c= Neurospecific Enolase

DISCUSSION

This is the first study focusing on organ protective effects of helium in patients after OHCA. We found that helium ventilation is feasible and can be used safely in patients treated with hypothermia after OHCA. No adverse events related to the helium ventilation occurred during the three hours of ventilation with this noble gas.

These results might open the door to a new treatment of brain injury following cardiac arrest. Helium might reduce the ischaemia-reperfusion injury, but our study was not designed to demonstrate this and subsequent studies on the potential therapeutic value of helium in organ protection following ischaemia and reperfusion are needed. Although the mortality rate is lower than values normally reported in the literature for ICU patients admitted after cardiac arrest, this is probably due to the patient selection.[3, 24] We included only patients who had a witnessed arrest, presented with VF or VT, and had a resuscitation time of thirty minutes or less, all factors that have a positive effect on outcome.

Comparison of our results to studies with helium or other noble gasses in patients after cardiac arrest is not possible, as this has never before been studied. Only animal studies have been performed showing conflicting results regarding neuroprotective properties of helium. In an *in vitro* model of traumatic brain injury, Coburn et al. found a protective effect of helium, and Pan et al. found a reduction in infarct size by helium inhalation in an *in vivo* rat model using middle cerebral artery (MCA) occlusion.[12, 13] More positive effects in a MCA occlusion model were reported by David et al. but this protective effect was only seen when the animals were allowed to cool down in a flow chamber.[16] The authors suggested that the protection was mediated by the induction of hypothermia. Finally, in neonatal rats in which one common carotid artery was temporally occluded, Liu et al. demonstrated neuroprotection by helium, and Zhuang et al. confirmed this protection for ischaemia of 90 minutes, but not for 120 minutes.[17, 25] Other studies did not find a beneficial effect of helium on cerebral injury. In an *in vitro* model using oxygen glucose deprivation to induce brain injury, Jawad et al. did not find any beneficial effect.[26] Pan et al. used a model of MCA occlusion and reported that helium only provided protection when given directly at the time of reperfusion, and in an inspired fraction of 70%.[27] Until today, the exact underlying mechanisms mediating possible organ protective effects of helium are unclear.[28]

Other noble gasses are also being investigated as neuroprotective agents. In a pig model of cardiac arrest, xenon was given after resuscitation and reduced brain injury.[29] The use of xenon in patients after cardiac arrest seems feasible and safe, although a specially designed

ventilator is required.[30] A subsequent randomised controlled trial of 110 patients was published very recently and found a reduction in white matter injury on MRI, but no improvement in survival or neurological outcome.[31] Xenon is also under investigation as a treatment for neonatal encephalopathy, but a first randomised controlled trial found no benefit on neurological injury in these patients.[32] Helium is less scarce than xenon and cheaper, and while xenon requires purposely-designed ventilators, helium can be administered using common ICU ventilators. If helium ventilation is an effective neuroprotective strategy in patients after OHCA, the application in daily clinical care in the ICU will be much easier than xenon ventilation.

It is known that the results of animal studies investigating neuroprotection in different animal models are difficult to translate to the human situation. Many neuroprotective drugs have been studied in stroke patients, based on positive animal experiments, but no effective drug has ever been found for humans.[33, 34] A large difference with focal ischaemic stroke models is that in patients after OHCA the vasculature of the brain is intact and open. As soon as circulation is restored, neuroprotective agents can easily reach the brain cells and perform their actions.

We chose to start with a small study, which makes conclusions about possible effectiveness insignificant and might underestimate the side effects of helium ventilation. Especially longer periods of helium ventilation, which might be needed for an optimal treatment effect, could lead to more problems. This would be the logical topic to address in a subsequent study.

Second, the open-label use of helium inadvertently introduces a risk for bias, however by using endpoints that are not influenced by observer interpretation (mortality, vegetative state and laboratory assessments) the risk for observer bias was reduced.

Third, the setting of a single ICU of a university hospital limits extrapolation of the results. However, since the objective of the study was to investigate the safety and feasibility, we feel that these limitations are of minor concern at this stage.

All patients were ventilated with 50% helium in order to give the same dosage. This also meant that all patients received 50% oxygen, regardless of their oxygenation status, which could lead to supernormal oxygen tensions in some patients. A high PaO_2 during or after cardiac arrest has been linked to an increase in mortality, and might influence a beneficial effect of helium.[35-37]

Prior to further clinical trials, subsequent research should include a relevant animal study, such as a porcine model of cardiac arrest, to strengthen the data on the efficacy of helium, and to answer questions on the required inspiration fraction, the optimal duration of treatment and the effect of delay between arrest and the start of helium treatment.

CONCLUSIONS

We demonstrated for the first time that helium ventilation for three hours is safe and feasible in patients after OHCA. This might open the route for further studies investigating the effectiveness of this new organ protective treatment modality.

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Chapter 7

Heliox improves carbon dioxide removal during lung protective mechanical ventilation

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ABSTRACT

Introduction: Helium is a noble gas with a low density and increased carbon dioxide (CO_2) diffusion capacity. This allows lower driving pressures in mechanical ventilation and CO_2 diffusion. We hypothesised that heliox facilitates ventilation in adult patients during lung–protective mechanical ventilation using low tidal volumes.

Methods: Prospective observational cohort sub study of an open label single arm intervention study. Twenty-four patients were included, who were admitted to the intensive care unit after a cardiac arrest. Patients were mechanically ventilated for 3 hours with heliox (50% helium; 50% oxygen) during a fixed protective ventilation protocol (6 ml/kg), with prospective observation for changes in lung mechanics and gas exchange. Statistics by Bonferroni post correction with statistical significance set at $P<0.017$.

Results: During heliox ventilation, respiratory rate was decreased (25±4 vs. 23±5 breaths min-1, P=0.010). Minute volume ventilation showed a trend to decrease compared to baseline (11.1±1.9 vs. 9.9±2.1 L min^{-1}, P=0.026), while reducing $PaCO_2$ levels (5.0±0.6 vs. 4.5±0.6 kPa, P=0.011) and peak pressures (21.1±3.3 vs. 19.8±3.2 cmH_2O, P=0.024).

Conclusions: Heliox improved CO_2 elimination while allowing reduced minute volume ventilation in adult patients without lung injury during protective mechanical ventilation.

INTRODUCTION

Helium is an inert gas with lower density than air [1], allowing for less turbulent flow through airways, leading to lower airway resistance. As a result, during mechanical ventilation with a helium-oxygen mixture (heliox), lower driving pressures are needed to distribute oxygen to the distal alveoli compared to ventilation with oxygen [2]. Furthermore, helium is known for its increased diffusion capacity of carbon dioxide (CO_2), which in addition might facilitate ventilation. Due to these properties, there may be a rationale to use heliox in patients with severe pulmonary disease with respiratory failure in whom protective mechanical ventilation with low tidal volumes is not feasible due to the development of respiratory acidosis e.g. in acute respiratory distress syndrome (ARDS) or chronic obstructive pulmonary disease (COPD). Nowadays, heliox is clinically applied during high frequency ventilation in paediatric patients [3, 4] and in patients with high airway resistance due to severe asthma or COPD, most often in children [5, 6]. Clinical data on adult patients during conventional mechanical ventilation are limited.

The aim of this study was to investigate the effect of heliox on gas exchange as part of a safety and feasibility study on the potential of heliox ventilation to improve neurological outcome after cardiac arrest [7]. We hypothesised that the use of heliox also allows for increased CO_2 elimination in adults during conventional mechanical ventilation with low tidal volumes.

METHODS

The study was approved by the local medical ethics committee of the Academic Medical Centre, University of Amsterdam, the Netherlands (protocol number NL 30466.018.09) and conducted in concordance with the principles of the declaration of Helsinki and good clinical practice. The study was registered with the Dutch Trial Registry (www.trialregister.nl) under NTR2257. From all patients or their legal surrogate written informed consent was obtained. It was a prospective observational cohort sub study of an open label single arm intervention study, performed in the mixed surgical-medical intensive care unit (ICU) of a tertiary referral centre in Amsterdam, the Netherlands. From April 2010 to October 2011, consecutive patients admitted to the ICU after cardiopulmonary resuscitation (CPR) because of a witnessed out–of–hospital cardiac arrest, were screened for inclusion in the study after informed consent was given by their relatives. Inclusion criteria were return of spontaneous circulation within 30 minutes of arrest and coma on admission. Exclusion criteria were hypoxaemia with a need for ventilation with a FiO_2 higher than 50% or more than 10 cmH_2O

positive end–expiratory pressure (PEEP), pregnancy, severe disability, a neurological disorder or co–morbidity with life expectancy of less than 6 months.

Before start of heliox ventilation, all patients were ventilated in a pressure-controlled mode, targeting a pH of 7.35 – 7.45 and $PaCO_2$ levels of 4.5 –5.5 kPa. Within 5 hours after the cardiac arrest patients received heliox ventilation. During heliox treatment patients were mechanically ventilated in a pressure-controlled mode, using a Servo–I ventilator, which was adjusted and calibrated for heliox ventilation. Helium (Linde Gas Therapeutics, Eindhoven, the Netherlands) was mixed with oxygen to achieve a concentration of 50% helium and 50% oxygen. Respiratory settings were modified using a study protocol. Inspiratory pressure and respiratory rate was adjusted to target a tidal volume of 6 ml/kg predicted body weight, a pH of 7.35 – 7.45 and $PaCO_2$ levels of 4.5 –5.5 kPa, with an inspiration to expiration (I:E) ratio of 1:2. No changes were made to I:E ratio, FiO_2 and PEEP levels during heliox treatment. After 3 hours, heliox was switched back to oxygen in air and patients were ventilated according to our standard ICU protocol with tidal volumes of 6 ml/kg predicted body weight. All patients were treated with therapeutic hypothermia (32°C–34°C) as part of standard care in patients with decreased consciousness after CPR. Target temperature had been reached by the time heliox ventilation was initiated and was maintained during heliox ventilation. For sedation, propofol and opiates were used. Neuromuscular relaxants were given as a bolus, but only during shivering.

Respiratory parameters were measured over time, starting just prior to heliox ventilation (T=–1), within 15 minutes after start heliox (T=0), during heliox treatment (T=1 – T=3) and until 3 hours after heliox was switched back to oxygen in air (T=4 – T=6). After the switch back, again all patients were ventilated in a pressure-controlled mode, targeting a pH of 7.35 – 7.45 and $PaCO_2$ levels of 4.5 –5.5 kPa. Dynamic lung compliance was measured during heliox ventilation (T=1 – T=3), as this was a read–out at the Servo–I ventilator only. Resistance was calculated by dividing the pressure difference by airflow per minute. Arterial blood gas analysis was determined hourly (Alpha stat, RAPIDLab 1200, Siemens, Deerfield, USA).

Statistical analysis

Data are expressed by mean and the standard error of the mean (SEM) in the figures. Time points within the same subjects were compared using paired T–test or Wilcoxon signed rank test, depending on distribution of the data. A total of three comparisons were made between several time points (T=–1 vs. T=0; T=0 vs. T=3; T=3 vs. T=6). Using Bonferroni post correction, statistical significance was set at $P<0.017$.

RESULTS

106 patients were screened, of whom 29 were eligible. Of these, informed consent was refused in four cases. Of 25 included patients, heliox was discontinued within 15 minutes in one patient due to hypoxaemia, requiring a PEEP level above 10 cm H_2O. This patient was excluded from further analyses. In the remaining 24 patients, PEEP requirements were 5-10 cm H_2O and 40-50% FiO_2. Of these, 83% was male with a mean age of 65±12 years, no acute infections were present at start of the study, one patient suffered from COPD, no other lung pathology was reported. During the study protocol, no changes in haemodynamics were observed.

Due to the switch of ventilation gas mixture from oxygen (T=–1) to heliox (T=0), respiratory settings needed adjustment according to the study protocol with limited tidal volume ventilation. Minute volume ventilation slightly rose after switching from oxygen to heliox, but no significant difference was found between before and right after the start of heliox ventilation (figure 1). Thereafter, during heliox ventilation, respiratory rates were adjusted to targeted pH and $PaCO_2$ levels, in accordance with the study protocol. This resulted in a significant decrease in respiratory rate and tended to decrease minute volume ventilation (figure 1). After discontinuation of helium these parameters did not change. Tidal volumes remained stable at 6 ml/kg according to study and standard ICU protocol and did not change over time (data not shown). Peak pressures tended to decrease, albeit in a non–significant manner (figure 1). Airway resistance and dynamic lung compliance by the ventilator did not change during heliox ventilation (figure 1).

Switch of oxygen in air to heliox ventilation resulted in a rapid decrease in $PaCO_2$ levels, which increased again at discontinuation of heliox (figure 2). $PaCO_2$ levels showed no changes during the 3 hours of heliox ventilation (figure 2). Also, an increase in pH to 7.37 was seen shortly after the application of heliox ventilation (figure 2). In the course of heliox ventilation, pH tended to increase further, but showed no change after discontinuation of heliox. Oxygenation was not altered significantly after start of heliox or after switching back to oxygen (figure 2). Applied FiO2 levels remained between 40-50%, whereas PaO2 levels tended to decrease.

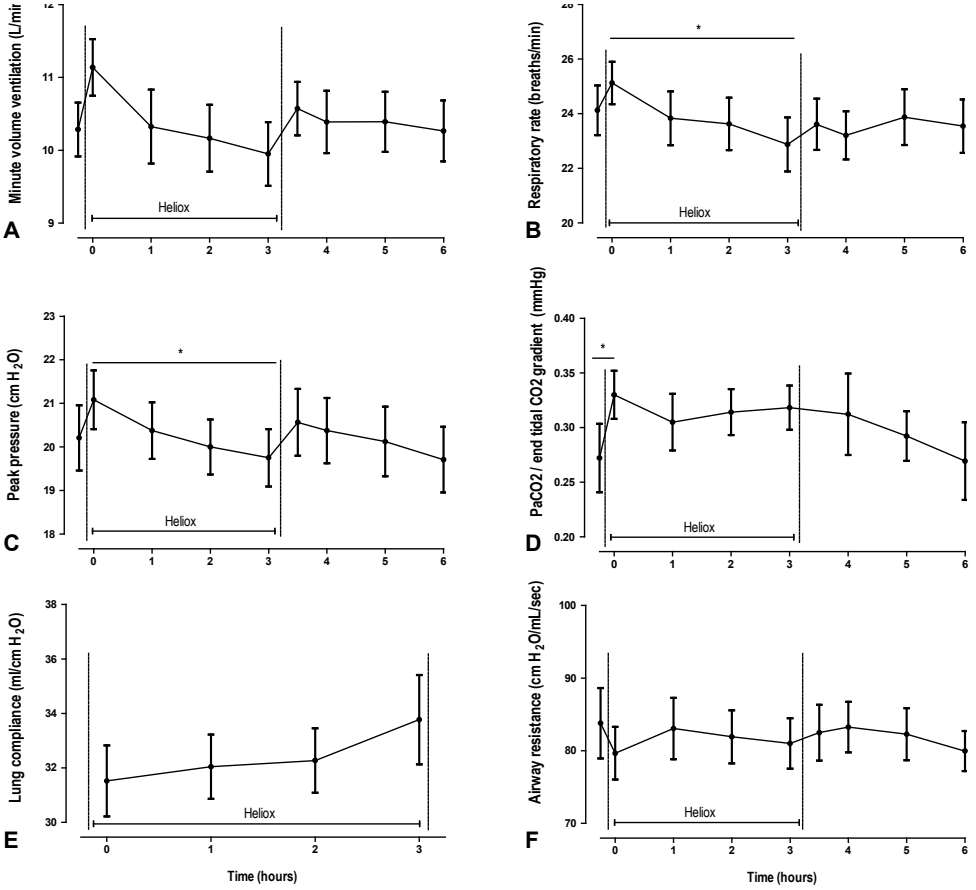

Figure 1. Respiratory parameters during heliox ventilation for 3 hours (T=0 to T=3) and after switch to normal oxygen in air mixture (T3 to T6). Measurements started prior to heliox administration (T= –1). Data are MEAN ± SEM. (A) Minute volume ventilation (L min^{-1}); (B) respiratory rate (breaths min^{-1}); (C) peak pressure (cm H_2O); (D) $PaCO_2$ / end tidal CO_2 gradient (mmHg); (E) airway resistance (cm H2O mL^{-1} sec^{-1}) and (F) lung compliance (ml cm^{-1} H_2O). *: P < 0.02

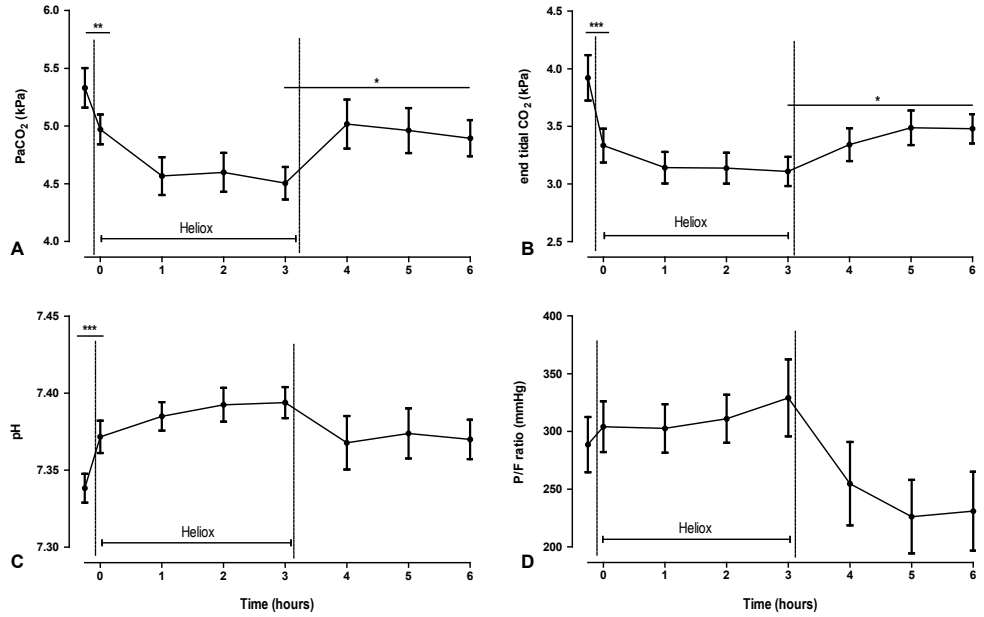

Figure 2. Gas exchange during ventilation with heliox for 3 hours (T=0 to T=3). Measurements started just prior to heliox administration (T= –1) until 3 hours after heliox discontinuation (T=3 to T=6). Data are MEAN ± SEM. (A) $PaCO_2$ (kPa); (B) end tidal CO_2 measurements (kPa); (C) pH measured hourly and (D) PaO_2/Fi O_2 ratio (mmHg).

*: P < 0.02; **: P < 0.01; ***: P < 0.001.

DISCUSSION

In adult patients ventilated with protective mechanical ventilation strategy according to current ventilation guidelines [8], use of heliox improved ventilation, by allowing lower minute volume ventilation while $PaCO_2$ levels decreased.

The use of heliox ventilation has been mostly investigated in respiratory conditions such as upper–airway obstruction, asthma, bronchiolitis and croup. Results indicate that heliox improves gas exchange and reduces work of breathing [4-6]. Most of the studies were performed in the paediatric population. In this study, we focussed on adult patients. Cardiac arrest patients are obviously not the patients who are expected to benefit most from lowering minute volume ventilation, because these patients do not have obstructed airflow. Nevertheless this population was studied, since the feasibility study investigating neuroprotective properties of heliox [7], enabled us to investigate the response to heliox

ventilation in adult patients ventilated with pressure controlled ventilation modes and currently recommended protective settings. The reduction of respiratory rate with the concomitant decrease of peak pressures during heliox ventilation are promising results. Given that changes were small, it can however be questioned whether these results have clinical relevance. Effects of heliox may have been mild because baseline resistance and compliance in these patients without lung injury was not severely hampered. It remains to be determined whether heliox is beneficial in patients with respiratory failure in whom protective ventilation is hampered by the development of respiratory acidosis.

Our study has several limitations. As this study was a secondary analysis of a safety and feasibility study on the use of heliox in cardiac arrest patients, the study was not primarily powered to investigate the effects of heliox on ventilation. This may explain observed trends but absence of statistical significance. Also, no control group was present. Thereby, the influence of time on findings is unknown. However, our data clearly show an increased CO_2 removal and improved ventilation, starting immediately after start of heliox ventilation. Long-term effects could not be studied as heliox ventilation was limited to 3 hours. Another limitation may be that all patients received therapeutic hypothermia, which is known to decrease $PaCO_2$ levels [9]. However, throughout the whole study period, temperatures were in the range of therapeutic induced hypothermia. Thereby, observed effects could not be due to hypothermia.

CONCLUSIONS

Heliox ventilation improved CO_2 elimination and allowed for a, although non-significant, decrease in minute volume ventilation, in a selected group of patients ventilated in a pressure-controlled mode according to the guidelines of the ARDS network[8]. Results may generate new hypotheses for future research considering heliox as a therapeutic possibility in patients in whom protective mechanical ventilation is hampered by the development of respiratory acidosis.

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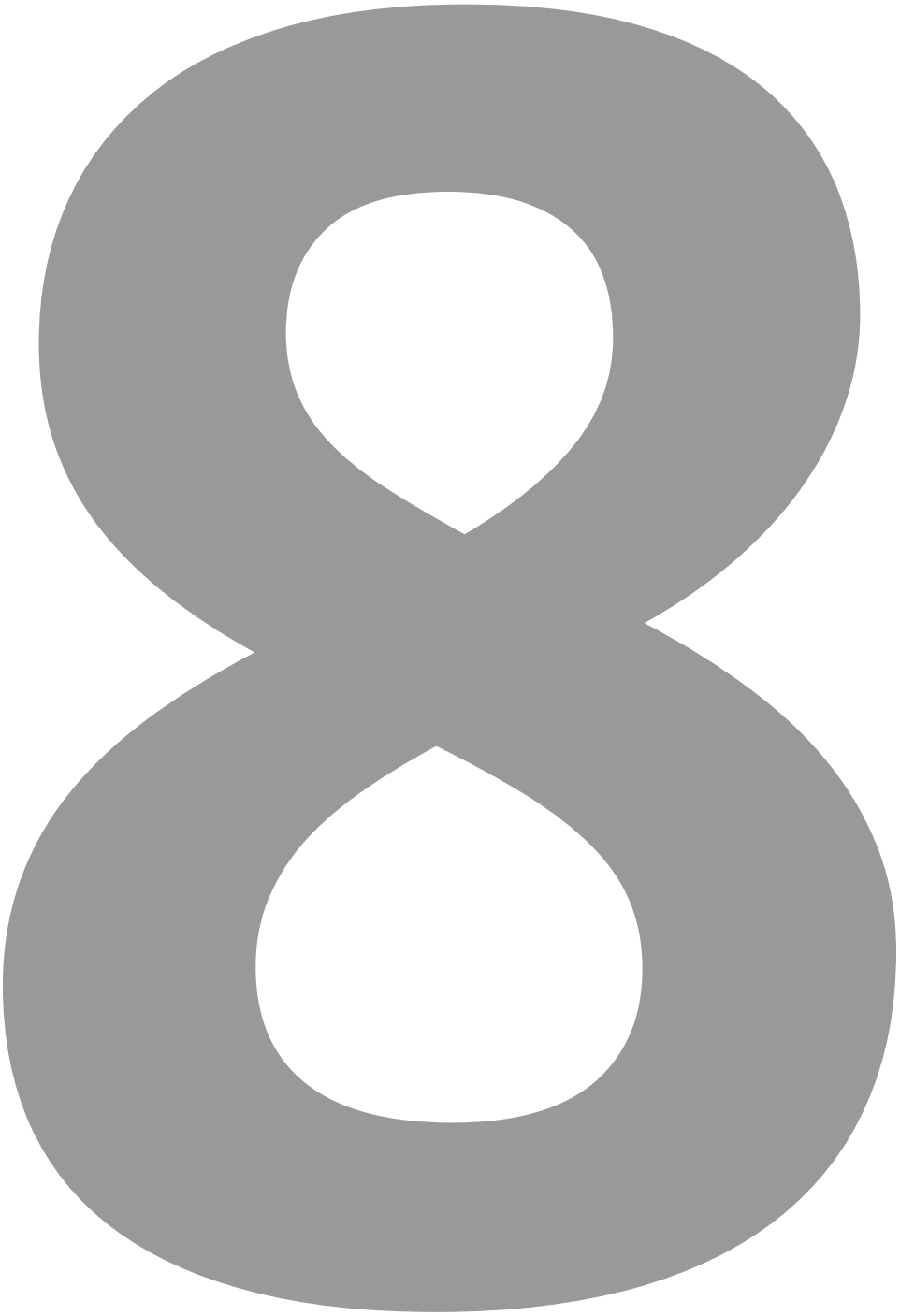

Chapter 8

Summary and general discussion

SUMMARY

In this thesis we explored two promising forms of conditioning for organ protection: remote ischaemia in a distant tissue and helium inhalation. Focusing on these two topics we evaluated whether these methods of conditioning can be translated into clinical practice; and what the possible underlying mechanism is for these protective strategies.

Part I Remote ischaemia

In **chapter 2** we summarised the results of 23 different randomised clinical trials (published up to July 2011) on remote ischaemic conditioning including almost 2000 patients.[1] These trials investigated remote ischaemic conditioning in multiple clinical settings, including cardiac and vascular surgery, as well as interventional cardiology. In this early meta-analysis, we found that remote ischaemic conditioning reduced cardiac injury and possibly myocardial infarction, but not death after myocardial infarction.

In **chapter 3** we describe a randomised controlled trial of remote ischaemic conditioning in cardiac surgery patients.[2] During surgery cardiac tissue samples were collected for molecular analysis in order to elucidate parts of the underlying molecular mechanism of possible cardioprotective effects. We found no effect of remote conditioning on the signal molecules extracellular regulated kinase 1 or 2 (ERK-1, ERK-2) or signal transducer and activation of transcription 3 or 5 (STAT3 or STAT5). This was the case in patients with or without diabetes mellitus. Nor did we see a reduction in myocardial injury as measured by troponin T release after surgery. However, the number of tissue samples available for analysis was much lower than initially planned.

We demonstrated that remote ischaemia reduced myocardial injury as measured by biomarker release and reduced the incidence of myocardial infarction, although the latter was largely driven by one study in particular.[1] We did not see a reduction in other clinical endpoints such as death, or length-of-stay or even the combined endpoint of major cardiovascular events. There was a large variety in the clinical setting of included trials, and some variation in the way the remote conditioning stimulus was administered as well. For example, the number of cycles of induced ischaemia and reperfusion varied and even the tissue used to apply the remote stimulus to differed. Most studies used skeletal muscle of the arm or leg, but in two trials direct clamping of an artery was used. Also, most studies were small and only reported clinical outcome as secondary parameters. So, even though we did find a reduction in cardiac injury, this did not translate into an improvement in clinical outcome in these early trials.

In our own randomised trial, we could not find an effect on molecular markers in myocardial tissue after remote ischaemic conditioning. However, this study was impaired by a smaller than planned sample size and we cannot say with confidence that there is indeed no effect. Also, since there was no reduction in biomarker release following surgery, it could be argued that there was no protective effect of remote ischaemia, and therefore molecular effects would not be expected either.

Diabetes mellitus probably interferes with the protective response following ischaemic conditioning. Therefore, we were specifically interested in whether the molecular mechanisms would be different in subjects with diabetes mellitus as compared to those without diabetes mellitus. However, due to the number of tissue samples available for analysis, this study did not clarify why patients with diabetes respond differently to remote ischaemic conditioning compared to patients without diabetes mellitus.

Part II Helium inhalation

In **chapter 4** we demonstrated that helium conditioning prevents endothelial dysfunction in healthy volunteers after forearm ischaemia. We used acetylcholine-induced vasodilation as a marker of endothelial function and measured the release of cytokines and microparticles after ischaemia. The protective effect was seen when helium was administered either 15 minutes or 24 hours before the ischaemic event. There was no effect on the level of circulating cytokines or microparticles.

Chapter 5 reports the results of a randomised controlled trial in 125 patients undergoing coronary artery bypass grafting (CABG) surgery, investigating the hypothesis that helium inhalation would activate certain signal molecules and would reduce myocardial injury. To assess myocardial injury, troponin T levels were measured for 48 hours after surgery. Additionally, during surgery samples of the myocardium were collected and analysed for activation of protective signal molecules. We did not see an effect of helium pre- or postconditioning on ERK-1, ERK-2, p-38 mitogen-activated protein kinase (p38 MAPK) or protein kinase C-epsilon (PKC-epsilon) activation. Nor did conditioning with helium reduce troponin T release after surgery.

In **chapter 6** we used heliox to ventilate 25 patients post-cardiac arrest in the intensive care unit, demonstrating that it was safe to use this inhalational agent in this patient population, and we encountered no major side effects while doing so.

In **chapter 7** we showed that using heliox ventilation improves carbon dioxide removal and reduces inspiratory pressure and minute ventilation in mechanically ventilated patients in the intensive care unit.

In one the first studies in humans we were able to demonstrate a protective effect by helium conditioning in healthy volunteers.[3] Helium inhalation preserved the vascular dilatation response to acetylcholine after induced ischaemia. We hypothesised that such an effect might be mediated by endothelial nitric oxide synthase (eNOS), however, inhibition of eNOS did not alter the effects on forearm blood flow. Data from another study suggested a role for the inflammatory response system,[4] but we did not find any effect of helium conditioning on the levels of circulating cytokines, such as interleukin-ß, interleukin-6, and interleukin-8. So, we could translate the protective effects of helium on ischaemia-reperfusion injury to humans, however, this study did not clarify how this protection is mediated.

In our randomised controlled trial investigating helium pre- and postconditioning in cardiac surgery, we could not replicate these results.[5] No effect on the investigated molecular markers was seen in tissue samples collected during surgery. Of note, we included a group of patients treated with sevoflurane preconditioning, but no effect was seen in these patients either, in contrast to a previous study.[6] As a secondary endpoint we investigated myocardial injury. Helium conditioning, when administered as preconditioning, postconditioning or both, did not reduce myocardial injury. Neither did anaesthetic conditioning by sevoflurane. Although the anaesthetic management was protocolised, both blood and crystalloid cardioplegia were used and multiple surgeons performed the procedures. These factors might have created to much heterogeneity within the groups to detect a difference in myocardial injury.

We demonstrated that helium can safely be administered to critically ill patients admitted to the intensive care unit after cardiac arrest. This study was not designed to show a beneficial effect of helium on ischaemia-reperfusion injury, so it is not possible to draw any conclusions on possible neuro- or cardioprotective effects of helium in this population. Focussing on the specifics of mechanical ventilation in the same patients, we see that helium reduces peak airway pressure and facilitates carbon dioxide removal, and allows for a reduction in respiratory rate and minute volume ventilation.

GENERAL DISCUSSION

Remote ischaemia

Although the protective effects of ischaemic conditioning were impressive in experimental settings, ischaemic conditioning itself was never incorporated into clinical care. Remote ischaemic conditioning appeared to be ideally suited to provide this translation into various clinical settings. Indeed, this safe and non-invasive way to implement a conditioning protocol in patients seemed very appealing.

In 2007 in a landmark trial on remote ischaemic conditioning, Hausenloy and co-workers randomised 57 patients scheduled for coronary artery bypass grafting surgery to be treated either by three cycles of ischaemia and reperfusion of the arm, or no arm ischaemia.[7] This conditioning protocol was implemented after induction of anaesthesia but before the start of surgery. They found a significant reduction in the primary endpoint “myocardial injury”, as measured by postoperative troponin release.

This study let to an increased interest in the subject and several studies were conducted in cardiovascular patients. We performed the first large systematic review and meta-analysis, summarising 23 clinical trials published before July 2011 on remote ischaemic conditioning. We found that remote ischaemic conditioning did indeed reduce myocardial injury assessed by biomarker release, but conclusions on clinical endpoints were not as robust.

Subsequently, our results were confirmed in a larger trial in Germany.[8] In this study 329 patients undergoing coronary artery bypass grafting surgery were included; myocardial injury was again assessed by troponin release. In this trial, patients were followed for up to four years for the assessment of secondary endpoints. Remote ischaemic conditioning was found to reduce not only the amount of myocardial injury, but also the occurrence of major cardiovascular events and death.

These results from earlier clinical studies needed to be confirmed in large multi-centre trials and most of those were conducted after our review was published. We will discuss three of them in more detail.

Hong and co-workers analysed 1280 patients undergoing cardiac surgery in two hospitals in Seoul.[9] Participants were randomly assigned to undergo four cycles of arm ischaemia before and after bypass, or before and after grafting of the coronary arteries in case of off-pump coronary artery bypass grafting surgery. The rate of the primary composite endpoint, including death, myocardial infarction and stroke, was similar in the conditioning group and the control group (38.0% vs 38.1%).

The ERICA trial was a multi-centre trial set in 30 hospitals in the United Kingdom, including 1612 patients undergoing coronary artery bypass grafting surgery, with or without concomitant valve surgery.[10] The trial focused on high-risk patients, including only those with a predicted perioperative mortality of 5% or higher. Participants were randomised to either receive four cycles of five minutes of fore arm ischaemia or sham conditioning. There was no effect of remote ischaemic conditioning on the occurrence of compound endpoint of major cardiac and cerebral events up to one year (26,5% in the conditioning group versus 27,7% in the control group). Nor was there any benefit on the secondary endpoints, such as myocardial infarction, stroke, death or cardiac injury as measured by troponin release post-operatively.

Similarly, the RIP Heart study was conducted in 14 German hospitals.[11] A total of 1403 patients scheduled to undergo a variety of different cardiac surgical procedures were included. Patients were randomised to either undergo four cycles of five minutes of forearm ischaemia or sham conditioning. Remote ischaemic conditioning did not reduce the composite primary endpoint of death, new acute myocardial infarction, stroke or kidney failure, within 14 days after surgery. Nor did remote conditioning show any beneficial effect on the secondary endpoints, including myocardial injury as measured by biomarker release, measurements of myocardial function during surgery, or survival up to one year postoperatively. This finding did not change when the population was analysed according to the preoperative predicted risk as calculated by EuroSCORE.

These results were confirmed in recent systematic reviews. In 2017, Benstoem et al. analysed 29 randomised controlled trials including more than 5000 patients.[12] In these trials, remote ischaemic preconditioning was compared with sham or control in patients undergoing coronary artery bypass grafting surgery. No effect was observed on a composite endpoint of death, myocardial infarction or stroke.

It is disappointing that these early results did not translate into a clinical benefit in larger trials. A small trial suggests there might be an effect of the choice of anaesthetics on the ability of remote ischaemic conditioning to provide cardioprotection.[13] Specifically, that propofol-based anaesthesia could inhibit remote ischaemic conditioning. However, a recent trial exclusively using volatile anaesthetics also failed to demonstrate a benefit of remote ischaemic conditioning.[14] No large trials exist that address this question directly and a systematic review concluded that there is not enough data available to clarify whether the use of propofol interferes with clinical conditioning.[15, 16] There are trials, however, investigating the effect of anaesthetic regime on myocardial injury after cardiac surgery, but without remote conditioning. Previous trials and meta-analyses suggested a benefit of volatile anaesthetics over intravenous anaesthesia.[17, 18] Recently, in the largest trial to date, including 5400 patients, the use of volatile anaesthetics did not reduce the rate of death or non-fatal myocardial infarction.[19] A recent meta-analysis found a reduction of

myocardial injury with the use of sevoflurane or desflurane, but no effect on 30-day mortality.[20]

We can conclude that despite very encouraging results in early trials, which were confirmed by our meta-analysis, subsequent larger trials and meta-analyses did not show a beneficial effect of remote ischaemic conditioning on clinical outcome.

Helium inhalation

Early experimental studies showed that helium-induced conditioning follows a similar mechanism as ischaemic preconditioning. Experiments using blockers of phosphatidylinositol-3-kinase (PI3K), ERK-1 and 70-kDa ribosomal proteins s6 kinase, all components of the reperfusion injury salvage kinase pathway (RISK pathway), demonstrated that these three enzymes were indeed part of the conditioning response by helium.[21] Follow-up experiments confirmed these findings and also established the mitochondrial permeability transition pore (mPTP) as a critical part of the signal transduction pathway.[22] Other upstream mediators such as opioid receptors were shown to be involved as well.[23] Helium conditioning prevented opening of the mPTP by maintaining intra-cellular acidosis,[24] and induced mild mitochondrial uncoupling via mitochondrial calcium sensitive potassium ($mKCa^2$) channels.[25]

In a rat model investigating gene expression, helium postconditioning increased ribonucleic acid (RNA) levels of several genes involved in autophagy, and decreased the level of RNA for genes linked to apoptosis.[26]

Recently, studies focused on caveolae and caveolins as a novel part of the conditioning process.[27] Caveolae are small dents or sacs in the cell membrane; caveolin proteins help to shape these structures and support their function.[28, 29] The caveolins contain a scaffolding domain for other proteins to bind to, regulating protein binding at the cellular membrane. Interestingly, some of the proteins known to bind at this scaffold site are also known to be involved in conditioning, such as PI3K, eNOS, PKC and ERK.[29, 30] Caveolins have been shown to be a part of the protective response following ischaemic conditioning or anaesthetic conditioning by isoflurane.[31, 32] In different animal models, helium conditioning reduced the membrane bound caveolins and increased the amount of circulating caveolins in serum.[33-35] This suggests that these caveolins have been secreted from the cell conditioning and this molecular mechanism could be integral in helium induced conditioning. The role of caveolins in helium-induced conditioning was further explored in a study using the plasma of helium-conditioned healthy volunteers. When this plasma was transferred to human endothelial cells ex vivo, it reduced hypoxic cell injury. Knockdown of caveolin-1 abolished this protective effect.[36]

The protective effects of helium conditioning are predominantly seen in healthy animals and human volunteers. For example, in aged, hypertensive, or obese animals, the effect of helium on ischaemia-reperfusion injury seems to be lost or severely reduced.[25, 37-39]

In our studies, we were able to confirm the presence of helium-induced conditioning in healthy volunteers, using induced forearm ischaemia as a safe and accessible model of ischaemia-reperfusion in humans.[3] Here, helium preserved endothelial responsiveness to acetylcholine, indicating protection of the endothelium from ischaemia-reperfusion injury. In contrast, another study did not find a protective effect on endothelial function.[4] In this study, helium was administered in a continuous fashion and endothelial function was assessed by post-ischaemic hyperaemia. No preservative effect of helium could be demonstrated. However, a modest anti-inflammatory effect was observed. A different study explored this anti-inflammatory effect in healthy volunteers, in which blood samples were collected after breathing a mixture of helium and oxygen or air.[40] The blood samples were stimulated and analysed for factors such as tumour necrosis factor alpha (TNF-a), interferon-gamma (IFN-g) and multiple interleukins. No effect of helium inhalation was seen on the levels of TNF-a, interleukin-1b, interleukin-6, IFN-g or interleukins-2. In the clinical setting of coronary artery bypass grafting surgery, helium inhalation did not ameliorate the ischaemic injury to the heart, as determined by postoperative troponin release.[5]

Animal experiments investigating the neuroprotective properties of helium yielded mixed results. In a rat model of brain ischaemia and reperfusion helium-oxygen reduced the volume of infarction, and improved neurological function at 24 hours.[41] In a study inducing brain ischaemia by temporally occluding the common carotid artery in new-born rats, helium preconditioning reduced the amount of injury and improved neurological function.[42] However, other studies did not find any protective effect of helium after neurological injury. For example, in a model of neonatal hypoxia, helium had no effect on infarct volume, whereas other noble gasses such as xenon and argon were found to be protective.[43] In a model of cardiac arrest, helium inhalation did not improve neurological function 24 hours after the arrest.[44]

We showed in **chapter 6** that helium could be used safely in patients admitted to the Intensive Care Unit and being mechanically ventilated after an out-of-hospital cardiac arrest. However, this study did not assess the potential of helium to reduce brain damage following a cardiac arrest.

In conclusion we were able to translate helium conditioning to human volunteers but not to patients in a clinically relevant setting. Animal experiments demonstrated cardioprotection by helium in healthy animals, but not in those more closely resembling the target patient population. Experiments on neuroprotection have yielded mixed results. At

this time there is no data supporting the use of helium in clinical ischaemia reperfusion situations.

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Chapter 9

Nederlands samenvatting voor niet-medici

ISCHEMIE EN REPERFUSIE SCHADE

Onderbreking van de doorbloeding van een orgaan leidt tot schade aan dit orgaan; dit noemen we ischemie. Dit kan optreden als een bloedvat is afgesloten, maar ook als bijvoorbeeld de bloeddruk heel laag is of het hart het bloed slecht rondpompt. De eerste behandeling van ischemie is dan ook gericht op het herstellen van dit onderliggende probleem.

Echter, ook na herstel van de bloedtoevoer stopt de verdere beschadiging van het weefsel niet meteen. Sterker, sommige schade processen worden versterkt. Deze fase wordt de reperfusie fase genoemd en aangezien de twee fasen niet los van elkaar gezien kunnen worden, wordt dit hele proces ischemie en reperfusie schade genoemd.

Omdat verschillende problemen uiteindelijk kunnen leiden tot ischemie en reperfusie schade, gebeurt dit ook in verschillende scenario's. Denk hierbij bijvoorbeeld aan patiënten die een hartinfarct krijgen, een hartoperatie moeten ondergaan of een hartstilstand hebben en gereanimeerd moeten worden. Een behandeling van ischemie en reperfusie schade kan dan ook veel patiënten helpen.

Conditioneren

Het is mogelijk om organen te "trainen" door ze een aantal keer kortdurend bloot te stellen aan ischemie. Dit training effect heet conditioneren en maakt organen minder gevoelig voor ischemie en reperfusie schade na een langere periode van ischemie. Interessant genoeg kan dit trainen ook als de doorbloeding weer hersteld wordt. Conditioneren door ischemie is bij patiënten echter niet toe te passen en er wordt gezocht naar andere vormen van conditioneren, die ook bescherming geven en tegelijk veel makkelijker toe te passen zijn. In dit proefschrift richten we ons op twee van zulke andere vormen van conditioneren.

Recent is duidelijk geworden dat het beschermende effect van conditioneren middels ischemie zich niet beperken tot het getrainde orgaan, maar ook andere organen op afstand bescherming biedt. Dit noemen we ischemisch conditioneren op afstand en het aantrekkelijke is dat bijvoorbeeld ook spierweefsel van een arm of been hiervoor gebruikt kan worden. Dit weefsel is veel beter bestand tegen ischemie dan bijvoorbeeld het hart en kan makkelijk gebruikt voor conditioneren door met een tourniquet de bloed toevoer tijdelijk te onderbreken. De eerste studies bij patiënten die hartchirurgie ondergaan laten zien dat ischemisch conditioneren op afstand de schade aan het hart vermindert.

Een andere interessante manier van conditioneren is het toedienen van helium. Helium vermindert in dier experimenteel onderzoek de schade aan het hart bij een hartinfarct. Het toedienen van helium gebeurt in een mengsel van helium met zuurstof wat ingeademd wordt. In het ziekenhuis wordt het soms toegepast voor het beademen van patiënten met ernstige longproblemen.

Deel I Ischemisch conditioneren op afstand

In **deel een** van dit proefschrift onderzoeken we conditioneren door ischemie op afstand om kwetsbare organen zoals het hart te beschermen. In **hoofdstuk 2** beschrijven we een literatuurstudie naar alle in juli 2011 beschikbare gerandomiseerde onderzoeken naar conditioneren door ischemie op afstand. Dit zijn 23 studies met in totaal bijna 2000 patiënten. Deze studies onderzoeken patiënten die hart- of vaatoperaties ondergaan of een dotterbehandeling moesten krijgen. Als we kijken naar klinische resultaten zien we geen verschillen. Patiënten die behandeld zijn met conditionering door ischemie op afstand krijgen niet minder vaak een hartinfarct, beroerte of schade aan de nieren en er is ook geen verschil in sterfte. Wel zien we dat conditioneren door ischemie op afstand de schade aan het hart bij deze procedures vermindert, gemeten aan het verminderd vrijkomen van biologische markers van hartschade.

Hoofdstuk 3 beschrijft een gerandomiseerde studie naar het effect van ischemie op afstand bij patiënten die een hartoperatie ondergaan. Tijdens de operatie zijn weefselmonsters afgenomen van het hart om die te analyseren op bepaalde signaal eiwitten, waarvan we denken dat die betrokken zijn bij het beschermende effect. We willen de effecten vergelijken tussen patiënten met diabetes mellitus en patiënten zonder diabetes mellitus. Dit doen we omdat patiënten met diabetes mellitus minder goed beschermd lijken te worden door conditionering door ischemie op afstand. We zien geen duidelijk effect op de onderzochte signaal eiwitten. Echter, de hoeveelheid beschikbare monsters was kleiner dan aanvankelijk gepland en dat maakt dat we niet met zekerheid kunnen zeggen dat er inderdaad geen effect was. Daarnaast vermindert ischemie op afstand in onze studie niet de schade aan het hart, zoals gemeten aan de hoeveelheid vrijgekomen biologische markers na de operatie.

Deel II Conditioneren door helium

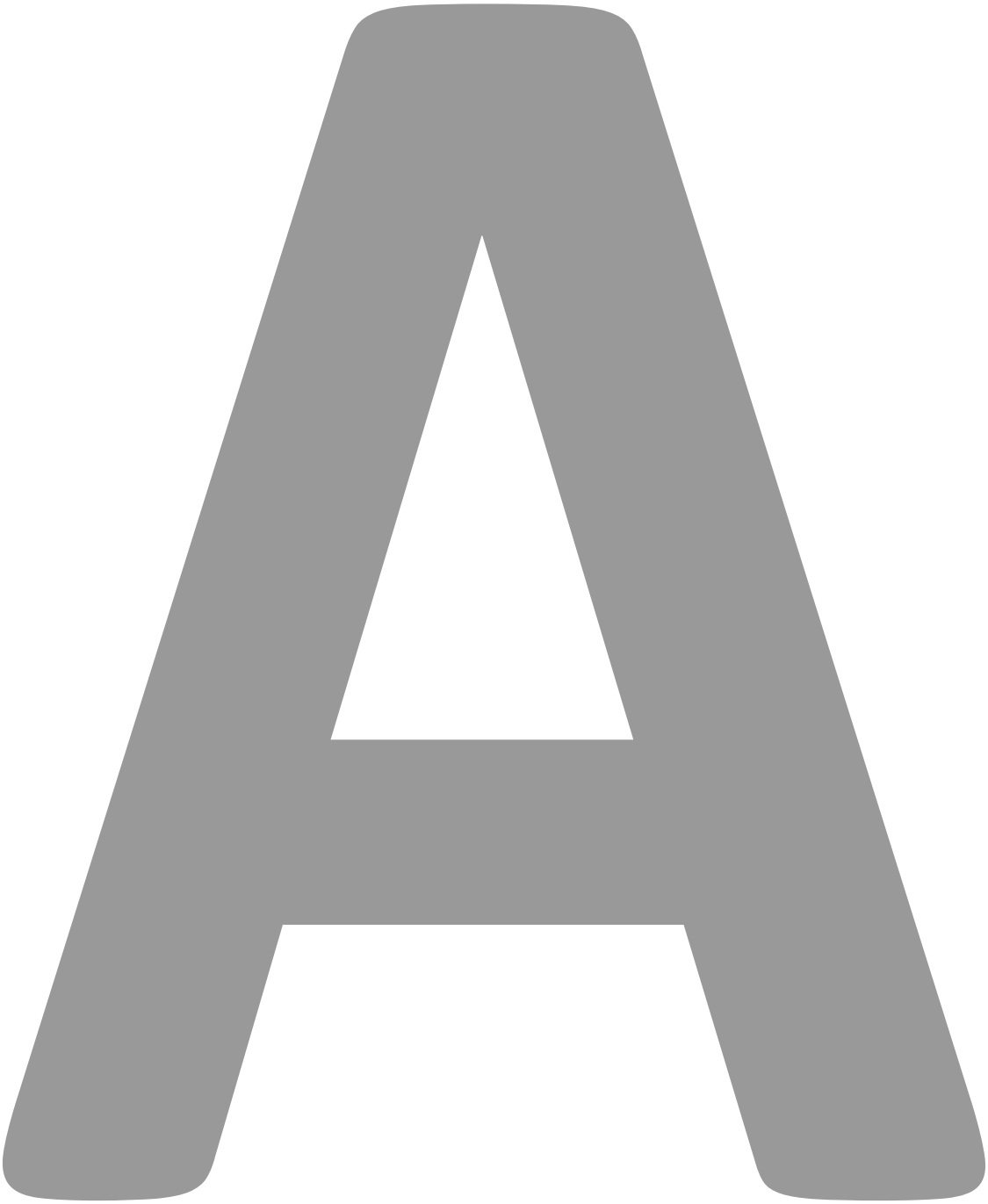
Deel twee van dit proefschrift richt zich op het gebruik van helium als bescherming middel tegen ischemie en reperfusie schade. In **hoofdstuk 4** maken we de stap van dieren naar mensen door helium te testen bij proefpersonen waarbij wij een arm kortdurend

ischemisch maken. Dit doen we om de functie van de bloedvaten te testen, deze wordt minder na ischemie maar blijft behouden als de proefpersonen eerst helium inademen. We denken dat dit mogelijk komt door een enzym dat vaatverwijdende stoffen maakt in de binnen bekleding van bloedvaten. Maar als we dit enzym remmen dan blijft het beschermende effect behouden.

In **hoofdstuk 5** beschrijven we een gerandomiseerde studie bij patiënten die hartchirurgie ondergaan. Tijdens de operatie worden weefsel biopten afgenomen om te kijken naar signaal eiwitten waarvan we denken dat die betrokken zijn bij bescherming door helium. We zien echter geen duidelijke verandering door het gebruik van helium. Ook de schade aan het hart gemeten in het bloed na de operatie is niet minder bij de patiënten die helium hebben gekregen. Mogelijk dat de chirurgische factoren een te grote invloed hebben gehad om en verschil aan te tonen.

In **hoofdstuk 6** beschrijven we het gebruik van helium in patiënt die op de intensive care zijn opgenomen na een hartstilstand buiten het ziekenhuis. Het doel was om ervaring hiermee op te doen en te kijken of dit veilig kan. Dit lijkt het geval te zijn, we zien geen nadelige effecten van het gebruik van helium. De studie was niet opgezet om te kijken naar eventuele conditionerende effecten van helium. Daarnaast hebben we naar de effecten van het gebruik van helium op de beademing gekeken, dit staat in **hoofdstuk 7**. De beademingsdrukken zijn lager met helium en het is makkelijker om koolstofdioxide af te blazen, wat leidde tot een lager adem minuut volume en een lagere ademarbeid.

We kunnen concluderen dat ischemie op afstand de ischemie en reperfusie schade vermindert en dat conditioneren met helium bescherming biedt bij gezonde vrijwilligers. Echter, geen van beide onderzochte manieren van conditioneren leidt tot een verbeterde uitkomst voor patiënten in situaties van ischemie en reperfusie.



Appendices

Contributing authors

Bas A. de Mol
Contributed in conceiving of research (Chapter 5)

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Contributed in conduction of experiments (Chapters 3 and 5) and conceiving of research, data analysis and supervision of the writing of the manuscript (Chapters 2, 3, 4, 5 and 6)

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PhD student: D. Brevoord

PhD supervisors: prof. dr. dr. M.W. Hollmann, prof. dr. B. Preckel, dr. N.C. Hauck-Weber and dr. J. Horn

PhD Teaching

	Year	Workload (ECTS)
Courses		
Good clinical practice / BROK	2009	0.9
Practical biostatistics	2010	1.1
Basic laboratory safety	2011	0.3
Scientific writing	2011	0.5
Specific courses		
Laboratory animals course	2009	3.9
Cardiac function and adaptation	2009	2
Seminars, workshops and master classes		
Laboratory research meeting (weekly)	2009 – 2011	6
Journal club (monthly)	2009 – 2011	3
Anaesthesia evening seminars (monthly)	2009 - 2017	3

International conferences

German Congress of Anaesthesiology, Hamburg	2011	0.5
Euroaenesthesia, Amsterdam	2011	1
EACTA Annual Congress, Amsterdam	2012	0.5
Euroaenesthesia, Paris	2012	1
Anesthesiology, Washington	2012	1
ISICEM, Brussels	2015	0.75

Presentations

German Congress of Anaesthesiology, Hamburg	2011	0.5
Poster presentation: "*Comparison of Somanetics INVOS and NONIN Medical Equanox NIRS systems for measurement of cerebral oxygenation"*		
Euroaenesthesia, Amsterdam	2011	0.5
Poster presentation: "*Cerebral oxygenation depends on flow and not vascular resistance in patient on cardiopulmonary bypass"*		
EACTA Annual Congress, Amsterdam	2012	0.5
Poster presentation: *"Does remote ischemic preconditioning improve outcome? A systematic review"*		
EACTA Annual Congress, Amsterdam	2012	0.5
Poster presentation: *"Helium pre- or postconditioning does not affect troponin release in patients undergoing coronary artery bypass grafting surgery"*		

Euroaenesthesia, Paris Poster presentation: *"Helium ventilation is safe and feasible in patients admitted to the ICU after cardiac arrest"*	2012	0.5

Teaching

Student coaching and mentoring, Research Internship, Medicine, University of Amsterdam	2011, 2012	2

Parameters of Esteem

Prizes

- Second place in 'best poster presentation' EACTA Annual Congress	2012	

Publications

M. A. Van Geer, **D. Brevoord**, K. F. D. Kuhlmann, C. T. Bakker, H. Mizuguchi, J. G. Wesseling, F. J. Ten Kate, D. J. Gouma, R. P. J. Oude Elferink and P. J. Bosma
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N. H. Sperna Weiland, **D. Brevoord**, D. A. Jobsis, E. M. de Beaumont, V. Evers, B. Preckel, M. W. Hollmann, S. van Dieren, B. A. de Mol and R. V. Immink
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Curriculum vitae

Daniel Brevoord was born in Amsterdam on the 4th of December 1980. He studied Medicine at the University of Amsterdam and completed his internships with rotations in Intensive Care Medicine and Anaesthesia. He became interested in scientific research during his research internship at the Liver Centre of the Academical Medical Centre in Amsterdam, which he voluntarily extended for half a year. He started his professional career in December 2006, as a registrar (Anios) in Medicine in the Tergooi Ziekenhuizen. In 2008 he worked as a registrar in Cardiology, in the Diakonessenhuis in Utrecht.

In 2009 he started his PhD research on organ protection by remote ischaemic conditioning and helium at the Laboratory of Experimental Intensive Care and Anaesthetics of the Academical Medical Centre in Amsterdam, under supervision of prof. dr. dr. M.W. Hollman, prof. dr. B. Preckel and dr. N.C. Hauck-Weber. During the following years dr. J. Horn joined as supervisor.

In 2012 he started as a specialty registrar in Anaesthesia at the Academical Medical Centre in Amsterdam, finishing his trainings in 2017, after which he stayed on as a senior registrar. In February 2018 he moved to London, for a fellowship in Cardiothoracic Anaesthesia and Intensive Care at St. Bartholomew's Hospital (Barts Health NHS Trust). Since May 2019 he's working as a fellow in Cardiothoracic Anaesthesia at St. Antonius Hospital in Nieuwegein and Utrecht.

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