

Remote Ischaemic PrEconditioning of Human Myocardium (RIPE): study protocol for a double-blinded randomised controlled trial

Marek A. Deja^{1,2}, Ryszard Wiaderkiewicz³, Piotr Czekaj³, Ewa Czech³, Marcin Malinowski^{1,2}, Leszek Machej^{4,5}, Andrzej Węglarzy⁵, Adam Kowalówka^{1,2}, Magda Piekarska^{1,2}, Bartosz Szurlej¹, Tomasz Latusek¹

¹Department of Cardiac Surgery, School of Medicine in Katowice, Medical University of Silesia, Katowice, Poland

²Department of Cardiac Surgery, Upper-Silesian Heart Centre, Katowice, Poland

³Chair of Histology and Embryology, School of Medicine in Katowice, Medical University of Silesia, Katowice, Poland

⁴Department of Anaesthesia and Intensive Care Nursing, School of Health Sciences, Medical University of Silesia, Katowice, Poland

⁵Department of Cardiac Anaesthesia, Upper-Silesian Heart Centre, Katowice, Poland

Abstract

Background: Remote preconditioning has been shown to be a potent protective phenomenon in many animals. Several studies aimed to demonstrate it was feasible in humans by trying to show its protective effect during cardiac surgery. Of these, some small studies and one larger trial were positive while two other bigger studies showed no effectiveness of remote preconditioning as assessed by levels of postoperatively released cardiac markers. Recently, two large clinical trials also failed to prove the benefit of remote preconditioning in cardiac surgery. No study showed that remote preconditioning actually increases resistance of human myocardium to standardised ischaemic and reperfusion stimulus in experimental settings. In animal studies, remote preconditioning was shown to improve mitochondrial function and structure, but such data on human myocardium are scarce.

Aim: The aim of the study is to determine whether remote preconditioning protects human myocardium against ischaemia-reperfusion injury in both in vivo and in vitro conditions.

Methods: The trial is designed as a single-centre, double-blinded, sham-controlled trial of 120 patients. We randomise (1:1) patients referred for coronary artery bypass grafting for stable coronary artery disease to remote preconditioning or “sham” intervention. The remote preconditioning is obtained by three cycles of 5 min inflation and 5 min deflation of a blood pressure cuff on the right arm. Postoperative course including myocardial enzymes profile will be analysed. Moreover, in the in-vitro arm the clinically preconditioned myocardium will be assessed for function, mitochondria structure, and mitochondria-dependent apoptosis. The informed consent of all patients is obtained before enrolment into the study by the investigator. The study conforms to the spirit and the letter of the declaration of Helsinki.

Results and conclusions: In case the effect of remote preconditioning is not measurable in ex-vivo assessment, any future attempt at implementing this phenomenon in clinical practice may be futile and should not be continued until the effect can be confirmed in a controlled experimental setting. The study might therefore indicate future directions in trials of clinical implementation of remote preconditioning.

Trial Registration: Clinical Trials Register (Clinicaltrials.gov) identifier: NCT01994707. The study was approved by Institutional Review Board of the Medical University of Silesia (KNW/0022/KB1/160/12).

Key words: remote ischaemic preconditioning; coronary artery bypass graft surgery, troponin T, apoptosis, cardioprotection

Kardiol Pol 2018; 76, 1: 136–143

Address for correspondence:

Prof. Ryszard Wiaderkiewicz, Chair of Histology and Embryology, School of Medicine in Katowice, Medical University of Silesia, ul. Medyków 18, 40–752, Katowice, Poland, e-mail: wiader@sum.edu.pl

Received: 21.07.2017

Accepted: 02.08.2017

Available as AOP: 20.09.2017

Kardiologia Polska Copyright © Polskie Towarzystwo Kardiologiczne 2018

INTRODUCTION

No experimental study showed that human myocardium can be remotely preconditioned against standardised ischaemic/hypoxic insult. We aim to remove this major knowledge gap by applying remote preconditioning to the patient and studying ex-vivo the myocardium obtained thereafter. We assume that we will be able to show that remote preconditioning by brief periods of ischaemia of the arm protects segments of human right atrial appendage myocardium subjected to simulated hypoxia and reoxygenation in-vitro.

This proof of principle is crucial. In case the effect of remote preconditioning is not measurable in ex-vivo assessment, any future attempt at implementing this phenomenon in clinical practice may be futile and should not be continued until the effect can be confirmed in a controlled experimental setting.

On the other hand, if we manage to prove that the remote preconditioning truly protects human myocardium, we will have clinical data, and the results from myocardial biopsies of the very same patients to correlate and possibly reconcile any apparent discrepancy between ex-vivo and in vivo studies. The study might therefore indicate future directions in trials of clinical implementation of remote preconditioning.

Finally, it is believed that remote preconditioning acts through its influence on mitochondria [1, 2] similarly to ischaemic preconditioning. Our study will be one of very few trials examining its influence on mitochondrial structure and induction of apoptosis and certainly the first to study this phenomenon in human myocardium.

METHODS

Trial design

The trial is a single-centre, randomised, double-blinded, sham-controlled trial of patients subjected to coronary artery bypass grafting (CABG) for stable coronary artery disease. Due to the assumption that some patients will be excluded from the study because of intraoperative events (see below), we planned to continue recruitment of patients into the study until we have enrolled 120 patients undergoing surgery according to the protocol and not complicated by a perioperative myocardial infarction. Patients, surgeons, the treatment team and data analysts are blinded to treatment allocation.

The study is conducted both in-vivo and ex-vivo (Fig. 1).

Inclusion and exclusion criteria

The study was approved by the Institutional Review Board of the Medical University of Silesia (KNW/0022/KB1/160/12). The study conforms to the spirit and the letter of the declaration of Helsinki. The informed consent of all patients is obtained before enrolment into the study by the investigator. Patient inclusion and exclusion criteria are presented in the Table 1.

Exclusions after randomisation include (1) perioperative myocardial infarction (MI), (2) perioperative technical

difficulties possibly leading to myocardial damage, and (3) breaches of protocol related to operative procedure (no operation performed, no cardiopulmonary bypass use, no aortic cross-clamp applied).

The patients who develop postoperative MI and those in whom technical problems during surgery might lead to myocardial damage will be excluded from the study. We believe that perioperative MI is predominantly caused by vessel occlusion (plaque rupture, microembolism, graft occlusion, surgical error) and should not influence the assessment of remote preconditioning protective effect against global myocardial ischaemia/reperfusion injury. The perioperative MI (type 5) will be diagnosed based on the Third Universal Definition of Myocardial Infarction based on data collected within two days of surgery [3]. The diagnosis based on cardiac marker levels, electrocardiogram (ECG), and echo results will be made by an independent cardiologist who will not have been involved in other study-related tasks.

Primary and secondary endpoints

The primary endpoint of the study is the postoperative release of cardiac troponin T. The area under the curve of the marker level over time will be compared between the groups.

The secondary endpoints include: creatine kinase isoenzyme MB (CK-MB), haemodynamic assessment with oxygen metabolic assessment and creatinine clearance (CKD-Epi method), and the results from in vitro study: right atrial muscle inotropism, Western-blot, immunohistochemistry, and electron microscopy.

Sample size calculation

To calculate the size of the study group we used the troponin T level (area under the curve [AUC]) as a primary endpoint, similarly to Rahman et al. [4]. We estimated, based on the previous positive studies of remote preconditioning in CABG, that remote preconditioning should decrease the troponin T AUC by 40% (a standardised difference of 0.8). Thus, with the hypothetical standardised difference of 0.6, we need 120 patients to be able to show lower troponin T release in the remote preconditioning group with $p < 0.05$ and a power of 90%.

Randomisation and intervention

Patients referred for CABG for stable coronary artery disease are recruited and randomised (1:1) by random digit generator to one of two groups: remote preconditioning or sham intervention. The mechanism of implementing the allocation sequence is based on sealed envelopes. On the day of surgery, after induction of anaesthesia and before the skin incision, remote preconditioning is elicited by three cycles of 5 min inflation (ischaemia) and 5 min deflation (reperfusion) of blood pressure cuff on the right arm. The same method of eliciting remote preconditioning was used in other studies that claimed

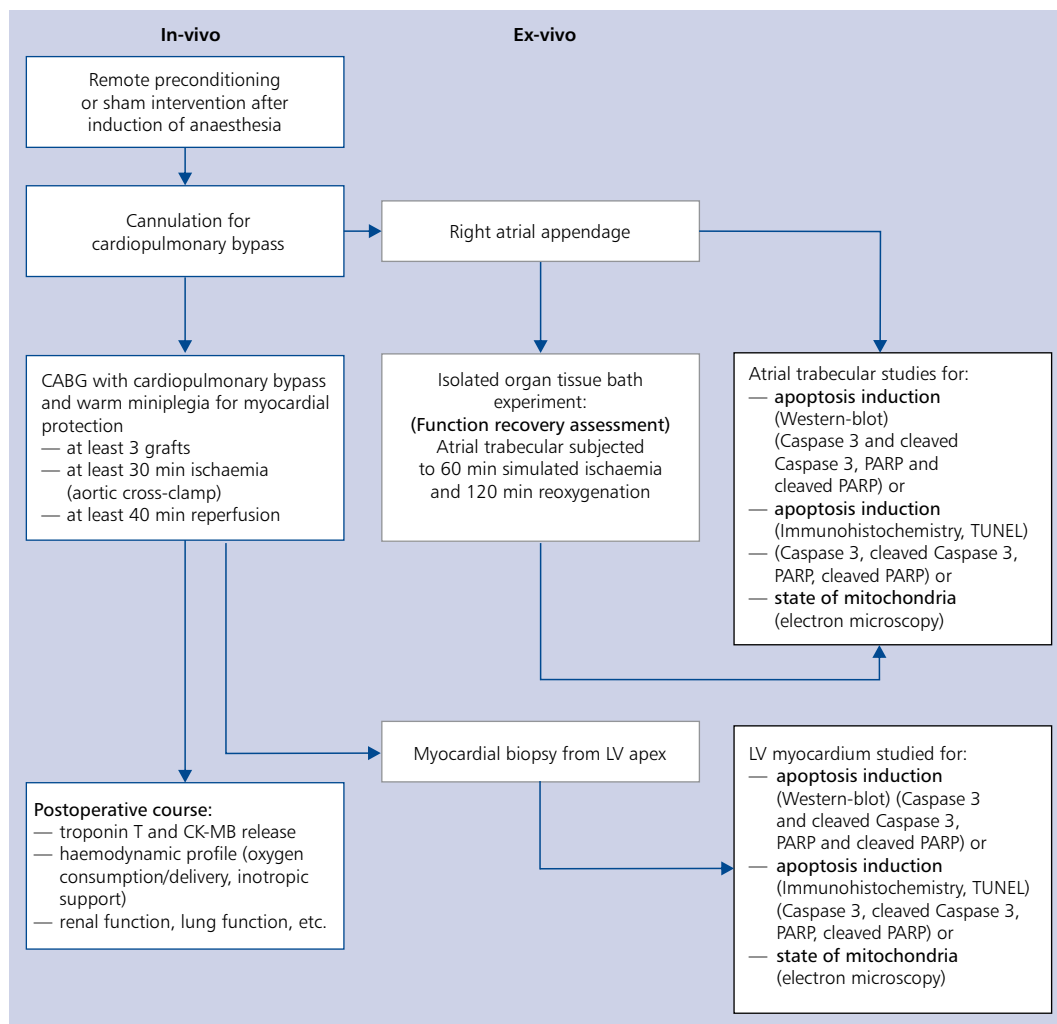


Figure 1. The scheme of the study; CABG — coronary artery bypass graft; CK-MB — creatine kinase-myocardial band; LV — left ventricle; PARP — poly-ADP ribose polymerase; TUNEL — terminal deoxynucleotidyl transferase dUTP nick end labelling

positive results [5–7]. The “sham” group has the pressure cuff placed on the right arm but no inflations are performed.

To obtain blinding, the inflation occurs under surgical drapes, and is always performed by the same person, who is not involved in the care of the patient at any stage, or other research related tasks. Our “intervention” consists of an inflation and deflation of blood pressure cuff, so we did not consider it necessary to establish a Data and Safety Monitoring Committee.

Anaesthesia

Anaesthesia is standardised and consists of midazolam 15 mg orally 1 h before surgery, etomidate 0.2 mg/kg, fentanyl 5 g/kg, and pancuronium 0.1 mg/kg IV for anaesthesia induction and propofol 0.5–1.0 mg/kg/h and fentanyl 4 g/kg/h infusion for anaesthesia maintenance. No anaesthetic gases are allowed. Full haemodynamic monitoring is utilised with a Swan-Ganz

catheter. First haemodynamic measurements and oxygen supply/consumption calculations are performed preoperatively before the remote preconditioning procedure.

The in-vivo trial

The operation is performed with the use of cardiopulmonary bypass in normothermia by an experienced cardiac surgeon. Intermittent warm-blood (37°C) antegrade cardioplegia (miniplegia) is used for myocardial protection. Oxygenated blood is infused through aortic a root needle at a rate of 300 mL/min. Using a syringe pump, potassium chloride is added to keep the potassium concentration at 20 mEq/L at induction and 10 mEq/L during maintenance. The patient is given a 3-min cardioplegia infusion at induction and 1.5-min infusion as maintenance dose every 15–20 min.

We expect aortic cross-clamp time to last at least 30 min. After cross clamp removal, the proximal anastomoses are

Table 1. Inclusion and exclusion criteria for the study

Inclusion criteria
Both genders
Stable coronary artery disease referred for surgical revascularisation in whom at least three coronary artery bypass grafts are planned with the use of cardiopulmonary bypass
Exclusion criteria
Age below 18 and above 80 years
Plan to use radial artery as a graft, the plan to perform other concomitant cardiac procedure in addition to coronary artery bypass grafting
Diabetes mellitus
Troponin T level before surgery in excess of 99 th percentile of the upper reference limit
Acute coronary syndrome in last 14 days before surgery
Angina pectoris in last 48 h before surgery
Significant peripheral arterial disease
Renal disease with either creatinine level ≥ 2 mg/dL or estimated glomerular filtration rate < 30 mL/h/1.73 m ²
Renal replacement therapy
Clinically relevant hepatic insufficiency with bilirubin level at least 1.5 times above upper limit of normal or aspartate transaminase, alanine transaminase levels at least 2 \times above upper limit of normal
Advanced lung disease with forced expiratory volume in 1 s (FEV ₁) $< 40\%$ of predicted value
Pregnancy
Psychiatric disease
Drug or alcohol abuse
Post-randomisation exclusions
Perioperative myocardial infarction
Perioperative technical difficulties possibly leading to myocardial damage
Breaches of protocol related to operative procedure:
— no operation performed
— no cardiopulmonary bypass used
— no aortic cross-clamp applied

performed using an aortic side clamp. The patient is weaned off cardiopulmonary bypass, haemostasis is secured, and the chest is closed over chest tubes. Just before closing the chest a 16 G needle-true cut biopsy of the left ventricular myocardium is obtained from the apex. We expect the reperfusion time from the cross-clamp removal to obtaining the biopsy to last at least 40 min. The exact ischaemia (cross-clamp) time and reperfusion time (until obtaining the biopsy) are measured.

When disconnecting cardiopulmonary bypass, all patients receive dopamine at a dose of 5 $\mu\text{g}/\text{kg}/\text{min}$. Thereafter, the dose is adjusted to the haemodynamic status. After the opera-

tion, the patient is transferred to an intensive care unit and treated as per routine.

We measure serum concentration of cardiac troponin T (electrochemiluminescence "ECLIA", Roche) preoperatively and the next 6 h, 12 h, 18 h, 24 h, 36 h, 48 h, and 72 h after cross-clamp removal. At the same time points the level of CK-MB is assessed (enzymatic assay, Roche).

To assess the myocardial function, all patients have a pulmonary artery catheter (Swan Ganz catheter) inserted preoperatively. Full haemodynamic assessment (thermodilution method) as well as oxygen metabolism status based on arterial and mixed venous gas analysis is performed preoperatively and the next 1 h, 3 h, 6 h, 12 h, 18 h, 24 h, 36 h, and 48 h after aortic cross-clamp removal. The serum is collected for biochemical analysis (S100 β concentration, neuronal enolase NSE, NGAL, kidney injury molecule KIM and zonulin concentration) at the same time points. As well as measuring the cardiac index we calculate left and right cardiac work indices (LCWI = CI \times MAP \times 0.0144; RCWI = CI \times MPAP \times 0.0144). The oxygen delivery index and extraction ratio are calculated as follows:

$$\text{DO}_2\text{I} = \text{CI} \times \text{C}_a\text{O}_2 \times 10;$$

$$\text{O}_2\text{ER} = \frac{\text{CaO}_2 - \text{CMVO}_2}{\text{CaO}_2} \times 100.$$

The lactate and creatinine (estimated glomerular filtration rate) levels are also measured.

The need for inotropic support will be assessed at the same time points using inotropic index. It is calculated as follows: dopamine dose + dobutamine dose + 100 \times epinephrine dose + 100 \times norepinephrine dose + 100 \times isoproterenol dose + 15 \times milrinone dose (all doses in $\mu\text{g}/\text{kg}/\text{min}$). All patients have postoperative ECG done on the 1st, 2nd, and 4th postoperative days.

The ex-vivo trial

Functional in vitro assessment

On cannulation for cardiopulmonary bypass, the right atrial appendage, which is routinely removed and discarded for venous cannula placement, is harvested in all patients. The tissue is transferred in ice-cold Krebs-Henseleit solution to the isolated organ laboratory in our department. One pectinate muscle trabecula is harvested for baseline assessment of apoptosis or mitochondria (see below). Another single trabecula less than 1 mm in diameter is mounted in the organ chamber — Schuler Organbath (Hugo Sachs Elektronik, March-Hugstetten, Germany [HSE]) containing Krebs-Henseleit solution of the following composition [M]: NaCl 118.0, KCl 4.70, CaCl₂ 2.52, MgSO₄ 1.64, NaHCO₃ 24.88, KH₂PO₄ 1.18, glucose 11.0, sodium pyruvate 2.0 (pH 7.4). It is oxygenated via glass frit with carbogen (95% oxygen, 5% carbon dioxide) and maintained at 37°C. The trabecula is driven with 1 Hz

50 ms square stimuli using platinum field electrodes and the potential of 150% of the threshold for given preparation. The stimulator Type 215 (HSE) is used. The contraction force is measured with F30 isometric force transducer Type 372 (HSE). The signal is enhanced with TAM A PlagSYS transducer amplifier module Type705 (HSE) and recorded using PowerLab/4SP system and Chart software (AD Instruments).

The trabecula is gradually stretched to 90% of optimal tension according to Frank-Starling relationship and left for 30 min of stabilisation and washout. 60 min ischaemia is simulated by substituting oxygen with argon in carbogen (95% argon, 5% carbon dioxide) and replacing Krebs-Henseleit solution with one containing no glucose or pyruvate. On reoxygenation the carbogen is added again and the tissue bath solution is replaced with the one used initially. The tissue is washed several times and left for 120 min of reoxygenation with washout every 15 min. This functional model of hypoxia reoxygenation has been used in our laboratory previously [8, 9]. Replacement of oxygen with argon results in the drop of tissue bath oxygen partial pressure from 475 ± 52 mm Hg to 51 ± 1.8 mm Hg ($p < 0.001$) [7–8]. It is accompanied by significant and rapid decline in isometric contraction force. During reoxygenation the contraction force returns initially and then we observe a slow decline of muscle inotropism, which we interpret as the development of reoxygenation injury. At the end of reoxygenation, we use 10^{-4} M norepinephrine ([–]Arterenol Bitartrate) to test for stunning.

Continuously recorded contractility is expressed as a percentage of the initial contraction force for a given preparation. We compare the maximal contraction force recovered, the contraction force after 30 min and 120 min of reoxygenation, and the one evoked by norepinephrine.

We also look for the signs of ischaemic contracture development defined as an increase of resting tension of the trabecula. This increase, if present, starts shortly after the onset of hypoxia and continues steadily throughout the whole hypoxia period (reference needed). We compare the increase in resting tension (in mN/mg tissue mass) at the end of hypoxia, i.e. at the time of maximal contracture. Contractility and lusitropism of the myocardium expressed as the first derivative of force over time during contraction and relaxation will also be studied. All measurements will be compared between trabeculae from remotely preconditioned and “sham” patients.

Myocardial apoptosis

Two atrial trabeculae from the same appendage, one harvested at baseline, and another after functional experiment (60 min hypoxia + 120 min reoxygenation) are studied each time for apoptosis induction (Western blotting and immunohistochemistry) or mitochondria structure (electron microscopy).

The left ventricle biopsies are assessed in a similar way. We randomly perform Western blotting in 60 (30:30) biopsies

harvested on liquid nitrogen, immunohistochemistry including TUNEL (terminal deoxynucleotidyl transferase dUTP nick and labelling) in 40 (20:20) biopsies harvested on 10% neutral buffered formalin, and electron microscopy in 20 (10:10) biopsies harvested on cacodyl buffer with 2% glutaraldehyde.

Western blot. Two trabeculae: baseline and after hypoxia/reoxygenation, and left ventricle biopsies from 30 preconditioned and 30 “sham” patients.

For Western blot immunoassay the samples are placed in liquid nitrogen. The expression of analysed proteins (Caspase 3, cleaved Caspase 3, poly[ADP-ribose] polymerase [PARP] and cleaved PARP) is measured. The specimens are stored in liquid nitrogen until used. After defreezing they are homogenised in RIPA buffer (3–5 mL/g tissue; ultrasound homogeniser Heidolph DIAX 900, Germany) and the protein content is estimated by BCA method using bovine serum albumin as a standard (Bicinchoninic Protein Assay, Sigma). Homogenate samples (5 mg of protein) are subjected to polyacrylamide gel electrophoresis (8% or 12%, 60 V, 60–120 min) in the presence of sodium dodecyl sulphate. After electrophoresis proteins are blotted onto PVDF membrane (30 mV, 90 min) and stained immunohistochemically. The binding of anti Casp3, anti-cleaved Casp3, anti-PARP, and anti-cleaved PARP antibodies is detected with secondary antibody conjugated with biotin — ABC technique. To visualise ABC complex we use peroxidase substrate containing DAB and hydrogen peroxide according to the manufacturer’s instructions (Vector Laboratories). The molecular weight and intensities of stained bands are analysed with One D-scan software (Scanalytics).

Immunohistochemistry. Two trabeculae: baseline and after hypoxia/reoxygenation, and left ventricle biopsies from 20 preconditioned and 20 “sham” patients.

Expression of Caspase 3, cleaved Caspase 3, PARP and cleaved PARP proteins in tissue sections are detected immunohistochemically. Tissue samples are fixed for 6 h in 4% buffered paraformaldehyde (phosphate buffer), subsequently passed through graded alcohol solutions, processed three times in xylene, and finally embedded in paraffin blocks. Slices of 5 μ m thickness are placed on silane-coated slides, deparaffinised, and rehydrated. For antigen retrieval, 10 mM citrate buffer, pH 6 (30 min) is used.

For quenching of endogenous peroxidase activity, tissue sections are blocked with 3% (vol/vol) H_2O_2 for 10 min. Before incubation with the primary antibodies, the sections are washed two times (5 min each) in TBST and pretreated with 5% normal goat serum for 60 min to prevent nonspecific binding of antibodies. The sections are incubated with the primary anti Casp3, anti-cleaved Casp3, anti-PARP, or anti-cleaved PARP antibodies overnight at 4°C, washed in TBST and then incubated for 30 min with Signal Stain Boost Detection Reagent (Cell Signaling Tech.). Bound antibodies are visualised with diaminobenzidine (DAB). Negative controls

are performed by using the Rabbit(DA1E)mAb XP Signal Stain Isotype Control (1:500). The sections are counterstained with haematoxylin, dehydrated in alcohols, and clarified in xylene, and coverslips are mounted with mounting medium.

The numbers of cells with positive reaction for Caspase 3, cleaved Caspase 3, PARP and cleaved PARP protein are determined using cellSens Entry programme (Olympus). The documentation of immunohistochemical reactions is performed with DP-26 camera (Olympus) coupled with a Nikon Eclipse E600 optical microscope.

TUNEL. Two trabeculae: baseline and after hypoxia/reoxygenation, and left ventricle biopsies from 20 preconditioned and 20 “sham” patients.

Tissue sections are deparaffinised in p-xylenes and rehydrated with decreasing concentrations of ethanol. Then, they are washed in distilled water and PBS, pretreated with proteinase K (17 min, room temperature) and washed twice in PBS. To block endogenous peroxidase activity specimens are incubated in 3% H₂O₂ in methanol for 10 min and then washed in PBS (3 × 2 min). After preincubation in TdT Labelling Buffer (5 min) the sections are incubated in TdT Labelling Reaction Mixture for 1 h at 37°C in a humidified chamber. The reaction is terminated in TdT Stop Buffer and then the sections are washed in PBS and incubated with Streptavidin-HRP solution for 10 min at room temperature. To visualise apoptotic cells the sections are incubated with TACS Blue Label (2 min) and counterstained with Fast Red for 90 s. The sections are dehydrated in alcohols and clarified in xylene, and coverslips are mounted with mounting medium.

The number of cells with positive TUNEL reaction is determined using cellSens Entry programme (Olympus). The documentation of TUNEL reactions is performed with a DP-26 camera (Olympus) coupled with a Nikon Eclipse E600 optical microscope.

Electron microscopy. Two trabeculae: baseline and after hypoxia/reoxygenation, and left ventricle biopsies from 10 remotely preconditioned and 10 “sham” patients.

Tissue specimens are placed in cacodyl buffer with 3% glutaraldehyde and/or in buffered (phosphate buffer, pH 7.2) 3% glutaraldehyde. Glutaraldehyde fixed samples are post-fixed with 1% osmium tetroxide and subsequently dehydrated with a series of ethanol, followed by propylene oxide (room temperature). The samples are embedded in epoxy resin mixture (about 48 h). After polymerisation (72 h) in increasing temperatures (35°C–45°C–60°C), the semi-thin sections are obtained with an ultramicrotome (Reichert, Vienna, Austria) and stained with toluidine blue. Ultra-thin sections are obtained and placed on copper grids and stained with uranyl acetate and lead citrate. The mitochondria are micrographed with a JEOL-JEM 100CX transmission electron microscope (JEOL Inc., Peabody, MA) and with TECNAI™ G2 12 Spirit BioTWIN (FEI, Eindhoven, the Netherlands)

equipped with a Morada CCD camera (Olympus Soft Imaging System Solutions GMBH, Germany) with magnification from 5000× to 20,000×. The electron micrographs are next saved and mitochondria size and structure analysed using cellSens Entry programme (Olympus).

Study objectives

We aim to definitively show if human myocardium can be remotely preconditioned. The study is undertaken to assess:

1. Resistance of isolated right atrial pectinate muscle trabeculae to simulated hypoxia/reperfusion in a functional organ bath model.
2. Resistance of isolated right atrial pectinate muscle trabeculae to induction of apoptosis by simulated hypoxia/reperfusion.
3. Resistance of mitochondria in isolated right atrial pectinate muscle trabeculae to changes induced by simulated hypoxia/reperfusion.

Simultaneously we assess:

1. Amount of myocardial necrosis in-vivo induced by periods of ischaemia and reperfusion during CABG as assessed by postoperative myocardial necrosis marker release profile.
2. Myocardial function in-vivo after the period of ischaemia and reperfusion during CABG as assessed by haemodynamic measurements (thermodilution method), oxygen supply/consumption, and inotropic support requirements.
3. Induction of apoptosis and status of mitochondria after the period of ischaemia, and reperfusion during CABG as assessed in left ventricular myocardial biopsies.

We will correlate the in vitro and in vivo findings from the same patient.

We hypothesise that we will be able to prove the remote preconditioning of human myocardium in in vitro experiments and show to what extent this phenomenon can be translated into clinical practice.

Statistical analysis

The data are collected using a Microsoft Access 2010 database, and statistical analysis will be performed using SigmaPlot 12.5 and IBM SPSS Statistics 22 software. The qualitative patient characteristics will be presented as mean with standard deviation. The frequencies will be expressed as percentages. The outcome data will be presented as an arithmetic mean with 95% confidence interval (CI) if normally distributed and a geometric mean with 95% CI if log-normally distributed. Results will be compared using a t-test. Otherwise the outcomes will be presented as median with quartiles and compared using the Mann-Whitney test. The AUC of troponin T concentration in serum will be calculated according to the trapezoid rule. The results will be log-transformed and compared using a one-way ANOVA. The ratios (with 95% CI)

of remote ischaemic preconditioning to control were obtained by back transformation of the ANOVA results.

The frequencies (e.g. numbers of cells counted on immunohistochemistry) will be compared using χ^2 test or Fisher exact test. Spearman's rank correlation will be used to test the association between ranked variables. In the case of repeated measurements (e.g. haemodynamic data) the two way repeated measures ANOVA will be used with remote preconditioning as one factor and time as another. In the case of some visible differences in preoperative values mixed effect modelling may be used, with the preoperative value as a random effect and the treatment group as a fixed effect.

RESULTS

Recruitment commenced in October 2013 for the 120 participants needed for the trial.

DISCUSSION AND CONCLUSIONS

Remote ischaemic preconditioning is the phenomenon by which short periods of ischaemia and reperfusion in one organ can protect the distant organ from prolonged periods of ischaemia, and subsequent reperfusion [10, 11]. Although it was first described in the 1990s and was shown in multiple species to be a universal phenomenon with systematic protective effects affecting multiple organs, no exact mechanisms have been defined [11, 12]. In many ways, it resembles local ischaemic preconditioning with the same kinases [13] and changes in mitochondrial function [14] being involved; however, the exact nature of signal transduction from remote tissue to target organ remains to be fully elucidated. Both humoral and neuronal pathways have been proposed [15].

The phenomenon, although not fully understood may easily be elicited by upper or lower limb ischaemia and potentially used clinically to protect vital organs from injury. One clinical application is cardiac surgery, which involves planned periods of myocardial ischaemia and reperfusion. Current methods of myocardial protection (cardioplegia) appear to work reasonably well. However, prediction of the requirement for inotropic support and the release of various amounts of myocardial necrosis markers in the postoperative period are difficult and indicate that the protection methods are far from optimal.

The first attempt of remote preconditioning in cardiac surgery occurred in 2006 when it was found to reduce postoperative troponin release in children. Other studies found a similar effect in CABG [6, 7, 16]. These studies were, however, small and underpowered. The initial enthusiasm was shaken when two relatively large studies failed to demonstrate any benefit of remote preconditioning in CABG [17, 18]. The failure was attributed to the fact that the ischaemic insult occurring during CABG is relatively small and with current myocardial protection methods additional protection provided by remote preconditioning

may be difficult to demonstrate. Still, in Rahman et al.'s study [17], the postoperative troponin T release was actually higher in the remote preconditioning group. In September 2013, after the beginning of our study, Thielmans' group published their findings showing that remote ischaemic preconditioning provides perioperative myocardial protection and improves the prognosis of patients undergoing elective CABG surgery [19]. Later, two large randomised trials revealed that remote ischaemic preconditioning did not improve clinical outcomes in patients undergoing elective on-pump cardiopulmonary bypass grafting with or without valve surgery [20, 21]. Meanwhile, no study has ever truly shown that remote preconditioning of human myocardium is at all feasible. Indeed, there are many studies on human myocardium showing that ischaemic preconditioning can be elicited in experimental conditions, and protects against standardised ischaemic and reperfusion insult [22–24]. The same was shown with various forms of pharmacological preconditioning [25–28]. Currently, no experimental study has shown that human myocardium can be remotely preconditioned against standardised ischaemic/hypoxic insult. We are aiming to fill this major knowledge gap by eliciting a remote preconditioning state in the patient, and studying ex vivo the myocardium obtained thereafter.

Strengths and limitations of this study

Currently, no experimental study has shown that human myocardium can be remotely preconditioned against standardised ischaemic/hypoxic insult. We aim to definitively show if human myocardium can be remotely preconditioned. The influence of remote ischaemic preconditioning on heart muscle protection may be, however, too small to be measured with the methods used in our study.

Acknowledgements

We would like to thank Ms. Anna Urdzoń for her superb technical assistance and all of the staff from the Department of Cardiac Surgery for their help.

Funding: *This work is supported by National Science Centre (POLAND) grant [UMO-2012/07/B/NZ5/02549]. The Centre has had and will have no influence on the study design, collection, management, and interpretation of data; writing the report; or submitting the report for publication.*

Conflict of interest: none declared

References

1. Freude B, Masters TN, Robicsek F, et al. Apoptosis is initiated by myocardial ischemia and executed during reperfusion. *J Mol Cell Cardiol.* 2000; 32(2): 197–208, doi: [10.1006/jmcc.1999.1066](https://doi.org/10.1006/jmcc.1999.1066), indexed in Pubmed: [10722797](https://pubmed.ncbi.nlm.nih.gov/10722797/).
2. Yeh CH, Chen TP, Lee CH, et al. Inhibition of poly(adp-ribose) polymerase reduces cardiomyocytic apoptosis after global cardiac arrest under cardiopulmonary bypass. *Shock.* 2006; 25(2):

- 168–175, doi: [10.1097/01.shk.0000188709.04777.48](https://doi.org/10.1097/01.shk.0000188709.04777.48), indexed in Pubmed: [16525356](https://pubmed.ncbi.nlm.nih.gov/16525356/).
3. Thygesen K, Alpert JS, Jaffe AS, et al. Third universal definition of myocardial infarction. *Eur Heart J*. 2012; 33(20): 2551–2567, doi: [10.1093/eurheartj/ehs184](https://doi.org/10.1093/eurheartj/ehs184), indexed in Pubmed: [22922414](https://pubmed.ncbi.nlm.nih.gov/22922414/).
 4. Rahman IA, Mascaro JG, Steeds RP, et al. Remote ischemic preconditioning in human coronary artery bypass surgery: from promise to disappointment? *Circulation*. 2010; 122(11 Suppl): S53–S59, doi: [10.1161/CIRCULATIONAHA.109.926667](https://doi.org/10.1161/CIRCULATIONAHA.109.926667), indexed in Pubmed: [20837926](https://pubmed.ncbi.nlm.nih.gov/20837926/).
 5. Hausenloy DJ, Mwamure PK, Venugopal V, et al. Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. *Lancet*. 2007; 370(9587): 575–579, doi: [10.1016/S0140-6736\(07\)61296-3](https://doi.org/10.1016/S0140-6736(07)61296-3), indexed in Pubmed: [17707752](https://pubmed.ncbi.nlm.nih.gov/17707752/).
 6. Venugopal V, Hausenloy DJ, Ludman A, et al. Remote ischaemic preconditioning reduces myocardial injury in patients undergoing cardiac surgery with cold-blood cardioplegia: a randomised controlled trial. *Heart*. 2009; 95(19): 1567–1571, doi: [10.1136/hrt.2008.155770](https://doi.org/10.1136/hrt.2008.155770), indexed in Pubmed: [19508973](https://pubmed.ncbi.nlm.nih.gov/19508973/).
 7. Thielmann M, Kottenberg E, Boengler K, et al. Remote ischemic preconditioning reduces myocardial injury after coronary artery bypass surgery with crystalloid cardioplegic arrest. *Basic Res Cardiol*. 2010; 105(5): 657–664, doi: [10.1007/s00395-010-0104-5](https://doi.org/10.1007/s00395-010-0104-5), indexed in Pubmed: [20495811](https://pubmed.ncbi.nlm.nih.gov/20495811/).
 8. Deja MA, Golba KS, Kolowca M, et al. Diazoxide provides protection to human myocardium in vitro that is concentration dependent. *Ann Thorac Surg*. 2004; 77(1): 226–232, doi: [10.1016/S0003-4975\(03\)01295-5](https://doi.org/10.1016/S0003-4975(03)01295-5), indexed in Pubmed: [14726066](https://pubmed.ncbi.nlm.nih.gov/14726066/).
 9. Deja MA, Golba KS, Malinowski M, et al. Diazoxide provides maximal KATP channels independent protection if present throughout hypoxia. *Ann Thorac Surg*. 2006; 81(4): 1408–1416, doi: [10.1016/j.athoracsur.2005.11.045](https://doi.org/10.1016/j.athoracsur.2005.11.045), indexed in Pubmed: [16564282](https://pubmed.ncbi.nlm.nih.gov/16564282/).
 10. Ali ZA, Callaghan CJ, Lim E, et al. Remote ischemic preconditioning reduces myocardial and renal injury after elective abdominal aortic aneurysm repair: a randomized controlled trial. *Circulation*. 2007; 116(11 Suppl): 198–105, doi: [10.1161/circulationaha.106.679167](https://doi.org/10.1161/circulationaha.106.679167), indexed in Pubmed: [17846333](https://pubmed.ncbi.nlm.nih.gov/17846333/).
 11. Kharbanda RK, Nielsen TT, Redington AN. Translation of remote ischaemic preconditioning into clinical practice. *Lancet*. 2009; 374(9700): 1557–1565, doi: [10.1016/S0140-6736\(09\)61421-5](https://doi.org/10.1016/S0140-6736(09)61421-5), indexed in Pubmed: [19880021](https://pubmed.ncbi.nlm.nih.gov/19880021/).
 12. Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev*. 2003; 83(4): 1113–1151, doi: [10.1152/physrev.00009.2003](https://doi.org/10.1152/physrev.00009.2003), indexed in Pubmed: [14506302](https://pubmed.ncbi.nlm.nih.gov/14506302/).
 13. Weinbrenner C, Nelles M, Herzog N, et al. Remote preconditioning by infrarenal occlusion of the aorta protects the heart from infarction: a newly identified nonneuronal but PKC-dependent pathway. *Cardiovasc Res*. 2001; 55: 590–601, doi: [10.1016/j.cardiores.2003.10.008](https://doi.org/10.1016/j.cardiores.2003.10.008).
 14. Kristiansen SB, Henning O, Kharbanda RK, et al. Remote preconditioning reduces ischemic injury in the explanted heart by a KATP channel-dependent mechanism. *Am J Physiol Heart Circ Physiol*. 2005; 288(3): H1252–H1256, doi: [10.1152/ajp-heart.00207.2004](https://doi.org/10.1152/ajp-heart.00207.2004), indexed in Pubmed: [15498829](https://pubmed.ncbi.nlm.nih.gov/15498829/).
 15. Pickard MJ, Bøtker HE, Crimi G, et al. Remote ischemic conditioning: from experimental observation to clinical application: report from the 8th Biennial Hatter Cardiovascular Institute Workshop. *Basic Res Cardiol*. 2015; 110(1): 453, doi: [10.1007/s00395-014-0453-6](https://doi.org/10.1007/s00395-014-0453-6), indexed in Pubmed: [25449895](https://pubmed.ncbi.nlm.nih.gov/25449895/).
 16. Liu X, Yi S, Liao B, et al. Clinical study of limb ischemic preconditioning on myocardial ischemia-reperfusion injury during open heart surgery. *Chinese J Cardiovas Rehab Med*. 2007; 16: 347–349.
 17. Rahman IA, Mascaro JG, Steeds RP, et al. Remote ischemic preconditioning in human coronary artery bypass surgery: from promise to disappointment? *Circulation*. 2010; 122(11 Suppl): S53–S59, doi: [10.1161/CIRCULATIONAHA.109.926667](https://doi.org/10.1161/CIRCULATIONAHA.109.926667), indexed in Pubmed: [20837926](https://pubmed.ncbi.nlm.nih.gov/20837926/).
 18. Karuppasamy P, Chaubey S, Dew T, et al. Remote intermittent ischemia before coronary artery bypass graft surgery: a strategy to reduce injury and inflammation? *Basic Res Cardiol*. 2011; 106(4): 511–519, doi: [10.1007/s00395-011-0185-9](https://doi.org/10.1007/s00395-011-0185-9), indexed in Pubmed: [21544683](https://pubmed.ncbi.nlm.nih.gov/21544683/).
 19. Thielmann M, Kottenberg E, Kleinbongard P, et al. Cardioprotective and prognostic effects of remote ischaemic preconditioning in patients undergoing coronary artery bypass surgery: a single-centre randomised, double-blind, controlled trial. *Lancet*. 2013; 382(9892): 597–604, doi: [10.1016/S0140-6736\(13\)61450-6](https://doi.org/10.1016/S0140-6736(13)61450-6), indexed in Pubmed: [23953384](https://pubmed.ncbi.nlm.nih.gov/23953384/).
 20. Meybohm P, Bein B, Brosteanu O, et al. A multicenter trial of remote ischemic preconditioning for heart surgery. *N Engl J Med*. 2015; 373(15): 1397–1407, doi: [10.1056/NEJMoa1413579](https://doi.org/10.1056/NEJMoa1413579), indexed in Pubmed: [26436208](https://pubmed.ncbi.nlm.nih.gov/26436208/).
 21. Hausenloy DJ, Candilio L, Evans R, et al. ERICCA Trial Investigators. Remote Ischemic Preconditioning and Outcomes of Cardiac Surgery. *N Engl J Med*. 2015; 373(15): 1408–1417, doi: [10.1056/NEJMoa1413534](https://doi.org/10.1056/NEJMoa1413534), indexed in Pubmed: [26436207](https://pubmed.ncbi.nlm.nih.gov/26436207/).
 22. Lu EX, Chen SX, Yuan MD, et al. Preconditioning Improves Myocardial Preservation in Patients Undergoing Open Heart Operations. *Ann Thoracic Surg*. 1997; 64(5): 1320–1324, doi: [10.1016/s0003-4975\(97\)00838-2](https://doi.org/10.1016/s0003-4975(97)00838-2).
 23. Wu ZK, Tarkka MR, Pehkonen E, et al. Ischaemic preconditioning has a beneficial effect on left ventricular haemodynamic function after a coronary artery biopass grafting operation. *Scand Cardiovasc J*. 2000; 34(3): 247–253, indexed in Pubmed: [10935770](https://pubmed.ncbi.nlm.nih.gov/10935770/).
 24. Teoh LK, Grant R, Hulf JA. A comparison between ischemic preconditioning, intermittent cross-clamp fibrillation and cold crystalloid cardioplegia for myocardial protection during coronary artery bypass graft surgery. *Cardiovasc Surg*. 2002; 53: 251–255, doi: [10.1177/096721090201000312](https://doi.org/10.1177/096721090201000312).
 25. Mentzer R, Birjiniuk V, Khuri S, et al. Adenosine Myocardial Protection. *Annals of Surgery*. 1999; 229(5): 643, doi: [10.1097/00000658-199905000-00006](https://doi.org/10.1097/00000658-199905000-00006).
 26. Wei M, Wang X, Kuukasjärvi P, et al. Bradykinin preconditioning in coronary artery bypass grafting. *Ann Thorac Surg*. 2004; 78(2): 492–497, doi: [10.1016/j.athoracsur.2003.11.039](https://doi.org/10.1016/j.athoracsur.2003.11.039), indexed in Pubmed: [15276504](https://pubmed.ncbi.nlm.nih.gov/15276504/).
 27. Mangano DT, Miao Y, Tudor IC, et al. Post-reperfusion myocardial infarction: long-term survival improvement using adenosine regulation with acadesine. *J Am Coll Cardiol*. 2006; 48(1): 206–214, doi: [10.1016/j.jacc.2006.04.044](https://doi.org/10.1016/j.jacc.2006.04.044), indexed in Pubmed: [16814669](https://pubmed.ncbi.nlm.nih.gov/16814669/).
 28. Deja MA, Malinowski M, Golba KS, et al. Diazoxide protects myocardial mitochondria, metabolism, and function during cardiac surgery: a double-blind randomized feasibility study of diazoxide-supplemented cardioplegia. *J Thorac Cardiovasc Surg*. 2009; 137(4): 997–1004, doi: [10.1016/j.jtcvs.2008.08.068](https://doi.org/10.1016/j.jtcvs.2008.08.068), indexed in Pubmed: [19327530](https://pubmed.ncbi.nlm.nih.gov/19327530/).

Cite this article as: Deja MA, Wiaderkiewicz R, Czekaj P, et al. Remote Ischaemic PreConditioning of Human Myocardium (RIPE): study protocol for a double-blinded randomised controlled trial. *Kardiologia Polska*. 2018; 76(1): 136–143, doi: [10.5603/KP.a2017.0180](https://doi.org/10.5603/KP.a2017.0180).