HEART FAILURE IS ASSOCIATED WITH EXAGGERATED ENDOTHELIAL ISCHAEMIA-REPERFUSION INJURY AND ATTENUATED EFFECT OF ISCHAEMIC PRECONDITIONING

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ABSTRACT

Reperfusion is mandatory after ischaemia, but also triggers ischaemia–reperfusion (IR)-injury. It is currently unknown whether heart failure (HF) alters the magnitude of IR-injury. Ischaemic preconditioning (IPC) can limit IR-injury. Since IPC is typically applied in subjects at risk for cardiovascular complications, it is of clinical importance to understand the efficacy of IPC in HF patients.

Objective. To examine the magnitude of endothelial IR-injury, and the ability of IPC to protect against endothelial IR-injury in HF.

Methods. We included 15 subjects with HF (67±10 years, NYHA-class II/III) and 15 healthy, age- and sex-matched controls (65±9 years). We examined brachial artery endothelial function using flow mediated dilation (FMD) before and after arm IR (induced by 5-minute ischaemic handgrip exercise +15 minutes reperfusion). IR was preceded by IPC (consisting of 3 cycles of 5-minute upper arm cuff inflation to 220 mmHg) or no inflation.

Results. A significant interaction-effect was found for the change in FMD after IR between groups (2-way ANOVA interaction-effect: *P*=0.01). Whilst post-hoc analysis revealed a significantly decline in FMD in both groups (*P*<0.05), the decline in FMD in HF patients (6.2±3.6% to 3.3±1.8%) was significantly larger than that observed in controls (4.9±2.1 to 4.1±2.0). In HF patients nor in controls, the decrease in FMD after IR was altered by IPC (3-way ANOVA interaction: *P*=0.87).

Conclusion. We found that patients with HF are associated with exaggerated endothelial IR-injury compared with age- and sex-matched, healthy controls, which may contribute to the poor clinical prognosis in HF. Furthermore, we found no protective effect of IPC (3x5-minutes forearm ischaemia) against endothelial IR-injury in HF patients.

KEYWORDS: cardiovascular disease; endothelial function; flow-mediated dilation; ischaemia; cardiovascular risk

INTRODUCTION

Heart failure (HF) is a major cause of death in developed countries and represents a growing public health problem, partly due to the ageing population, and is responsible for an increasing proportion of hospital admissions [1]. A potential explanation for the poor prognosis of HF patients may relate to an exaggerated ischaemia-reperfusion (IR)-injury in HF as demonstrated in rats [2]. Such an increased vulnerability to IR-injury is clinically relevant, as this may contribute to worsening of the clinical outcome after a cardiovascular event. DeVan and colleagues demonstrated that traditional cardiovascular risk factors, such as advanced age, are associated with a greater magnitude and delayed recovery from endothelial IR-injury in humans [3]. Also experimental studies suggest that the presence of cardiovascular risk factors or disease is associated with exaggerated IR-injury [2, 4, 5], although some studies suggest otherwise [6, 7]. Accordingly, we examined the hypothesis that HF patients demonstrate an increased endothelial IR-injury compared to healthy peers *in vivo*.

Originally described in animals, ischaemic preconditioning (IPC; intermittent episodes of nonlethal ischaemia) is a powerful strategy to limit or even prevent IR-injury [8]. Previous human *in vivo* studies found that IPC effectively prevents endothelial IR-injury, with putative mechanisms for protection related to the sympathetic nervous system and the production of reactive oxygen species [9-11]. Despite successful pre-clinical studies, clinical trials implementing IPC have demonstrated somewhat disappointing results [12]. One potential explanation relates to the interaction between the efficacy of IPC and the presence of cardiovascular risk factors or disease [9]. Indeed, some preclinical studies provide evidence that HF is associated with an attenuated efficacy of IPC to prevent injury [13-15]. Since no previous study in humans has explored this hypothesis, the second aim of the study is to examine the efficacy of IPC to prevent or attenuate endothelial IR-injury in patients with HF. To study

these questions, we examined brachial artery flow-mediated dilation (FMD) before and after

ischaemia (induced by 5-minute ischaemic handgrip exercise) and reperfusion (15-min) and

use the reduction in FMD as a well-validated marker of endothelial injury. This model is

frequently used as a surrogate endpoint for IR-injury [9, 10].

METHODS

Participants

We included 15 subjects with HF (67±10 years, NYHA-class II/III, ejection fraction ≤ 45%)

and 15 healthy, age- and sex-matched older subjects (65±9 years). HF patients were recruited

from the Department of Cardiology of the Radboud University Nijmegen Medical Centre. We

excluded pre-menopausal women (or women with hormone replacement therapy), subjects

with diabetes mellitus type 1 or 2, hypertension (systolic ≥140 or diastolic ≥90 mmHg),

chronic obstructive pulmonary disease and severe hepatic or renal insufficiency. Healthy

control subjects were free of any chronic disease and did not use any type of medication

known to interfere with the cardiovascular system. HF patients were categorized as New York

Heart Association (NYHA) class II/III. Patients were on stable optimized pharmacological

therapy for ≥3 months. All subjects signed an informed consent and study procedures were

approved by the local ethics committee and performed according to the Declaration of

Helsinki (2000).

Experimental Design

Subjects attended our laboratory twice (separated by at least 7 days). Brachial artery

endothelial function was measured with FMD in the right arm. Brachial artery FMD was

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measured before and after IR-injury. IR-injury was induced by a 5-minute ischaemic handgrip exercise stimulus followed by 15 minutes of reperfusion. Local ischaemia during handgrip exercise was induced with upper arm cuff inflation to 220 mmHg. This ischaemic handgrip protocol leads to a (near) maximal ischaemic stimulus and peak reactive hyperaemia [16]. The transient decrease in FMD is believed to reflect IR-induced endothelial dysfunction, a finding supported by studies that successfully prevented this decline in FMD by well-established pharmacological (i.e. statins and physical (i.e. ischaemic preconditioning [9, 10]) interventions that protect against IR-injury. As such, studies have typically adopted protocols that induce significant exposure to ischemia and reperfusion to examine IR-injury in conduit arteries. Furthermore, brachial artery FMD correlates well with coronary artery endothelial function in humans [17], and predicts cardiovascular events in asymptomatic subjects and in those with established cardiovascular diseases [18, 19]. The assessment of FMD before and after IR-injury was performed with or without the preceding ischaemic preconditioning stimulus (IPC-intervention). IPC consisted of 3 cycles of 5-minute upper arm cuff inflation to 220 mmHg, with 5 minutes reperfusion time after each occlusion. This IPC-protocol is based on previous studies that have reported a protective effect of this stimulus in the heart or peripheral tissues [9, 10].

Measurements

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

circumference. Resting heart rate and blood pressure were measured twice in supine position, using a manual sphygmomanometer after 5-min of rest. Finally, with a finger stitch a small amount of blood was collected in order to assess glucose and blood cholesterol levels.

Flow mediated dilation (FMD). Before each experiment, participants refrained from food ingestion ≥6 hours, caffeine and products with high levels of vitamin C ≥18 hours, and from strenuous physical activity ≥24 hours. Subjects were tested at the same time of day to prevent diurnal variation in FMD response. All measurements were performed in a temperature-controlled room (22.5°C) and using recent guidelines of FMD [20].

Subjects rested in a supine position with the right arm extended and immobilized, supported at an angle of ~80° abduction from the torso. Heart rate and mean arterial pressure were determined with a manual sphygmomanometer. For the assessment of FMD, a rapid inflation/deflation pneumatic cuff was placed distal to the olecranon process to provide an ischaemic stimulus distal from the brachial artery to provoke vasodilation and subsequent shear stress. A 10-MHz (T3000, Terason, Aloka, UK) multi-frequency linear array probe attached to a high-resolution ultrasound machine was used to perform imageing. The brachial artery was imaged in the distal third of the upper arm. Ultrasound parameters were set to optimize longitudinal B-mode images of the lumen/arterial wall interface. A continuous Doppler velocity assessment was obtained simultaneously, and data were collected using the lowest possible insonation angle (always <60°), which did not vary during each study [20]. After a resting period of at least 15 minutes, 1 minute of baseline recording of the arterial diameter and velocity was performed. Subsequently, the occlusion cuff was inflated to 220 mmHg for 5 minutes. The arterial diameter and velocity recordings were restarted at least 30 seconds before cuff deflation and continued for at least 3 minutes after deflation. Peak arterial diameter and flow, and the time to reach this peak after cuff deflation, were recorded.

Brachial artery diameter and blood flow analysis

Analysis of the brachial artery diameter was performed using custom-designed edge-detection and wall-tracking software, which is independent of investigator bias. Baseline data were calculated across the 1 minute preceding cuff inflation. Following cuff deflation, peak diameter was automatically detected according to an algorithm as described in detail elsewhere [21]. Within-subject reproducibility of the FMD using this semi-automated software is 6.7-10.5% (coefficient of variation) [22]. Post-deflation shear rate data, derived from velocity and diameter measures, was used to calculate the area under the shear rate curve (SR_{AUC}).

Statistical analysis

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

A recent study described that inadequate scaling for FMD would be present if the upper confidence limit of the regression slope of the relationship between logarithmically

transformed base diameter and peak diameter is less than one [23]. In such an event, FMD% may not be an appropriate measure to estimate endothelial function. Therefore, we repeated the analysis for FMD using the allometric modelling solution [23]. The level of statistical significance was set at 0.05.

RESULTS

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

Endothelial IR-injury

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

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

IPC and endothelial IR-injury

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

DISCUSSION

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

preconditioning in HF patients. Accordingly, the magnitude of decline in FMD after IR-injury when preceded with IPC, remains larger in HF patients than in their healthy age- and sex-matched controls. Therefore, our study revealed that HF is associated with an exaggerated decline in endothelial function after IR-injury, whilst IPC failed to protect against this decrease.

Endothelial IR-injury

In agreement with several previous studies, we found that IR-injury induces a transient, conduit artery endothelial dysfunction [9, 10]. A novel observation is that HF patients demonstrate an exaggerated decline in FMD after IR-injury compared with their healthy peers. To date, the impact of HF on IR-injury has only been examined in animal studies, which provided conflicting results ranging from an increased [6, 7] to a decreased tolerance [2, 4, 5] against prolonged ischaemia. Differences in the ischaemia-stimulus *within* and *between* studies may contribute to these conflicting results. In our study, both groups received the same ischaemic stimulus. Moreover, inter-species differences or the experimental procedures to induce HF may also have contributed to the conflicting results from animal studies. Nonetheless, our study provides support that, in humans, HF is associated with an exaggerated decline in endothelial function after endothelial IR-injury.

The larger decline in FMD in HF patients than controls after IR may relate to differences in antioxidative capacity between groups. Endothelial injury after IR is caused, at least partially, by excessive production of oxidative stress [24]. Whilst healthy individuals have a well-controlled balance between the production of reactive oxygen species (ROS) and antioxidative enzymes, patients with HF demonstrate less antioxidative capacity, resulting in increased oxidative stress [25, 26]. Nonetheless, our novel observations in HF patients warrants

future research to better understand the potential underlying mechanisms that contribute to the exaggerated IR-injury in HF.

IPC and endothelial IR-injury

In a recent study, we demonstrated that the well-established protective effects of IPC are abolished in healthy older men compared to younger control patients [9]. In addition, the present study provides evidence that the protective effect of IPC to attenuate endothelial IR-injury is also abolished in HF patients. This latter observation is in line with data from animals, supporting an emerging hypothesis of a reduced efficacy of IPC associated with cardiovascular disease or risk factors [12, 13, 27]. For example, a recent animal study revealed the inability of preconditioning to protect the old diabetic heart against an ischaemic insult [28]. In line with these findings, preclinical studies in patients with HF demonstrated an impaired effect of preconditioning to prevent ischaemia-induced tissue damage [13-15].

Our study supports the detrimental findings on the efficacy of IPC in HF patients. We can only speculate about possible mechanisms to explain this finding. Preclinical studies suggest that the impaired efficacy of IPC in cardiovascular disease is linked to morphological and biochemical alterations, which may impact on signal transduction [27]. For instance, it was recently demonstrated that the presence of post-infarction cardiac remodelling is closely linked to an abolished effect of IPC [15]. Whilst this finding supports a role for morphological changes underlying our findings, others have provided support for biochemical alterations in patients with HF. Indeed, IPC in HF failed to induce protein kinase C-ε translocation [29], which represents an important step in the protection through preconditioning. Finally, mitochondrial defects in the genesis and progression of HF have also been proposed to

contribute to the diminished effect of IPC. While mitochondria seem to serve as end-effectors of IPC, decreased enzyme activities of the electron transport seen in failing hearts may potentially negatively impact the efficacy of IPC [27].

Clinical implication. Our finding of a reduced efficacy of IPC in HF patients may have clinical implications. Various (non)pharmacological preconditioning interventions are currently applied in randomised controlled trials in patients, including those with HF. Despite some recent successful studies [30, 31], application of (remote) IPC in the clinical setting in general is often disappointing [32]. Especially the increased cardiovascular death rate in post-infarcted, failing hearts suggests that endogenous protective mechanisms in HF against IR-injury may be lost or attenuated [27]; a finding which is in line with the present study. Possible reasons relate to the inclusion of relatively young animals in preclinical studies with a relatively short disease duration, whilst clinical trials mostly involve patient groups such as HF (e.g. in heart transplantation) [27]. Therefore the majority of preclinical studies do not adequately reflect the clinical setting in which patients are included with lower efficacy of IPC. This should be taken into consideration when examining the impact of IPC in clinical groups, such as HF.

Limitations. A number of limitations must be discussed. First, our model to examine IR-injury involved measurement of endothelial function in the forearm. Although strong correlations have been reported between brachial artery FMD and coronary endothelial function [17], caution should be taken when extrapolating our findings to other vascular beds. Given the aetiology of HF as a disease affecting the heart, studying the heart muscle tissue directly might reveal even more pronounced results on the magnitude of IR-injury. Secondly, in contrast to previous studies that adopted 15-20 minutes of ischemia to induce endothelial IR-injury, our study used

5-min ischaemic handgrip. However, previous work demonstrated that 5-minutes of ischaemic handgrip exercise induces reperfusion that is at least similar to 15-minutes of ischemia. Moreover, FMD decreased in both groups using this protocol. Therefore, this approach is valid to examine endothelial IR-injury. Third, patients in our study continued their medication during testing. Continuing medication in HF patients may explain why we found no differences in FMD between groups. Indeed, our FMD-data matches with previous studies that included medicated HF patients [33]. We deliberately chose to continue (pharmacological) treatment, so that our results would reflect a 'real life' situation. This approach revealed, despite the intake of drugs with established preconditioning effects (i.e. statins), that an exaggerated endothelial IR-injury in HF patients could not be attenuated by IPC. These findings raise questions regarding the potential loss of preconditioning effects of statins with sustained intake, such as recently highlighted [34]. Finally, we only examined a single time-point after IR-injury. This limits insight into a potential difference between groups (or between interventions) in the time-course of restoration of FMD after IR injury. Such differences in time-course may have provided further insight to better understand our findings.

In conclusion, we provide data in humans *in vivo* that HF is associated with an exaggerated damage to the endothelium after an ischaemic insult, which cannot be prevented by IPC. These novel findings may contribute to the poor clinical outcome after cardiac injury in HF patients, and should be considered when examining the effects of traditional, non-pharmacological preconditioning in HF patients.

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FIGURE LEGENDS

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