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Rapid ventricular pacing-induced postconditioning attenuates reperfusion injury: effects on peroxynitrite, RISK and SAFE pathways

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BACKGROUND AND PURPOSE

Rapid ventricular pacing (RVP) applied before an index ischaemia has anti-ischaemic effects. Here, we investigated whether RVP applied after index ischaemia attenuates reperfusion injury and whether peroxynitrite, reperfusion injury salvage kinase (RISK) and survival activating factor enhancement (SAFE) pathways as well as haem oxygenase 1 (HO1) are involved in the mechanism of RVP-induced postconditioning.

EXPERIMENTAL APPROACH

Langendorff perfused rat hearts were subjected to 30 min regional ischaemia and 120 min reperfusion with or without ischaemic postconditioning (6 × 10/10 s reperfusion/ischaemia; IPost) or RVP (6 × 10/10 s non-pacing/rapid pacing at 600 bpm) applied at the onset of reperfusion.

KEY RESULTS

Meta-analysis of our previous studies revealed an association between longer reperfusion-induced ventricular tachycardia/fibrillation with decreased infarct size. In the present experiments, we tested whether RVP is cardioprotective and found that both IPost and RVP significantly decreased infarct size; however, only RVP attenuated the incidence of reperfusion-induced ventricular tachycardia. Both postconditioning methods increased the formation of cardiac 3-nitrotyrosine and superoxide, and non-significantly enhanced Akt phosphorylation at the beginning of reperfusion without affecting ERK1/2 and STAT3, while IPost alone induced HO1. Application of brief ischaemia/reperfusion cycles or RVP without preceding index ischaemia also facilitated peroxynitrite formation; nevertheless, only brief RVP increased STAT3 phosphorylation.

CONCLUSIONS AND IMPLICATIONS

Short periods of RVP at the onset of reperfusion are cardioprotective and increase peroxynitrite formation similarly to IPost and thus may serve as an alternative postconditioning method. However, downstream mechanisms of the protection elicited by IPost and RVP seem to be partially different.

LINKED ARTICLES
This article is part of a themed section on Conditioning the Heart – Pathways to Translation. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-8

Abbreviations
HO1, haem oxygenase 1; I/R, ischaemia/reperfusion; IPost, ischaemic postconditioning; LAD, left anterior descending coronary artery; RISK, reperfusion injury salvage kinase; RVP, rapid ventricular pacing; SAFE, survival activating factor enhancement; VF, ventricular fibrillation; VT, ventricular tachycardia

Tables of Links

TARGETS			
Acetylcholinesterase	ERK1	HO1	PKG
Akt (PKB)	ERK2	PKC	

LIGANDS	
cGMP	Nitric oxide (NO)
CGRP	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Ischaemic heart diseases, including acute myocardial infarction, are the leading cause of death in industrialized countries. Reperfusion therapy for infarction allows rapid return of blood flow to the ischaemic myocardium and decreases mortality rate. However, early reperfusion itself is accompanied by deleterious events: the occurrence of life-threatening arrhythmias, no-reflow phenomenon, myocardial stunning and additional cell death (Yellon and Hausenloy, 2007). This paradoxical reperfusion injury caused by the restoration of blood flow and oxygen supply (Yamada *et al.*, 1990) leads to increased infarct size, impaired contractile function and electrical vulnerability, largely compromising clinical outcomes.

Ischaemic postconditioning (IPost) has emerged in the last decade as a potential therapeutic intervention for limiting reperfusion injury (Zhao *et al.*, 2003; Ovize *et al.*, 2010). The procedure is based upon the application of brief cycles of ischaemia/reperfusion (I/R) immediately after a prolonged ischaemia and it has been reported to reduce myocardial damage both in animal studies and in human clinical trials (Ovize *et al.*, 2010). Nevertheless, some studies have reported the ineffectiveness of IPost both in animals and in humans (Dow and Kloner, 2007; Hahn *et al.*, 2013). A possible explanation for the controversial results could be that the outcome of postconditioning may depend on several factors, such as failure to achieve complete reperfusion during application of brief I/R cycles, the duration of index ischaemia, the algorithm of postconditioning manoeuvre, gender, age and temperature (Skyschally *et al.*, 2009b). In addition, co-morbidities, such as hyperlipidaemia (Kupai *et al.*, 2009) and diabetes (Miki *et al.*, 2012), may interfere with the infarct size-limiting effect of postconditioning. These confounding factors indicate the necessity to develop new alternative methods and models to induce postconditioning.

Heart rate is known to play a role in the development of I/R injury (Bernier *et al.*, 1989), and it was shown that either slowing or increasing heart rate before ischaemia limits myocardial injury (Tosaki *et al.*, 1988; Bernier *et al.*, 1989; Hearse *et al.*, 1999). Moreover, we have previously shown that short periods of rapid ventricular pacing (RVP) applied before an index ischaemia has anti-ischaemic effects (pacing-induced preconditioning) (Ferdinandy *et al.*, 1997a,b; 1998). However, the effect of short periods of RVP performed at the early phase of reperfusion has not been investigated so far.

The exact molecular mechanism of myocardial postconditioning is not entirely clear. Increasing evidence suggests that enhanced formation of cardiac peroxynitrite is involved in cardioprotection afforded by both pre- (Altug *et al.*, 2000; Altup *et al.*, 2001; Csonka *et al.*, 2001) and postconditioning (Kupai *et al.*, 2009; Li *et al.*, 2013). Kupai et *al.* reported first that IPost failed to decrease infarct size in the presence of a peroxynitrite decomposition catalyst, thereby suggesting essential triggering role of peroxynitrite in postconditioning-induced cardioprotection (Kupai *et al.*, 2009).

Therefore, here we aimed to investigate whether RVP applied after index ischaemia has any effect on the markers of reperfusion injury and we studied the role of peroxynitrite in the mechanisms of postconditioning. Furthermore, we looked at activation of reperfusion injury salvage kinase (RISK) and survival activating factor enhancement (SAFE) pathways and haem oxygenase 1 (HO1) as possible downstream targets of RVP-induced postconditioning.

Methods

Male Wistar rats were used in our previous and present (n = 74) studies. The studies conform to the 'Guide for the care and use of laboratory animals' published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by local ethics committees. The animals were kept at 12/12h light/dark cycle and had free access to standard laboratory chow and drinking water. All

studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Isolated heart preparation

Isolated heart preparation was carried out as described in our previous studies with slight modifications (Ferdinandy *et al.*, 1997a; Kocsis *et al.*, 2012; Varga *et al.*, 2014). Inhalation anaesthesia of rats was induced in a glass desiccator containing cellulose wadding soaked in diethyl ether, an anaesthetic not known to interfere with cardioprotection. During isolation of the heart, rats were removed from the chamber and a beaker containing wadding soaked in ether was held near the muzzle of rats in order to maintain anaesthesia. Rats were given 500 U·kg^{-1} heparin i.v. Hearts were then isolated and perfused according to Langendorff at 37°C with Krebs–Henseleit buffer containing 118 mM NaCl, 25 mM $NaHCO_3$, 4.3 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose, gassed with 95% O_2 and 5% CO_2. Hydrostatic perfusion pressure was kept constant at 100 cmH_2O (9.8 kPa) throughout the experiments. Coronary flow was measured by collecting coronary effluent for a period of time and was expressed as mL·min^{-1}.

A 3-0 silk suture was placed around the left anterior descending coronary artery (LAD) close to its origin and the snare was tightened by applying a 100 g hanging weight to induce regional index ischaemia. For IPost, brief no-flow global ischaemia was performed by turning off the perfusion cannula. The presence of ischaemia was verified by monitoring coronary flow. RVP (600 bpm; 10 Hz) was performed by an electric stimulator (Experimetria Inc., Budapest, Hungary) with double threshold square, 1 V, 1 mA and 5 ms impulses conducted by electrodes attached directly to the surface of the right ventricle close to the apex and to the aortic cannula as described previously (Ferdinandy *et al.*, 1997a,b; 1998). Heart rates were monitored (Isosys; Experimetria Inc.) by recording epicardial ECG throughout the whole duration of perfusion.

Relationship between the duration of reperfusion-induced ventricular tachyarrhythmia and infarct size: a meta-analysis

Meta-analysis was performed on ECGs and infarct size data from our six previous studies performed in our laboratory on isolated rat hearts subjected to 30 min regional ischaemia and 120 min reperfusion (Figure 1A). Reperfusion-induced arrhythmias were analysed in the first 10 min of reperfusion. Hearts presenting sustained (>10 min) tachyarrhythmia were excluded ($n = 14$). Three separate evaluations were performed based on the total duration of ventricular tachycardia (VT), ventricular fibrillation (VF) or VT + VF respectively. Infarct size data were presented on the basis of duration (shorter or longer than 60 s) of VT, VF or VT + VF. Infarct size data exceeding mean ± 2 SD were excluded from the analysis ($n = 6$).

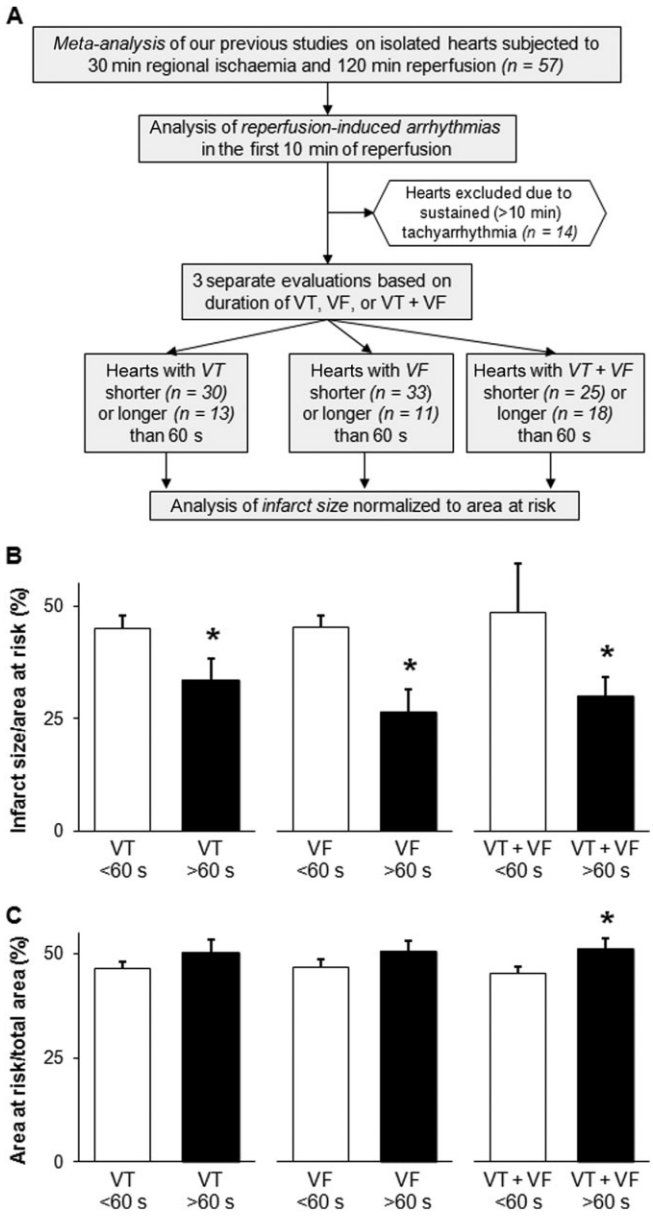

Figure 1

Duration of reperfusion-induced VT and/or VF is associated with decreased infarct size: a meta-analysis. Flow chart of the meta-analysis (A) indicates that reperfusion-induced tachyarrhythmias and infarct size data from our previous studies on isolated rat hearts subjected to 30 min regional ischaemia and 120 min reperfusion were analysed in three separate ways considering the duration of either VT, VF or both in the first 10 min of reperfusion. The results of the meta-analysis show infarct size normalized to area at risk (B) and area at risk (C) in the presence of shorter (<60 s) or longer (>60 s) total durations of VT, VF or VT + VF respectively. Values are expressed as mean ± SEM. *$P < 0.05$ versus corresponding <60 s groups, unpaired *t*-test.

Experimental design 1: testing the cardioprotective effect of RVP

To examine whether RVP applied at the onset of reperfusion induces cardioprotection, isolated hearts were perfused as shown in Figure 2A. Three experimental groups were designed: (i) I/R control; (ii) ischaemic postconditioning; and (iii) RVP groups ($n = 12$ in each group). The I/R control group was subjected to a 15 min equilibration period, followed by 30 min regional index ischaemia and 120 min reperfusion. IPost was induced by six consecutive cycles of 10 s

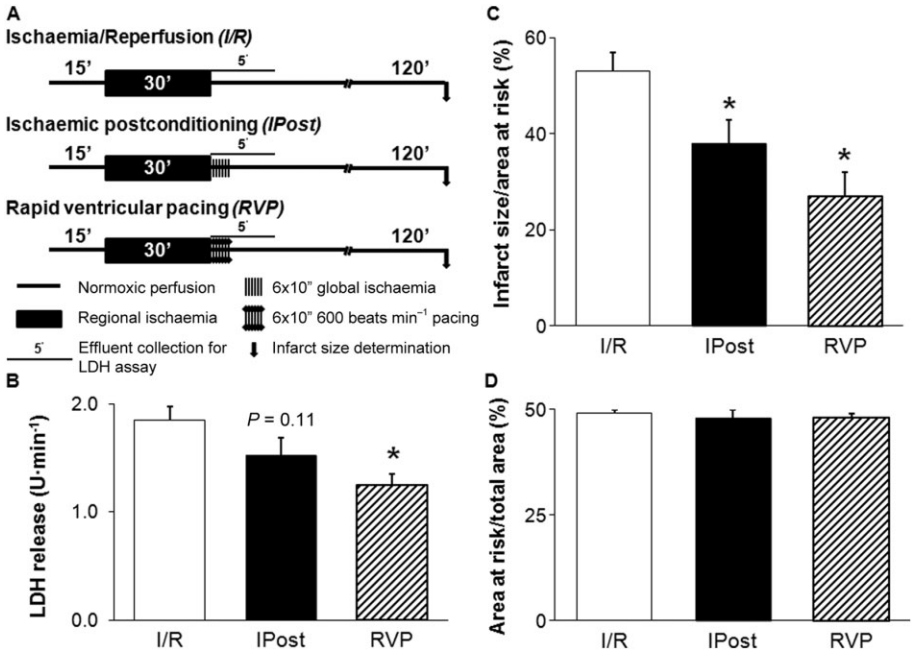

Figure 2

RVP reduces post-ischaemic LDH release and infarct size. Experimental protocol (A), post-ischaemic LDH release (B), infarct size normalized to area at risk (C) and area at risk (D). Hearts were subjected to a 15 min equilibration period, followed by 30 min regional ischaemia and 120 min reperfusion. Ischaemic postconditioning was induced by 6 × 10 s/10 s cycles of reperfusion/no-flow global ischaemia. In the RVP group, the autonomic rhythm of the hearts was replaced by 10 s pacing period (600 beats min^{-1}; 10 Hz) in six alternating cycles at the onset of reperfusion. Coronary effluent was collected during the first 5 min of reperfusion for LDH activity determination ($n = 5$ in each group), the measured activities were multiplied by the corresponding coronary flow to give LDH release. Infarct size was measured at the end of reperfusion ($n = 12$ in each group). Values are expressed as mean ± SEM. *$P < 0.05$ versus I/R, one-way ANOVA.

reperfusion and 10 s no-flow global ischaemia at the onset of reperfusion. In the RVP group, the spontaneous rhythm of hearts was replaced by a 10 s pacing period (600 beats min^{-1}; 10 Hz) in six alternating cycles during the first 2 min of reperfusion.

To assess the severity of cellular damage in the myocardium, the activity of LDH enzyme from coronary effluents (collected during the first 5 min of reperfusion) was measured using an LDH-P kit (Diagnosticum, Budapest, Hungary) ($n = 5$ in each group). The enzyme activity ($U \cdot mL^{-1}$) measured in an effluent was multiplied by the corresponding coronary flow ($mL \cdot min^{-1}$) to give LDH release expressed as $U \cdot min^{-1}$.

To determine infarct size, the LAD was re-occluded at the end of reperfusion and hearts were stained with 0.1% Evans blue to determine area at risk (Csonka *et al.*, 2010). Hearts were then frozen at –20°C and cut into approximately 2 mm thick slices. Each slice was incubated at 37°C for 10 min in 1% 2,3,4-triphenyl-tetrazolium-chloride solution dissolved in phosphate buffer (pH 7.4). Slices were then fixed in 10% formaldehyde and scanned. Infarct size was evaluated by planimetry (InfarctSize™ 2.4.b; Pharmahungary Group, Szeged, Hungary) and normalized to area at risk.

To assess reperfusion-induced tachyarrhythmias (VT and VF), ECG was recorded (Isosys; Experimetria Inc.) during the entire perfusion protocol. Analysis of arrhythmias was carried out according to the original Lambeth conventions (Walker *et al.*, 1988).

Experimental design 2: investigating the role of peroxynitrite and possible downstream targets in RVP-induced postconditioning

To assess the possible role of peroxynitrite in cardioprotection induced by ischaemic- or RVP-induced postconditioning, in separate experiments, cardiac 3-nitrotyrosine, a well-known peroxynitrite marker, was determined. To confirm increased peroxynitrite formation, cardiac superoxide anion was also measured. Furthermore, involvement of molecular mechanisms (i.e. RISK and SAFE pathways, HO1) that have been implicated in cardioprotection (Hausenloy and Yellon, 2004; Lecour, 2009; Bak *et al.*, 2010) was also investigated as possible downstream targets of RVP-induced postconditioning.

Hearts were subjected to 15 min equilibration period, followed by 30 min regional ischaemia and 7 min reperfusion with or without IPost or RVP (Figure 4A). At the end of reperfusion, myocardial samples were taken from the ischaemic zone of the left ventricle for 3-nitrotyrosine measurement and Western blot analysis ($n = 5$ in each group). Sampling was carried out by an oblique cut from the origin of the LAD towards the right side of the apical area that involves the majority of the anterior wall of the left ventricle as well as the apex of the heart. Samples were rapidly freeze-clamped, powdered with a pestle and mortar in liquid nitrogen and stored in cryovials at –80°C until further analysis. Sampling for *in situ* detection of superoxide anion was carried out in separate

experiments (n = 3 in each group) using the same perfusion protocol (Figure 4A). Approximately 3 mm thick transverse slices were cut from the middle of the ventricles, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, The Netherlands), carefully frozen in isopentane pre-cooled in liquid nitrogen and stored at –80°C until sectioning with a microtome.

Cardiac free 3-nitrotyrosine content, a marker of peroxynitrite, was measured by ELISA (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions (Kupai *et al.*, 2009; Kocsis *et al.*, 2012). Briefly, homogenates were incubated overnight with nitrotyrosine acetylcholinesterase tracer and anti-nitrotyrosine rabbit IgG in microplates pre-coated with mouse anti-rabbit IgG. Ellman's reagent was used for development. Free nitrotyrosine content was normalized to protein content of cardiac homogenate and expressed as ng mg^{-1} protein.

Superoxide anion (O_2^-) is a reactive oxygen radical that reacts with NO to form peroxynitrite. The *in situ* fluorescent dihydroethidium staining was performed to evaluate intracellular production of superoxide anion (Varga *et al.*, 2013). Unfixed frozen heart sections (30 µm) were placed on glass slides and incubated in 10^{-6} mol·L^{-1} dihydroethidium (Sigma, St. Louis, MO, USA) in PBS buffer (pH 7.4) at 37°C for 30 min in a dark humidified container. Fluorescence was then detected by a fluorescent microscope (Nikon, Tokyo, Japan) with a 590 nm long-pass filter. Images of the hearts were collected digitally (n = 20 in each heart); integrated density was evaluated by ImageJ 1.44p software and expressed in arbitrary unit.

The involvement of possible downstream targets in the mechanism of RVP-induced postconditioning was examined by standard Western blot techniques (Kocsis *et al.*, 2008; Fekete *et al.*, 2013). Tissue samples were homogenized with an ultrasonicator (UP100H Hielscher, Teltow, Germany) in RIPA buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 5 mM EDTA, 0.1% SDS, 1% NP-40] supplemented with protease inhibitor cocktail (Sigma), PMSF, NaF and Na_3VO_4. The crude homogenates were centrifuged at 10 000× g for 10 min at 4°C. After quantification of protein concentrations of the supernatants using BCA Protein Assay Kit (Pierce, Rockford, IL, USA), 20 µg (50 µg for HO1) reduced and denaturated protein was loaded and SDS-PAGE (10% gel, 90 V, 1.5 h) was performed followed by transfer of proteins onto nitrocellulose membrane (20% methanol, 35 V, 2 h). Membranes were blocked for 1 h in 5% (w v^{-1}) BSA at room temperature and then incubated with primary antibodies against phospho(Ser^{473})-Akt 1:500, Akt 1:2000, phospho(Thr^{202}/Tyr^{204})-ERK1/ERK2 1:2000, ERK1/ERK2 1:1000, phospho(Tyr^{705})-STAT3 1:2000, STAT3 1:2000 (Cell Signaling, Beverly, MA, USA; overnight, 4°C, 5% BSA) or HO1 1:2000 (Enzo Life Sciences, Plymouth Meeting, PA, USA; 2 h, room temperature, 1% milk) or GAPDH 1:10 000 (Cell Signaling, Beverly, MA, USA; 1 h, room temperature, 1% milk). After incubation with HRP-conjugated secondary antibody 1:5000 (1:20 000 for GAPDH) (Dako Corporation, Santa Barbara, CA, USA; 1 h, room temperature, 1% milk), membranes were developed using an enhanced chemiluminescence kit (Pierce).

To further prove that both IPost and RVP protocols (i.e. application of brief I/R or RVP) facilitate peroxynitrite formation, 3-nitrotyrosine was measured in the absence of index ischaemia. The effect of the protocols on possible downstream targets of peroxynitrite (i.e. RISK and SAFE pathways) was also examined in the absence of preceding index ischaemia.

In this set of experiments, the time course of the perfusion protocol was adjusted to the previous set-up without index ischaemia (Figure 5A). In the normoxic perfusion group (n = 8), hearts were perfused for 52 min. In the repeated brief I/R group (n = 7), hearts were subjected to 45 min perfusion followed by 6 × 10/10 s cycles of no-flow global I/R and 5 min reperfusion. In the repeated brief RVP group (n = 8), the spontaneous rhythm of the hearts was replaced by 10 s pacing period (600 beats min^{-1}; 10 Hz) in six alternating cycles after 45 min perfusion. At the end of perfusion, the cardiac free 3-nitrotyrosine level was determined and RISK as well as SAFE pathways were examined as described earlier.

Statistical analysis

Data are expressed as mean ± SEM and analysed by use of Student's unpaired t-test, one-way ANOVA, or Fisher's exact test as appropriate. If a difference was established in ANOVA, Fisher's least significant difference *post hoc* test was applied. Differences were considered significant at $P < 0.05$.

Results

Duration of reperfusion-induced VT and/or fibrillation is associated with decreased infarct size

Meta-analysis of six separate studies previously performed in our laboratory using the same experimental protocol (i.e. isolated rat hearts subjected to I/R) showed that the presence of VT, VF or VT + VF with a total duration of longer than 60 s in the first 10 min of reperfusion was associated with a markedly decreased infarct size (Figure 1B) respectively. In this analysis, a larger area at risk was associated with longer than 60 s total duration of VT + VF (Figure 1C).

RVP exerts cardioprotective effect: limits the infarction and reperfusion-induced arrhythmias

In order to assess the possible cardioprotective effect of RVP, the extent of myocardial infarction (LDH release and infarct size) was measured and reperfusion-induced arrhythmias were analysed.

The post-ischaemic LDH release was significantly reduced by RVP (Figure 2B). IPost also reduced LDH release; however, the difference did not reach the level of statistical significance (Figure 2B). Infarct size was significantly decreased by both IPost and RVP (Figure 2C). There was no difference in the area at risk of either experimental group (Figure 2D).

The incidence of VT and VF was not affected significantly by IPost in our present study (Figure 3). In contrast, short periods of RVP decreased the incidence of reperfusion-induced VT without having a significant effect on VF (Figure 3).

There was no difference in animal weight, heart wet weight, baseline heart rate and coronary flow (baseline,

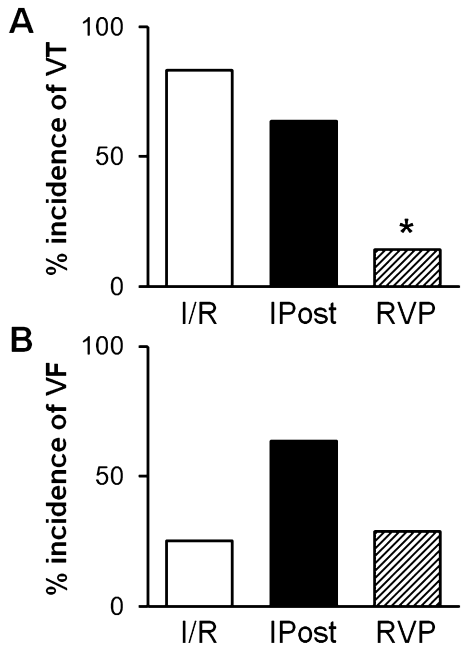

Figure 3

RVP attenuates reperfusion-induced arrhythmias. Incidence of reperfusion-induced VT (A) and VF (B) are shown. *$P < 0.05$ versus I/R, Fisher's exact test.

beginning of ischaemia, end of reperfusion) between the experimental groups (Table 1). In contrast to IPost, coronary flow at the onset of reperfusion was not changed by short periods of RVP compared with I/R control (Table 1).

Peroxynitrite is likely involved in RVP–induced postconditioning

To obtain some mechanistic insight into the beneficial effect of RVP, cardiac 3-nitrotyrosine and superoxide were measured at 7 min of reperfusion following the 30 min index ischaemia.

Postconditioning induced either by IPost or by RVP significantly increased free cardiac 3-nitrotyrosine level (a marker of peroxynitrite formation) (Figure 4B). Moreover, the peroxynitrite precursor superoxide anion was mildly, but significantly elevated in both postconditioning groups (Figure 4C).

To further prove that the postconditioning manoeuvres induce nitrative stress, cardiac 3-nitrotyrosine was measured after the postconditioning stimuli applied following normoxic perfusion without index ischaemia. The application of brief I/R cycles or periodic RVP increased the cardiac formation of 3-nitrotyrosine in the absence of index ischaemia (Figure 5B).

Downstream mechanisms of RVP-induced cardioprotection differs from that of ischaemic postconditioning

To elucidate the possible downstream targets of RVP, RISK and SAFE pathways as well as HO1 were investigated either in the presence or absence of index ischaemia.

Both postconditioning methods non-significantly enhanced Akt phosphorylation after index ischaemia at the beginning of reperfusion without affecting phosphorylation of ERK1/2 and STAT3 (Figure 4E,F). Protein level of HO1 was increased by IPost but not RVP (Figure 4E,F). In the absence of index ischaemia, applying short periods of RVP protocol increased STAT3 phosphorylation, in contrast to brief cycles of I/R (Figure 5C,D). Phosphorylation of Akt and ERK1/2 was not affected significantly by any of the interventions in the absence of index ischaemia (Figure 5C,D).

Discussion and conclusion

In our present study, using an isolated perfused rat heart model, we confirmed that IPost beneficially affects I/R injury. Moreover, we demonstrated for the first time in the literature that applying short periods of RVP at the onset of reperfusion also exerts a cardioprotective effect as it attenuates reperfusion injury by decreasing infarct size and reperfusion-induced arrhythmias. We showed that RVP increased peroxynitrite formation either in the presence or absence of index ischaemia in a way similar to IPost. These findings suggest that the formation of peroxynitrite in early reperfusion is a key event in the development of cardioprotection elicited by IPost or RVP. However, we also demonstrated that the downstream mechanisms of RVP-induced cardioprotection and IPost seem to be partially different.

In a meta-analysis of our previous studies on isolated hearts subjected to I/R, we analysed if there is an association between the duration of reperfusion-induced ventricular tachyarrhythmias (VT, VF or VT + VF) and infarct size. It is well accepted in the literature that I/R induces cellular damage that makes the myocardium more susceptible to arrhythmogenesis, and thus reperfusion-induced arrhythmias are considered as indicators of I/R injury (Engelen *et al.*, 2003; Majidi *et al.*, 2009). For instance, Majidi *et al.* reported that the presence of reperfusion arrhythmia bursts in STEMI patients is associated with a worse outcome (larger infarct size and decreased ejection fraction) (Majidi *et al.*, 2009). However, here we found surprisingly that longer than 60 s reperfusion-induced VT/VF was associated with a decreased infarct size. In this analysis, a larger area at risk was associated with longer total duration of VT + VF in accordance with the literature data (Curtis and Hearse, 1989). The interpretation of these results is difficult since causality was not examined in these studies. A possible explanation for the results of our meta-analysis is that the size of infarction affects the occurrence of sustained VT and/or VF, while another possibility is that longer tachyarrhythmias at the beginning of reperfusion somehow attenuate infarct development. To the best of our knowledge, this latter approach has not been investigated in the literature and, therefore, these findings served as a basis for our current experimental study to investigate if exogenous application of controlled tachycardia induced by RVP at the onset of reperfusion is able to elicit cardioprotection.

Heart rate is known to play a role in the development of I/R injury (Bernier *et al.*, 1989) and its controlled modification may elicit cardioprotection. For instance, pharmacologically-induced bradycardia (Tosaki *et al.*, 1987), slow (Tosaki *et al.*, 1988) or rapid (Ferdinandy *et al.*, 1998;

Table 1

Morphological and *ex vivo* haemodynamic parameters

	I/R	IPost	RVP
Animal weight (g)	367 ± 8	358 ± 10	345 ± 10
Heart wet weight (g)	1.28 ± 0.03	1.22 ± 0.04	1.30 ± 0.06
Basal heart rate (beats min^{-1})	301 ± 11	291 ± 12	304 ± 8
Coronary flow ($mL \cdot min^{-1}$)			
Before ischaemia	18.8 ± 1.5	16.7 ± 1.2	18.7 ± 1.1
Beginning of ischaemia[a]	10.7 ± 1.0	9.0 ± 0.8	11.5 ± 1.0
Beginning of reperfusion[b]	16.5 ± 1.0	8.7 ± 0.6*	17.9 ± 0.7
End of reperfusion	11.5 ± 1.5	9.9 ± 0.9	11.8 ± 1.5

Results are expressed as mean ± SEM. *$P < 0.05$ versus I/R and RVP, one-way ANOVA.
[a]Regional ischaemia.
[b]6 × 10 s global ischaemia was applied to induce IPost in the first 2 min of reperfusion. Coronary flow was measured by collecting coronary effluent for 2 min and then was expressed as $mL \cdot min^{-1}$.

Hearse *et al.*, 1999) pacing before ischaemia was reported to limit myocardial injury. Since the presence of longer reperfusion-induced tachyarrhythmias was associated with lower infarct size in our meta-analysis, we wanted to test whether exogenous rapid pacing exerts protection. To the best of our knowledge, we demonstrated for the first time in the literature that the application of short periods of rapid (600 beats min^{-1}) ventricular pacing at the beginning of reperfusion reduces infarct size and reperfusion-induced arrhythmias.

In the present study, both RVP and classic IPost decreased infarct size. The beneficial effect of RVP on infarct size was further confirmed by a reduction in LDH release into the coronary effluent. Infarct size is a key determinant of major clinical outcomes (mortality and morbidity of consequent heart failure) (Gibbons *et al.*, 2004); therefore, development of procedures that effectively decrease infarct size along with reperfusion therapy is in the focus of preclinical and clinical studies (Ovize *et al.*, 2010). IPost is a widely studied approach, and the infarct size-reducing effect of this procedure was confirmed in various mice, rat, rabbit, dog and swine animal models (Skyschally *et al.*, 2009b) as well as in clinical trials (Ovize *et al.*, 2010). However, some studies reported the ineffectiveness of IPost in animal models (Dow and Kloner, 2007; Skyschally *et al.*, 2009b) and in clinical trials (Hahn *et al.*, 2013). A possible explanation for the controversial results could be that the cardioprotective effect of IPost depends upon several factors such as (i) species, strain, gender and age of research animal; (ii) experimental model and set-up; (iii) the duration of index ischaemia before reperfusion; (iv) number and duration of brief I/R cycles; (v) technical difficulty to achieve complete reperfusion; (vi) temperature; and (vii) presence of co-morbidities. These confounding factors indicate the necessity to develop alternative methods of IPost and we suggest that RVP-induced postconditioning is a simple method that eliminates technical problems associated with the induction of IPost.

Besides infarct size reduction, RVP-induced postconditioning decreased reperfusion-induced ventricular arrhythmias as well. Reperfusion therapy is accompanied by the occurrence of arrhythmias (Krumholz and Goldberger, 1991). Some of them are benign (e.g. accelerated idioventricular rhythm, the most common type) but others are potentially life-threatening malignant arrhythmias such as VT or VF that need to be managed in the clinical practice to avoid fatal consequences. Based on the literature data (Kloner *et al.*, 2006), IPost effectively decreases ventricular arrhythmias. However, in our present study, solely RVP-induced postconditioning reduced the incidence of reperfusion-induced VT with no significant effect on VF. The reason for the inability of RVP to improve post-ischaemic VF is not clear. However, one may speculate that some interacting triggers of reperfusion-induced VF (e.g. reactive oxygen intermediates and calcium) may interfere with the possible anti-VF effect of RVP (Hearse and Tosaki, 1988).

Here, we demonstrated that IPost and RVP-induced postconditioning enhanced peroxynitrite formation at the onset of reperfusion after an index ischaemia. In addition, postconditioning manoeuvres themselves (i.e. brief I/R and RVP) increased peroxynitrite formation in the absence of the index ischaemia. Since peroxynitrite is reported as a possible trigger of IPost (Kupai *et al.*, 2009), based upon our current results, we propose that the enhanced peroxynitrite formation also plays a role in triggering RVP-induced postconditioning. Back in 1997, Yasmin *et al.* reported that the level of peroxynitrite increases during reperfusion, which contributes to reperfusion injury in isolated rat hearts (Yasmin *et al.*, 1997). Further studies also confirmed that enhanced peroxynitrite formation plays a central role in numerous cardiovascular diseases by inducing oxidative, nitrative and nitrosative stress (Pacher *et al.*, 2007). However, peroxynitrite was demonstrated to have physiological functions (Lefer *et al.*, 1997) and to play a role in triggering ischaemic preconditioning (Altug *et al.*, 2000; Altup *et al.*, 2001; Csonka *et al.*, 2001). We have previously reported for the first time that peroxynitrite is a trigger of IPost since the peroxynitrite scavenger, FeTPPS, interfered with the cardioprotective effect of IPost (Kupai *et al.*, 2009). Our results were confirmed by Li *et al.* showing that perox-

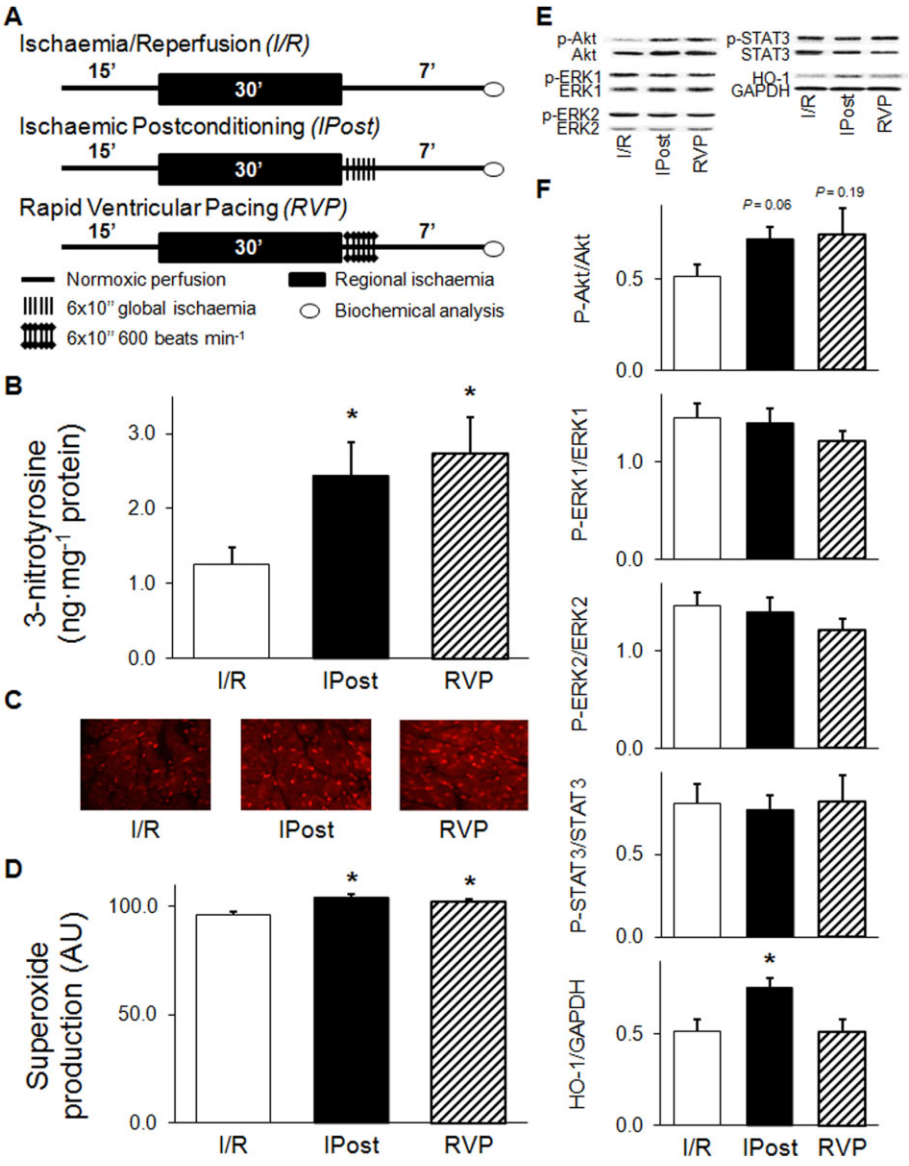

Figure 4

Postconditioning by RVP enhances formation of peroxynitrite and superoxide anion, effects on possible downstream targets. Experimental protocol (A), level of free cardiac 3-nitrotyrosine (B), representative images of *in situ* superoxide detection (C), quantification of *in situ* superoxide anion level (D), representative images (E) and quantification (F) of Western blots of possible downstream targets. Hearts were subjected to a 15 min equilibration period, followed by 30 min of regional ischaemia and 7 min reperfusion with or without ischaemic postconditioning or RVP. At the end of reperfusion, myocardial samples were taken from the ischaemic zone of the left ventricle for biochemical analysis. The peroxynitrite marker, 3-nitrotyrosine, was quantified by ELISA ($n = 5$ in each group). Transverse cardiac sections from three hearts per group were used for *in situ* detection of superoxide anion ($n = 60$ random images in each group). Activation of RISK (Akt, ERK1/2) and SAFE (STAT3) pathways as well as protein level of HO1 was assessed by Western blot. Values are expressed as mean ± SEM. $^*P < 0.05$ versus I/R, one-way ANOVA. p-Akt, phospho(Ser^{473})-Akt; p-ERK1, phospho(Thr^{202})-ERK1; p-ERK2, phospho(Tyr^{204})-ERK2; p-STAT3, phospho(Tyr^{705})-STAT3.

ynitrite is a key mediator of IPost *in vivo* (Li *et al.*, 2013). Nevertheless, the possible mechanisms lying downstream of peroxynitrite formation in postconditioning have not been elucidated.

Here, we also looked at possible targets of endogenous peroxynitrite formation induced by IPost or by RVP. Several studies have reported that the activation of RISK (Akt, ERK1/ERK2) and SAFE (STAT3) pathways at the onset of reperfusion might play a role in the cardioprotective effect of IPost (Hausenloy, 2009; Lecour, 2009). In other studies, overexpression of HO1 was shown to reduce infarct size in the heart (Bak *et al.*, 2010) and was implicated in pulmonary and hepatic IPost (Xia *et al.*, 2009; Zeng *et al.*, 2011). In our present study, both IPost and RVP-induced postconditioning non-significantly enhanced Akt phosphorylation without affecting ERK1/2 and STAT3 at the beginning of reperfusion. Although several studies showed increased phosphorylation of Akt and/or ERK due to IPost (Tsang *et al.*, 2004; Yang *et al.*,

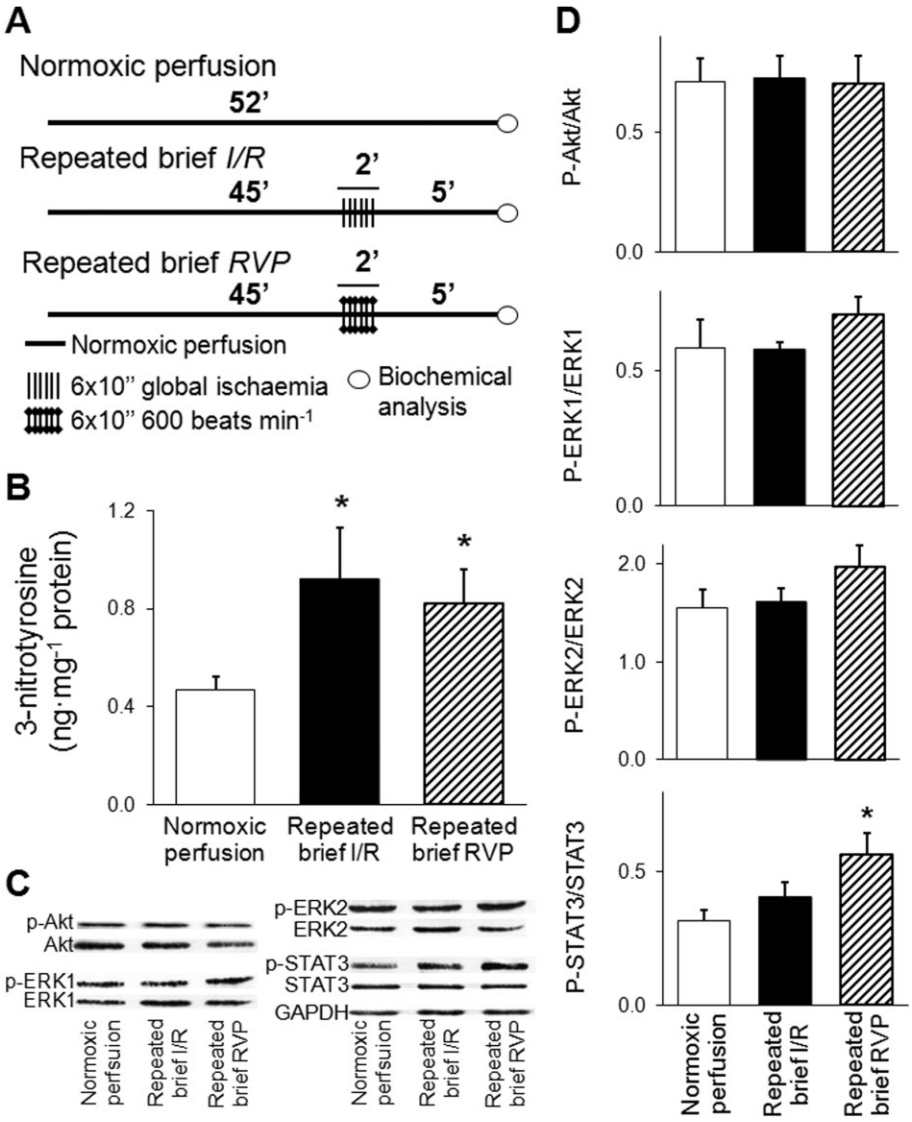

Figure 5

Postconditioning manoeuvres without a preceding index ischaemia enhance peroxynitrite formation, effects on possible downstream targets. Experimental protocol (A), level of free cardiac 3-nitrotyrosine (B), and representative images (C) and quantification (D) of Western blots of possible downstream targets. After 45 min normoxic perfusion, repeated (6 × 10/10 s) brief cycles of no-flow global I/R ($n = 7$) or RVP at 600 beats min^{-1}/spontaneous rhythm of the hearts ($n = 8$) were applied followed by 5 min perfusion. In the normoxic perfusion control group ($n = 8$), hearts were perfused for 52 min. At the end of perfusion, cardiac free 3-nitrotyrosine level was determined by ELISA and activation of RISK and SAFE pathways were examined by Western blots. Values are expressed as mean ± SEM. *$P < 0.05$ versus normoxic perfusion control, one-way ANOVA. p-Akt, phospho(Ser^{473})-Akt; p-ERK1, phospho(Thr^{202})-ERK1; p-ERK2, phospho(Tyr^{204})-ERK2; p-STAT3, phospho(Tyr^{705})-STAT3.

2004), some recent papers suggested that postconditioning did not activate the RISK pathway in the early phase of reperfusion (Skyschally *et al.*, 2009a; Fekete *et al.*, 2013). We also found here that IPost, but not RVP, increased HO1 protein in the heart. This effect of IPost on HO1 is in agreement with the findings of others in the lung and liver (Xia *et al.*, 2009; Zeng *et al.*, 2011). We also examined the effect of postconditioning manoeuvres (i.e. repeated brief cycles of I/R or RVP) in the absence of a preceding index ischaemia and found no activation of the RISK pathway. In these experiments, STAT3 phosphorylation was increased only by short periods of RVP protocol. Taken together, our present results indicate that (i) the downstream mechanisms of RVP-induced cardioprotection and IPost are partially different; (ii) HO1 is probably not involved in the cardioprotective effect of RVP-induced postconditioning; and (iii) the precise role of the RISK and SAFE pathways remains to be elucidated in future studies. The involvement of alternative pathways in the protective effect of RVP-induced postconditioning is likely and may include, for instance, activation of NO-cGMP-PKG, sphingosine-, PKC- or CGRP-mediated pathways (Heusch *et al.*, 2008; Bice and Baxter, 2014). Since endogenous NO-cGMP plays a role in protection against reperfusion injury by attenuating infarct size (Penna *et al.*, 2006) and

reperfusion-induced VF (Pabla *et al.*, 1995; Pabla and Curtis, 1996), investigation of the exact role of NO in RVP would be interesting.

Although we clearly demonstrated that RVP induces cardioprotection when applied at the onset of reperfusion, some further limitations of our study may be considered. Firstly, ventricular pacing was reported to have direct pro-arrhythmic effects caused by the stimulus itself independently of the heart rate (Nakata *et al.*, 1990). Although in our study ventricular pacing lasts only for short periods (6×10 s), and the incidence of reperfusion-induced VF was not increased in the RVP group when compared to I/R controls, consideration of pacing as an ectopic focus cannot be excluded. Secondly, in RVP-induced postconditioning, ventricles were activated in a non-physiological way in the present *ex vivo* study. Although the atrio-ventricular conduction system of rats was reported to be suitable for reaching 600 bpm heart rate by atrial pacing in an *in vivo* model (Gonzalez *et al.*, 1998), further *in vivo* studies are needed to investigate the infarct size-limiting effect of postconditioning induced by rapid atrial or ventricular pacing at different rates. Thirdly, our study suggests that rapid heart rate at the early phase of reperfusion may contribute to initiation of adaptive molecular mechanisms to prevent I/R-induced cellular damage. However, further studies are needed to analyse (i) the precise molecular nature of these mechanisms and (ii) if reperfusion-induced spontaneous arrhythmias also trigger adaptive mechanisms in the myocardium. Our findings may also suggest that reperfusion-induced tachyarrhythmias require attention in future studies focusing on cardioprotection assessed by infarct size.

In conclusion, the application of short periods of RVP at the onset of reperfusion beneficially affects the essential components of reperfusion injury: the infarct size and reperfusion-induced ventricular arrhythmias. In addition, RVP increases peroxynitrite formation, which likely plays a role in triggering cardioprotection similarly to IPost. Nevertheless, downstream mechanisms in RVP-induced protection seem to be partially different from that of IPost, and further research is needed to elucidate them. Since RVP exerted a cardioprotective effect similar to IPost, we feel that RVP-induced postconditioning may serve as an alternative experimental model of IPost. Moreover, RVP could be performed in a more controlled manner than applying brief I/R cycles in IPost, which is an important technical advantage compared with IPost.

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Author contributions

Z. V. V. and T. C. designed the experiments. M. P., Z. V. V., K. K., G. F. K. and R. G. performed the research. M. P., Z. V. V. and C. C. analysed the data. C. C. and T. C. interpreted the data. M. P. drafted the manuscript. M. P., Z. V. V. and T. C. revised the manuscript. M. P., Z. V. V., K. K., R. G., G. F. K., C. C. and T. C. approved the final version of the manuscript.

Conflict of interest

Not declared.

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