Vascular conditioning prevents adverse left ventricular remodelling after acute myocardial infarction: a randomised remote conditioning study

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Abstract

Aims: Remote ischemic conditioning (RIC) alleviates ischemia-reperfusion injury via several pathways, including micro-RNAs (miRs) expression and oxidative stress modulation. We investigated the effects of RIC on endothelial glycocalyx, arterial stiffness, LV remodelling, and the underlying mediators within the vasculature as a target for protection. **Methods & Results:** We block-randomised 270 patients within 48h of STEMI post-PCI to either one or two cycles of bilateral brachial cuff inflation, and a control group without RIC. We measured: a) the perfusion boundary region (PBR) of the sublingual arterial microvessels to assess glycocalyx integrity; b) the carotid-femoral pulse wave velocity (PWV); c) miR-144,-150,-21,-208, nitrate-nitrite (NOx) and malondialdehyde (MDA) plasma levels at baseline (T0) and 40 minutes after RIC onset (T3); and d) LV volumes at baseline and after one year. Compared to baseline, there was a greater PBR and PWV decrease, miR-144 and NOx levels increase ($p<0.05$) at T3 following single- than double-cycle inflation (PBR:ΔT0-T3=0.249±0.033 vs 0.126±0.034 μm, p=0.03 and PWV:0.4±0.21 vs -1.02±0.24 m/s, p=0.03). Increased miR-150,-21,-208 ($p<0.05$) and reduced MDA was observed after both protocols. Increased miR-144 was related with PWV reduction ($r=0.763$, $p<0.001$) after the first-cycle inflation in both protocols. After one year, single-cycle RIC was associated with LV end-systolic volume reduction (LVESV) >15% (odds-ratio of 3.75,p=0.029). miR-144 and PWV changes post-RIC were interrelated and associated with LVESV reduction at follow-up ($r=0.40$ and 0.37, $p<0.05$), in the single cycle RIC. **Conclusion:** RIC evokes "vascular conditioning" likely by upregulation of cardio-protective microRNAs, NOx production, and oxidative stress reduction, facilitating reverse LV remodelling.

Clinical Trial Registration: http://www.clinicaltrials.gov. Unique identifier: NCT03984123

Key words: ischemia-reperfusion injury; remote conditioning; arterial stiffness; oxidative stress; endothelial glycocalyx; remodelling

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Conflicts of interest/Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval; **consent to participate**

The study was approved by the University General Hospital "Attikon" Institutional Review Board, conforms to the principles outlined in the Declaration of Helsinki, and is registered at the US National Institutes of Health (ClinicalTrials.gov: #NCT03984123). In addition, all participants gave their written informed consent.

Consent for publication

Not applicable

Availability of data and material

The datasets generated during and/or analysed during the current study are not publicly available due to confidentiality reasons but are available from the corresponding author on reasonable request.

Code availability

Not applicable

Authors' contributions

II: Analysis and interpretation of data, critical content revision, and final version approval; DV: Analysis and interpretation of data, drafting and critical content revision, and final version approval; IA: Acquisition of biochemical data, Critical content revision, final version approval; MG: Acquisition of biochemical data; PE: Acquisition of biochemical data; MV: Acquisition of vascular function data; GM: Acquisition of echocardiography data; AK: Acquisition of biochemical data; JL: Critical content revision; JP: Critical content revision; SK: Acquisition of echocardiography data; DT: Acquisition of echocardiography data; DJH: Critical content revision; DA: Critical content revision; DVC: Critical content revision; H-EB: Critical content revision; EKI: Study design, interpretation of data, critical content revision, and final version approval.

Introduction

Ischemia-reperfusion injury (IRI) may limit the beneficial effects of primary percutaneous coronary intervention (PCI) on myocardial salvage in patients with ST-elevation myocardial infarction (STEMI) [21]. Remote ischemic conditioning (RIC), with application of brief episodes of ischemia and reperfusion in vascular beds distant to the organ at risk, activates a protective phenotype against IRI [17]. It confers reduced myocardial infarct size and improved myocardial salvage index, decreases the need for pharmacological and mechanical haemodynamic support, and induces superior recovery of left ventricular (LV) systolic function after STEMI [11], with mixed evidence regarding its impact on cardiac mortality and hospitalisation for heart failure [11, 15]. In addition, seven-day RIC improves local and systemic endothelial function and microcirculation in healthy humans [32]. However, the role of RIC on vascular function in STEMI post-PCI patients and the mechanism of its possible protective action has not been evaluated.

Endothelial glycocalyx consists of glycoproteins and proteoglycans that form a surface layer, preventing the direct contact between blood cells and vascular endothelium [44]. It is damaged after exposure to atherogenic risk factors, including hyperglycaemia, dyslipidaemia, hypertension, and smoking [36], and by IRI [20], contributing to coronary microvascular injury (resulting in oedema, vasomotion impairment, coronary microembolization, capillary destruction and haemorrhage). In turn, coronary microvascular injury adversely affects ventricular function and remodelling, and is associated with increased incidence of cardiovascular complications and mortality [20]. Increased oxidative stress appears to play an important role, since reactive oxygen species (ROS) induce an acute but reversible impairment of glycocalyx structure [51]. In addition to the coronary circulation, endothelial function of the peripheral arteries is also impaired following AMI with the maximal disturbance observed during the first 24 to 72 hours post AMI [2,12], while its assessment within 24 hours of the index event has been shown to predict infarct extension and adverse LV remodelling [2]. Furthermore, endothelial glycocalyx impairment assessed by sublingual microscopy has been associated with microvascular angina, providing additional evidence that the properties of the peripheral arterial system may reflect the state of the coronary microvasculature [31]. In the context of RIC investigation, this method of endothelial glycocalyx integrity measurement might be preferable to assessment of endothelial function using measurement of the flow-mediated dilation (FMD) of the brachial artery. This is because it obviates the need for an additional ischemia-hyperaemia cycle provoked by the extra cuff inflation needed for the FMD study, which would confound the total ischemic burden and stimulus timing of our intervention. Moreover, endothelial glycocalyx integrity is a measure

of vascular permeability, while FMD measurement mainly quantifies the capability of NO production by the endothelium. Although RIC has been demonstrated to improve peripheral [17] and coronary endothelial function [19,43] and to reduce the oxidative stress burden associated with IRI [40], its effects on glycocalyx properties have not been defined.

Following AMI, a non-contractile and expanding infarcted zone of scar tissue is formed. This expansion leads to an increased volume load, which in turn augments the pressure load exerted on non-infarcted regions resulting in adverse LV remodelling. Long-term LV remodelling after AMI may last for up to 2 years after the index event and is associated with cardiovascular mortality [18]. Endothelial dysfunction [2], NO bioavailability, and oxidative stress are recognised among the factors contributing to its progression [5]. Nitrate (NO_3^-) and nitrite (NO_2^-) have recently been shown to function as recycling substrates in a process of NO regeneration, which is independent of the classic L-arginine-NO-synthase (NOS) pathway [47]. Thus, the nitrate-nitrite (NOx) pool could be perceived as a reservoir of NO bioactivity that complements NOS in states of low-oxygen tension, such as during AMI and could further contribute to vascular protection and myocardial protection post-RIC [6].

RIC is associated with arterial stiffness alleviation in patients with stable ischemic heart disease [61]. Increased arterial stiffness augments LV afterload and decreases diastolic coronary perfusion [56], reducing oxygen supply to demand ratio. Thus, arterial stiffening may contribute to adverse LV remodelling and thus, to poor prognosis post-AMI [9]. However, the effects of RIC on arterial elastic properties and their interaction with LV remodelling in AMI patients remain unclear.

MicroRNAs (MiRs) are small, single stranded, non-coding RNA molecules that regulate post-transcriptional gene expression in response to cellular or environmental stimuli [60]. MicroRNA-144 (miR-144) has been recognised as an important mediator [39] implicated in RIC signalling both in vascular and myocardial cells. Moreover, miR-150 inhibits apoptosis and fibrosis in the setting of animal models of myocardial IRI [53]. Additionally, miR-21 has been demonstrated to mediate cardio-protection in coronary artery bypass graft (CABG) patients undergoing remote ischemic conditioning [10]. On the contrary, miR-208 exerts deleterious effects by way of hypertrophy and adverse remodelling induction [45]. However, the effects of RIC on miRs involved in cardiac and vascular function are not fully investigated in the clinical setting.

Oxidative stress enhancement constitutes an important component of IRI [4]. Biomembrane polyunsaturated lipid peroxidation by ROS generated during abrupt reperfusion generates malondialdehyde (MDA) [4]; indeed, increased MDA levels have been reported following PCI and thrombolysis for STEMI [40].

Studies in patients with systemic inflammatory disease have linked increased MDA with impaired LV function and its reduction after treatment with a concomitant LV function improvement [25]. In this respect, RIC reduces lipid peroxidation with attendant decreased MDA levels [40], and thus may induce improvement of myocardial function post-PCI in STEMI. In addition, IRI evokes a systemic inflammatory response with significantly increased IL-6 levels [49]. This results in enhanced neutrophil adherence to the cardiovascular endothelium, with deleterious effects [49]. Similarly, IL-6 pathway appears to mediate vascular inflammation in various disease processes [1]. RIC has been reported to decrease IL-6 levels in animal models of systemic inflammation and ischemia [34], but its effects on IL6 production post-STEMI have not been clearly defined.

Based on the above observations, the aim of the present study was to determine the "vascular conditioning" potential of RIC by investigating its effects on endothelial glycocalyx, arterial stiffness, and oxidative stress burden after primary PCI, as well as to identify the role of specific miRs , NOx and IL-6 production on vascular function early post MI. With regards to the implemented protocols, there is evidence supporting that a total ischemic period of 5 to 10 minutes may confer the optimum ischemic conditioning stimulus [3, 35], while 5 minutes of ischemic inflation induce maximal shear-mediated NO release and vasodilation in FMD studies [14]. Additionally, a single 5-minute inflation-deflation cycle has been demonstrated to confer increased nitrite levels and attendant cyto-protection, both of which progressively weaned following each additional ischemic cycle [7]. Based on the above, we utilised either a single or a double 5-minute cycle ischemic inflation to explore the potential effects of each RIC protocol on "vascular conditioning". Additionally, we intended to shed light on any possible contribution of "vascular conditioning" to long-term reverse LV remodelling.

Methods

Study design and population

The present study was a prospective, randomized trial conducted at the Second Department of Cardiology in Attikon University Hospital, which entailed an acute and a chronic phase. Two hundred seventy patients with STEMI after primary PCI (mean age 53±16 years, 84% male) were recruited and underwent block randomisation (block size 9) to either one (single-cycle, n=90), or two 5-min cycles of bilateral brachial cuff inflations, separated by 5 minutes (double-cycle, n=90), or no cuff inflation (control group, n=90) added to standard care. Randomisation was assigned to a team member who was unmasked to group allocation and was performed via a website generator (Sealed Envelope, London, UK). Data collection and outcome assessment was performed only

by members blinded to group allocation. We chose to apply RIC within 48 h after primary PCI (36±12h), in an effort to induce "vascular conditioning" during the period of maximum endothelial dysfunction [2]. Our first protocol utilized two ischemic stimuli by bilateral brachial cuff inflation at 200 mmHg for 5 minutes [35], separated by 5 minutes, after a baseline vascular function assessment (T0). Each ischemic stimulus was followed by a vascular function assessment (T1, T2), with a final assessment 25 minutes after the second cuff deflation (T3). The second protocol was identical to the first, except for the second ischemic stimulus omission; thus, the total ischemic stimulus was either 10 (double-inflation) or 5 minutes (single inflation). Both protocols were preceded by a sham procedure, by way of cuff placement around the ordinary brachial position without inflation. Blood samples were drawn at baseline (T0) and at protocol termination (T3). All patients were in sinus rhythm, while exclusion criteria included age>85 years, cardiogenic shock or Killip class>2 during the index event, administration of nitrates, history of previous known coronary artery or other cardiovascular disease, previous PCI or coronary artery bypass surgery (CABG), as well chronic inflammatory, systemic, or malignant disease. All patients were scheduled for 2-dimensional echocardiography examination at 12 months after the index hospitalisation to assess the extent of LV remodelling. The study was approved by the University General Hospital "Attikon" Institutional Review Board, conforms to the principles outlined in the Declaration of Helsinki, and is registered at the US National Institutes of Health (ClinicalTrials.gov: #NCT03984123). In addition, all participants gave their written informed consent.

Endothelial glycocalyx assessment

The perfusion boundary region (PBR) is the cell-poor layer that results from the phase separation of flowing red blood cells (RBC) and plasma on the microvessel luminal surface. It includes the glycocalyx component that allows cell penetration. An increased PBR is consistent with deeper penetration of erythrocytes into the glycocalyx, reflecting impairment of glycocalyx barrier properties and reduced glycocalyx thickness [44]; hence, it represents a standardized, reproducible, operator-independent method for assessment of arterial endothelium glycocalyx properties [44]. We measured the PBR of the sublingual arterial microvasculature (diameter span from 5 to 25 μm) using Sidestream Darkfield imaging (Microscan, Glycocheck, Microvascular Health Solutions Inc., Salt Lake City, UT, USA; figure 2).

Arterial stiffness assessment

Arterial stiffness was assessed by carotid-femoral pulse wave velocity (PWV) [42] using arterial tonometry (Complior, Alam Medical, Vincennes, France; normal values <10 m/s [42]). PWV was calculated as the distance

between the carotid and femoral arterial pulse palpation site, divided by the respective transit time (m/s). All measurements were performed by the same blinded examiner (intra-observer variability=5%).

Oxidative stress and inflammatory biomarkers

Malondialdehyde (MDA) was determined spectrophotometrically with a commercial kit (Oxford Biomedical Research, Rochester Hills, Mich, colorimetric assay for lipid peroxidation; measurement range 1-20 nmol/L; 3.39% and 4.75% intra-assay and inter-assay variability respectively) [27]. IL-6 was measured by a high-sensitivity immunoassay [human IL-6 Quantikinine (high sensitivity)] that detects values as low as 0.094 (intra-assay variability <5%) [24].

Plasma microRNA levels

Serum miRNAs were obtained from samples using the NucleoSpin miRNA Plasma Kit (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany**)** according to instructions of the manufacturer (Supplementary file 1).

Nitrate-nitrite-nitric oxide pathway

The concentration of nitrate/nitrite in blood plasma was determined using Griess reaction with a commercially available kit (Cayman's Nitrate/Nitrite Colorimetric Assay Kit 780001) (Supplementary file 1).

LV remodelling

LV remodelling was assessed by way of two-dimensional echocardiography, using a Vivid 7 or E95 (GE Medical Systems, Horten, Norway) phased array ultrasound system. All studies were digitally stored and analysed by two blinded observers, using a computerised station (Echopac 202 GE, Horten, Norway). LV end-diastolic (LVEDV) and end-systolic volumes (LVESV) were calculated from four-and two-chamber views using the modified Simpson biplane method within 48 hours post PCI before RIC and after 1 year. A cut-off of >15% decrease in LVESV was implemented as a criterium of reverse LV remodelling, as this constitutes a validated reverse remodelling marker in the context of ischemic cardiomyopathy [22, 58].

Statistical analysis

Power analysis

In a pilot study of 30 STEMI patients who underwent single-cycle, double-cycle, or no cuff inflation RIC protocol (1:1:1), the response within each subject group for ΔPWV (T0-T3) was normally distributed with a standard deviation of 1 and the calculated effect size was 0.13 with a correlation among repeated measures of 0.2. Thus,

we would need 80 patients in each group to reject the null hypothesis that the population means of the single-cycle, double-cycle, and no cuff inflation groups are equal with a probability (power) of 0.8. The Type I error probability associated with the test of this null hypothesis is 0.05 (ANOVA, repeated measures, between factors, G*Power version 3.1.9.6, University of Kiel, Germany). Assuming a 10% loss of patients during follow up and 5% poor echocardiography images, we decided to include 90 patients in each group.

STATA v.11 and SPSS v.22 were used to analyse the data. The Shapiro-Wilk test was used to examine whether the data were normally distributed, whereas the Levene test was used to examine the homoscedasticity of the data. All non-parametric variables were compared using the Wilcoxon test for comparisons between baseline and post-intervention values and were transformed into ranks for multivariate analysis. In all analyses, we used two tailed tests with $p<0.05$. We used parametric (Pearson r) and non-parametric (Spearman rho) correlation coefficients to examine cross-sectional associations. Analysis of variance (ANOVA) for clinical and biological data was performed to test the differences among groups and all non-parametric variables were transformed into ranks before entering the analysis using a previously published methodology [27]. Two-way ANOVA (general linear model, SPSS 22, SPSS Inc, Chicago, Ill) for repeated measurements was applied on the examined vascular function and biochemical markers (at T0, T1, T2, and T3 for the vascular markers and T0 and T3 for biomarkers) with the parameter of time used as a within-subject factor and the applied protocol, age, sex, BMI, dyslipidaemia, diabetes, hypertension, concomitant medical treatments, MI location, and number of diseased coronary vessels (>70% stenosis) used as between-subject factors; ANCOVA (analysis of covariance) was applied to investigate the effect of the baseline values of the investigated marker and myocardial enzyme elevation. The Greenhouse-Geisser correction was used when the sphericity assumption, as assessed by Mauchly's test, was not met. Post hoc comparisons were performed with Bonferroni correction. A p-value of <0.05 was considered as statistically significant. Inter- and intra-observer variabilities (%) of vascular and biochemical markers were calculated as the SD of the differences between the first and second measurements, and expressed as a percentage of the average value in 30 healthy volunteers. Logistic regression analysis using the presence of LV remodelling at 1-year follow-up as the dependent variable and the application or not of RIC as the independent variable was performed. Similar to the acute phase measurements, ANOVA and ANCOVA were used to examine the effects of age, sex, BMI, dyslipidaemia, diabetes, hypertension, concomitant medical treatments, MI location, myocardial enzymes, number of diseased coronary vessels (>70% stenosis), and baseline values.

RESULTS

Study population characteristics

Out of the 270 patients, 126 (47%) suffered from an anterior STEMI, 119 (44%) had single-vessel, 123 (45%) two-vessel, and 28 (11%) three-vessel CAD. Median high-sensitivity (hs)-troponin T was 3886 [807-9779] ng/mL. Patients undergoing RIC had similar clinical characteristics to the control patients ($p>0.05$, Table1). All except three single-cycle, two double-cycle inflation, and 3 control patients had achieved TIMI 3 flow by PCI at angiographic reperfusion assessment. All were free from angina, arrhythmias, and any significant ECG change during PCI. There were no differences in the angiographic or biochemical characteristic of STEMI, time from onset of symptoms to hospital admission, and time from admission to reperfusion (total ischemic time) between the groups assigned to single-, double-cycle inflation, or no RIC (Table 1).

Medical treatment included antiplatelet therapy (aspirin and clopidogrel) and anticoagulation (enoxaparin). Additional bolus infusions of unfractionated heparin (UFH) were given during PCI. β-blockers, ACE inhibitors, and statins were given to all patients. No difference was noted between the drug therapy given to the study groups (Table 1). A participant flow diagram has been submitted as Figure S2.

Glycocalyx barrier properties

Compared to baseline, all patients had decreased PBR at T2 and T3 ($p<0.05$, Figure 3, Table 2). By ANOVA there was a statistically significant interaction between the changes of PBR and the RIC protocol ($p=0.03$), suggesting that the magnitude of PBR changes along time was different between the 2 protocols and controls. The single-cycle inflation group achieved a greater improvement of PBR than the double-cycle inflation group at termination of the protocol (T3) ($\Delta T1=-0.259\pm0.031$ vs -0.3 ± 0.029 μm, $p=0.7$; $\Delta T2=-0.245\pm0.025$ vs -0.149 ± 0.02 μm, $p=0.04$; $\Delta T3=-0.249\pm0.033$ vs -0.126 ± 0.034 μm, $p=0.03$, for the single versus the double-cycle inflation protocol, respectively). A greater improvement of glycocalyx properties was observed after the first inflation cycle at T1 in patients with baseline PBR> 2.1 μm ($n=50$; mean difference in PBR improvement=0.5 ± 0.03 μm at T1, $p<0.001$). No changes in PBR were induced by sham inflation ($p=0.7$, data not shown). No changes in PBR were observed in the control group of no inflation ($p=0.9$, Table 2).

Biochemical markers

There was no statistically significant difference in the baseline MDA, NOx, or IL-6 levels among the studied groups ($p>0.05$). Compared to baseline, MDA was significantly reduced at T3 ($p<0.001$) in both protocols ($\Delta MDA=-0.49\pm0.29$ vs -0.48 ± 0.21 nmol/L, $p=0.9$, for the single- versus the double-inflation protocol, respectively) (Figure 4). Additionally, the single-inflation protocol promoted an increase in NOx levels, in contrast to the double-inflation protocol, which resulted in reduced NOx levels at protocol termination (T3) ($\Delta NOx=$

2.85±0.81 μmol/l vs -1.88±0.62 μmol/l, p= 0.01, for the single- versus the double-inflation protocol, respectively). IL-6 levels were not affected by any intervention (p>0.05). No changes in the examined biomarkers were observed following sham (p>0.05, data not shown) or no inflation (p>0.05, Table 3).

MiRs

Compared to baseline, all patients had increased Micro-RNA plasma concentration post-RIC (p<0.05, Table 3). There was a significantly greater increase in miR-144 concentration following the single- compared to the double-inflation protocol (Δmir144= 48.5±15.3 vs 32.3±12.1 /U6sn, p=0.02, for the single- versus the double-cycle protocol, respectively). The increase in miR-144 levels correlated with PWV reduction measured 5 minutes following the first cuff deflation in both protocols (T1; r=0.763, p<0.001). Both RIC protocols induced a similar increase in miR -150, -21, and -208 levels compared to baseline (p<0.05, Table 3) (Δmir-150=1.6±0.4 vs 1.5±0.5 /U6sn, p=0.9; Δmir-21=0.9±0.3 vs 0.9±0.4 /U6sn, p=0.99; and Δmir-208=0.5±0.2 vs 0.37±0.19 /U6sn, p=0.6, for the single- versus the double-cycle protocol, respectively) (Table 3, Figure 5). No changes in miRs were observed in the control group (p=0.9, Table 3).

Arterial stiffness

PWV was significantly affected by our intervention (p<0.05; table 2, figure 4). By ANOVA, there was a statistically significant interaction between PWV changes and the RIC protocol exploited (p=0.03). Compared to baseline, ΔT1=-0.55±0.19 vs -0.49±0.17 m/s, p=0.7; ΔT2=-0.7±0.2 vs -0.69±0.21 m/s, p=0.9; ΔT3: -0.4±0.21 vs +1.02±0.24 m/s, p=0.03, for the single- versus the double-inflation protocol, respectively (Figure 6). Thus, there was a decrease of PWV at T1 and T2 in both protocols (P<0.05), but at protocol termination (T3, 25 min after the second inflation) there was as a net PWV decrease in the single- compared to an increase in the double-inflation group. Regardless of the protocol used, patients with baseline PWV >11 m/s benefited from a larger aortic elasticity improvement than patients with lower baseline PWV after the first inflation cycle (T1: mean difference in PWV improvement= 3.5±0.6 m/s, p< 0.002). No changes were observed following sham (p=0.7, data not shown) or no inflation (p=0.8, Table 3).

LV remodelling

We assessed every patient at 1-year follow-up be echocardiography and compared the LV volumes changes in the single- and double-cycle group with the respective changes in the control group. Out of 180 patients who underwent RIC, 85 of the single-cycle group and 87 of the double-cycle group were found for follow–up

echocardiography and were compared with 85 patients without RIC. Single-cycle was associated with a significantly greater decrease in LVEDV and LVESV within 12 months of the index event compared with double- and no inflation (ΔLVEDV= -23±3 vs -7±2 vs -6±2 ml, p<0.001; ΔLVESV: -10±2 vs -3±1 vs -2±1, p<0.001, respectively; table 4, figure 7). By binary logistic regression, single-inflation RIC was related to reverse LV remodelling (LVESV change>15%) with an odds ratio of 3.75 (95% CI: 1.120-8.675, p=0.03), after adjusting for patient age, sex, BMI, dyslipidaemia, diabetes, hypertension, concomitant medical treatments, MI location, myocardial enzymes, number of diseased coronary vessels (>70% stenosis), and baseline values of the LVEDV and LVESV. Interestingly, within this group, the increase in miR-144 post-RIC was significantly correlated with the respective decrease in LVESV (r=-0.40, p=0.001). Additionally, compared to baseline, the reduction of PWV at protocol termination (PWV T0-PWV T3) in the single-inflation group was related with the respective LVESV reduction at follow-up (r=0.37, p=0.002), after adjustment for age, sex, BMI, dyslipidaemia, diabetes, hypertension, concomitant medical treatments, MI location, myocardial enzymes, number of diseased coronary vessels (>70% stenosis), and baseline PWV.

Discussion

In this study, we have shown that RIC with a single 5 min cycle of bilateral brachial cuff inflation conferred improvement of endothelial glycocalyx properties and reduction of aortic stiffness, at 5 and 35 minutes post-inflation in STEMI patients. This improvement in vascular function was in parallel with upregulation of protective miRs, namely miR-144, -150, and -21, oxidative stress burden reduction, and increase in NOx levels. More specifically, increased miR-144 concentration was closely associated with PWV improvement after RIC. These changes were not evident in the control group without cuff inflation. Single-cycle protocol was demonstrated to be superior regarding improvement of endothelial glycocalyx properties, miR-144 levels, aortic stiffness reduction, and increased NO bioavailability. Furthermore, the RIC-induced increase of miR-144 levels and improvement of PWV in the early phase of AMI were interrelated and both associated with a greater decrease of LVESV at 1 year of follow-up. Moreover, RIC by a single cuff inflation cycle was associated with a 3-fold higher probability of reverse LV remodelling within 12 months of the index event compared to the double-cycle or no RIC.

IRI induces coronary microcirculation injury with endothelial glycocalyx shedding, resulting in myocardial oedema, resistant vasoconstriction, platelet-leukocyte aggregation, coronary microembolization, and capillary destruction [17,19,20]; peripheral endothelial function is similarly affected [17]. RIC has been

demonstrated to ameliorate coronary endothelial dysfunction, reducing myocardial oedema and infarct size [19,20,43], as well as peripheral endothelial dysfunction, preserving flow-mediated dilation [17]. Interestingly, coronary microvascular dysfunction is reflected on the peripheral endothelial glycocalyx impairment as assessed by sublingual microscopy [31]. Further expanding this notion, our study is the first in our knowledge to provide direct evidence of improved endothelial glycocalyx properties in patients undergoing RIC after primary PCI. This could be at least partially mediated by the attendant oxidative stress alleviation, which has been shown to cause a rapid glycocalyx cadherin externalisation and gap junction restoration [51], normalising glycocalyx permeability.

Nitrate-nitrite-NO pathway has been demonstrated to mediate myocardial protection from IRI by modulating mitochondrial membrane electron transfer and inhibiting apoptosis [47]; RIC increases circulating nitrite levels in both human and animal models of IRI [47]. Interestingly, one 5-minute inflation-deflation cycle has been found to induce superior levels of plasma nitrite and associated cyto-protection compared with multiple cycles, with progressively diminished effects after every successive ischemic stimulus [7]. In agreement with the above-mentioned findings, our single-cycle intervention conferred increased nitrate and nitrite levels; on the contrary, the double-cycle protocol caused a net decrease in the measured concentrations, suggesting a possible consumption of the NOx pool by the ischemic insult of the second inflation-deflation (T2) cycle.

Enhanced oxidative stress with attendant membrane phospholipid peroxidation plays a major role in IRI and MDA constitutes a breakdown product of lipid peroxide β-cleavage [4]. Indeed, increased MDA levels have been reported following thrombolysis and PCI for STEMI [40]. In this respect, RIC has been demonstrated to ameliorate oxidative stress and reduce MDA concentration following PCI [40]. In agreement with this, our intervention resulted in MDA levels reduction, irrespective of the protocol applied. Moreover, IRI evokes a systemic inflammatory response with significantly increased IL-6 levels. Interestingly, myocytes produce increased IL-6 levels in response to hypoxia, resulting in enhanced neutrophil adherence to the cardiovascular endothelium with deleterious effects [49]. Similarly, activation of the NFκB-IL-6 pathway appears to mediate vascular inflammation in various disease processes [1]. On the one hand, RIC has been reported to activate HO-1, thereby inhibiting NFκB and decreasing IL-6 levels in animal models of systemic inflammation and ischemia [34]. On the other hand, remote preconditioning applied in patients undergoing coronary artery bypass surgery under cardioplegic arrest resulted in increased IL-6 levels. It could therefore be hypothesised that IL-6 could be a circulating factor of RIC instead of a solely deleterious mediator, but evidence has been inconclusive. In our study, IL-6 levels were not affected by any of the applied protocols; this remains an interesting area for future investigation.

MiR-144 is a key effector of RIC [39], whose non-coding nature, small size, and direct effects on ribosomal function [60] allow it to rapidly modulate multiple cascades that abrogate IRI. It serves as a pivotal mediator of cellular adaptation to hypoxia [52] and experimental studies have demonstrated that its expression is upregulated in aortic endothelial and/or smooth muscle cells in response to stress [50]. Our finding suggests a possible vascular source of miR-144 production during RIC. One of its main mechanisms of action is the rapid - within 60 minutes [39] - activation of the reperfusion injury salvage kinase (RISK) pathway which constitutes a common pro-survival signalling pathway of remote pre- and post-conditioning [16]. Moreover, miR-144 evokes a crucial vascular antioxidative mechanism, in the form of Rac-1 downregulation [57]. To this end, modulation of ROS endothelial signalling appears to be one of its main mechanisms of action [48]. Indeed, we demonstrated that RIC results in increased miR-144 concentration, in parallel with oxidative stress reduction as assessed by MDA reduction, and in correlation with arterial elasticity improvement as assessed by PWV reduction.

Increased NO bioavailability [55] and oxidative stress alleviation [32] are promoted by miR-144 expression and have been found to reduce arterial stiffness. Indeed, in our study increased miR-144 levels were correlated with PWV reduction. This finding suggests that our single-cycle intervention through increased miR-144 expression, increased NOx levels, reduced oxidative stress and improved glycocalyx properties likely resulted in PWV reduction [16,39,57]; on the contrary, double-cycle inflation caused a net decrease in the measured NOx concentrations, suggesting a possible consumption of the NOx pool by the ischemic insult caused by the second inflation-deflation cycle, possibly contributing to the increased PWV values at protocol termination. RIC has been previously demonstrated to improve arterial elasticity in patients with CAD [61], but our study is the first to describe PWV improvement within 48 hours of primary PCI.

Increased arterial stiffness increases LV afterload while reducing diastolic coronary perfusion with resultant subendocardial ischemia [56,61] and ventricular-arterial decoupling [23]. These effects within 48 hours of AMI contribute to adverse LV remodelling and prognosis [26,37]. Moreover, it has been experimentally demonstrated that even transient increases in LV afterload may detrimentally affect remodelling [46]. In support of these findings, our single inflation protocol was associated with a greater reduction of LVESV and increased prevalence of reverse remodelling (LVESV decrease >15%) at 12 months post-AMI compared to double or no inflation likely through reduction of PWV at a critical for myocardial salvage time. Importantly, the reduction of PWV at protocol termination as well as the miR-144 concentration were associated with the LVESV decrease at follow-up. Similarly, per-conditioning by staccato reperfusion has been associated with reduced LV volumes

within 12 months of PCI [26], while post-conditioning has been found to confer improved LV remodelling, as assessed by LVESV, within 1 year of AMI [54].

The discrepancy between our single- and double-cycle protocol effects highlights the association between the number of ischemic cycles, the total ischemic time, and the underlying ischemic damage with RIC effectiveness [3]. In more detail, the second cycle may have crossed the ischemic burden threshold above which the beneficial effects of RIC on arterial stiffness are lost. This is concordant with previous findings of superior nitrite levels and cyto-protection with a single, compared to multiple inflation-deflation cycles [7]. Given the prominent role of NO in muscular arteries stiffness modulation [59], it can be postulated that the second ischemic stimulus consumed a component of the NOx pool, thereby reducing NO bioavailability with an attendant increase in aortic stiffness and failure to promote reverse LV remodelling. Similarly, the single-cycle protocol conferred superior restoration of the endothelial glycocalyx integrity. This could reflect coronary microvascular impairment amelioration to a greater extent compared to the double cycle RIC [31] and is in agreement with existing evidence of coronary microvascular injury adversely affecting LV remodelling [20]. The above mechanisms may explain the similar changes in LV volumes between the double- and no inflation group. These observations are also in accordance with the notion that one 5 min ischemic cycle may induce a favourable ischemic conditioning response [36], compared with multiple ischemic cycles [8,15,28-30].

There are some limitations to the interpretation of the results of our study, which are pertinent to the effects of PWV improvement on LV remodelling. On the one hand, it is known that increased LV afterload can adversely affect LV remodelling after AMI, even when it is transient in nature [46] and thus an early reduction of afterload may prohibit adverse LV remodelling. On the other hand, staccato reperfusion [26] and ischemic postconditioning [54] have also been demonstrated to have direct beneficial effects on the myocardium, leading to reverse LV remodelling. Our design does not permit to investigate the causality between LV remodelling and changes of vascular function caused after RIC versus the direct effects of RIC on the myocardium given the fact that aortic stiffness alleviation was an inherent result of our conditioning protocols. In addition, NOx levels represent the cumulative measurement of nitrates and nitrites, the levels of which (especially of nitrates) may be affected by food intake. The relatively high percentage of male subjects in our study should be also acknowledged as a limitation as female subjects may be underrepresented.

In conclusion, our findings suggest that RIC within 48 hours of STEMI acutely modulates the cardiovascular biochemical environment, evoking "vascular conditioning". More specifically, miR-144 is upregulated, nitrate-nitrite-NO pathway is activated, and oxidative stress burden is reduced. Consequently, endothelial glycocalyx properties are improved, resulting in arterial stiffness alleviation. Ultimately, reverse LV remodelling is encouraged. The above protective effects of "vascular conditioning" occur at a time window after the first critical 90 min needed to diagnose STEMI and rush the patient to primary PCI, facilitating the application of RIC in clinical practice at a more convenient time for both the patient and the medical team, to encourage positive LV remodelling. Another future implication of our study would be to apply interventions to restore NO bioavailability early post STEMI in order to investigate a possible reversal on PWV thus a positive effect on future LV remodelling

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Figure legends

Figure 1: Double-cycle, single-cycle, and no bilateral cuff inflation protocols

Figure 2: Sideview darkfield imaging assessment of endothelial glycocalyx Dper: diameter of perfusion; PBR: perfusion boundary region; RBC: red blood cell; RBCW: red blood cell width a) Sideview darkfield (SDF) imaging utilises the light emitting diode (LED) light reflected from haemoglobin to visualise the red blood cells (RBC) flowing in sublingual microvessels. The lateral distribution of the observed RBC columns demarks the boundaries of the perfused area of the vascular lumen, quantified by the Diameter of perfusion (Dper). Thus, an increased Dper infers deeper RBC penetration and a reduced non-permeable endothelial component, which signifies glycocalyx structural damage. b) The measurement process begins with image capturing, where the perfused luminal area is depicted as dark contrast flow. c) Thereafter, vascular segments are automatically identified. d) This is followed by RBC column width (RBCW) calculation, the distribution of which is used to calculate the perfused area diameter (Dperf) and the perfusion boundary region (PBR) according to the formula: PBR= (Dperf – RBCW) / 2.

Figure 3: Effects of remote conditioning on perfusion boundary region PBR: perfusion boundary region (μM); RIC: remote ischemic conditioning. The vertical lines represent the standard deviation of the mean. Two-way ANOVA for repeated measurements was applied with the parameter of time (T0,T1,T2,T3) used as a within-subject factor, and single- versus double-inflation, age, sex, BMI, dyslipidaemia, diabetes, hypertension, concomitant medical treatments, MI location, myocardial enzymes and the number of diseased coronary vessels (>70% stenosis) as between subject factors; ANCOVA was applied to investigate the effect of the baseline values of the respective markers. Post hoc comparisons were performed with Bonferroni correction and the adjusted p values for the comparison between T0 versus T1, T2, and T3 are shown. The interaction between RIC protocol [single- vs double-cycle vs no cuff inflation (controls)] and changes of PBR over time were also examined, and the p values of the interaction are shown.

Figure 4: Effects of remote conditioning on pulse wave velocity PWV (m/sec): pulse wave velocity; RIC: remote ischemic conditioning. The vertical lines represent the standard deviation of the mean. Two-way ANOVA for repeated measurements was applied with the parameter of time (T0,T1,T2,T3) used as a within-subject factor, and single- versus double-inflation, age, sex, BMI, dyslipidaemia, diabetes, hypertension, concomitant medical treatments, MI location, myocardial enzymes and the number of diseased coronary vessels (>70% stenosis) as between subject factors; ANCOVA was applied to investigate the effect of the baseline values of the respective

markers. Post hoc comparisons were performed with Bonferroni correction and the adjusted p values for the comparison between T0 versus T1, T2 and T3 are shown. adjusted p values are shown. The interaction between RIC protocol [single- vs double-cycle vs no cuff inflation (controls)] and changes of PBR over time were also examined, and the p values of the interaction are shown.

Figure 5: Effects of remote conditioning on serum malondialdehyde levels MDA: malondialdehyde (nmol/L); RIC: remote ischemic conditioning. The vertical lines represent the standard deviation of the mean. Two-way ANOVA for repeated measurements was applied with the parameter of time (T0,T1,T2,T3) used as a within-subject factor, and single- versus double-inflation, age, sex, BMI, dyslipidaemia, diabetes, hypertension, concomitant medical treatments, MI location, myocardial enzymes and the number of diseased coronary vessels (>70% stenosis) as between subject factors; ANCOVA was applied to investigate the effect of the baseline values of the respective markers. The interaction between RIC protocol [single- vs double-cycle vs no cuff inflation (controls)] and changes of PBR over time were also examined, and the p values of the interaction are shown.

Figure 6: Effects of remote post-conditioning in miR-144 expression RIC: remote ischemic conditioning. The fold change in expression level compared to the housekeeping gene, U6sn, was calculated using the $2^{-\Delta\Delta Ct}$ method. The vertical lines represent the standard deviation of the mean. Two-way ANOVA for repeated measurements was applied with the parameter of time (T0, T3) used as a within-subject factor, and single- versus double-inflation, age, sex, BMI, dyslipidaemia, diabetes, hypertension, concomitant medical treatments, MI location, myocardial enzymes and the number of diseased coronary vessels (>70% stenosis) as between subject factors; ANCOVA was applied to investigate the effect of the baseline values of the respective markers. The adjusted p values for the comparison between T0 and T3 are shown. The interaction between RIC protocol [single- vs double-cycle vs no cuff inflation (controls)] and changes of PBR over time were also examined, and the p values of the interaction are shown.

Figure 7: Effects of remote conditioning on LV remodelling LV: left ventricular, LVEDV: left ventricular end-diastolic volume, LVESV: left ventricular end-systolic volume, RIC: remote ischemic conditioning. The vertical lines represent the standard deviation of the mean. Two-way ANOVA for repeated measurements was applied with the parameter of time (baseline, 1 year follow-up) used as a within-subject factor, and single- versus double-inflation, age, sex, BMI, dyslipidaemia, diabetes, hypertension, concomitant medical treatments, MI location, myocardial enzymes and the number of diseased coronary vessels (>70% stenosis) as between subject factors;

ANCOVA was applied to investigate the effect of the baseline values of the respective markers. The interaction between RIC protocol [single- vs double-cycle vs no cuff inflation (controls)] and changes of PBR over time were also examined, and the p values of the interaction are shown.

Table 1: Baseline patient characteristics

Patient characteristics	**Single-cycle RIC (n=90)**	**Double-cycle RIC (n=90)**	**Control (n=90)**	**p-value**
Age (years)	53±16	54±16	52±16	0.70
Sex (Male %)	72 (80%)	73(82%)	75 (83%)	0.69
BMI	27±4	27±5	27±5	0.72
Hypertension	24 (27%)	26 (29%)	27 (30%)	0.70
Diabetes mellitus	15 (17%)	15 (17%)	16 (18%)	0.74
Dyslipidemia	21 (24%)	23 (26%)	22 (25%)	0.69
Smoking	47 (53%)	49 (55%)	51 (56%)	0.60
1 vessel disease	38 (42%)	41 (46%)	40 (44%)	0.50
2 vessel disease	41 (46%)	40 (44%)	42 (47%)	0.31
3 vessel disease	11 (12%)	9 (10%)	8 (9%)	0.22
Infarct related artery				
LAD	42 (47%)	43 (48%)	41 (46%)	0.40
Cx	23 (25%)	22 (24%)	21 (23%)	0.41
RCA	25 (28%)	25 (28%)	28 (31%)	0.21
hs-Troponin (ng/mL)	3843 [991-9338]	3926 [600-10000]	3890 [832-10000]	0.31
Symptom to balloon time (min)	179 [133-280]	181 [131-279]	180 [135-278]	0.12
First medical contact to balloon time (min)	104 [80-130]	102 [79-131]	105 [82-129]	0.18
WBC (/mcL)	8.790±2.577	8.540±2.588	8.680±2.592	0.20
CRP (mg/L)	191±3	170±4	185±4	0.30
EF (%)	44±13	46±14	46±10	0.50
Systolic BP (mmHg)	121±18	120±17	122±19	0.70
Diastolic BP (mmHg)	78±15	79±14	80±14	0.77

control: no inflation; BMI: body mass index; BP: blood pressure; CRP: C-reactive protein; Cx: circumflex artery; EF: ejection fraction; hs-Troponin: high sensitivity Troponin; LAD: left anterior descending artery; RCA: right coronary artery; RIC: remote ischemic conditioning; WBC: white blood cells count

Table 2: Effects of RIC on endothelial glycocalyx integrity and aortic stiffness

Vascular assessment	**Group**	**T0**	**T1**	**T2**	**T3**
PBR (μm)	Single	2.31±0.05	2.05±0.04*	2.06±0.06*	2.06±0.05*
	Double	2.34±0.04	2.04±0.06*	2.19±0.05*	2.21±0.06*
	Control	2.32±0.07	2.32±0.08	2.31±0.1	2.32±0.09
PWV (m/s)	Single	12.09±0.6	11.54±0.7*	11.39±0.7*	11.71±0.65*¶
	Double	12.06±0.5	11.57±0.6	11.37±0.7*	13.8±0.7*¶
	Control	11.7±0.8	11.6±1	11.6±1	11.7±1.5

PBR: perfusion boundary region; PWV: pulse wave velocity; T0: baseline; T1: after first cuff inflation; T2: after second inflation (or omission of 2nd inflation); T3: 20 min after second (or omission) inflation; controls=no inflation; *: $p<0.05$ for comparison with T0; ¶: $p<0.05$ for single- vs double-cycle protocol

Table 3: Effects of RIC on oxidative stress, cumulative nitrate-nitrite levels, and miRs expression

Biochemical assessment	**Group**	**T0**	**T3**
MDA (nmol/L)	Single	2.57±0.16	2.08±0.14*
	Double	2.61±0.15	2.13±0.15*
	Control	2.5±0.29	2.5±0.18
NOx (µmol/L)	Single	8.25±1.18	11.1±2*¶
	Double	10.79±1.18	8.91±2*¶
	Control	9.5±1	9.4±0.8
IL-6 (pg/ml)	Single	6.55±4.02	6.78±4.21
	Double	6.61±4.18	6.58±4.5
	Control	6.54±4,26	6.63±4.19
miR-144(/U6sn)	Single	7.4±0.7	55.9±0.8*¶
	Double	7.65±0.5	39.87±0.7*¶
	Control	5±0.6	4.8±0.5
miR-150(/U6sn)	Single	1.8±0.8	3.4±0.9*
	Double	2.05±0.5	3.53±0.6*
	Control	1.7±0.6	3.1±1.8
miR-499(/U6sn)	Single	1.6±0.4	3.5±0.4*
	Double	1.72±0.5	2.96±0.4*
	Control	1.4±0.3	1.8±0.3
miR-21(/U6sn)	Single	1.2±0.3	2.1±0.3*
	Double	1.28±0.4	2.18±0.5*
	Control	1.1±0.3	1.3±0.3
miR-208(/U6sn)	Single	1.9±0.5	2.4±0.4*
	Double	1.99±0.4	2.36±0.4*
	Control	1.8±0.4	2±0.3

T0: baseline; T3: 20 min after second (or omission of 2nd) inflation IL-6: interleukin-6; MDA: malondialdehyde; NOx: nitrate-nitrite; *: $p<0.05$ for baseline vs T3; ¶: $p<0.05$ for single- vs double-cycle protocol

Table 4: Progression of LV remodelling within 12 months of the index event

	group	**baseline**	**12 months**	**p-value**
LVEDV (mL)	single	105±34	83±31*¶	0.03
	double	107±29	100±32*¶	0.04
	control	110±29	104±32*¶	0.04
LVESV (mL)	single	59±31	49±25*¶	0.02
	double	60±25	57±25¶	0.06
	control	63±25	61±25¶	0.06
LVEF (%)	single	44±13	44±12	0.16
	double	45±10	43±12	0.1
	control	46±10	44±12	0.1

LVESV left ventricular end systolic volume LVEDV, left ventricular end diastolic volume LVEF left ventricular ejection fraction; *: $p<0.05$ for baseline vs 1 year follow up; ¶: $p<0.05$ for single-cycle vs double-cycle or control (no inflation)