

Original Article

Ischemic postconditioning reduces the anoxia/reoxygenation injury in human umbilical vein endothelial cells via p-Akt pathway

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Abstract: Object: To investigate the role of ischemic postconditioning (IPostC) in human umbilical vein endothelial cells (HUVECs) under anoxia/reoxygenation (A/R) injury and the potential protective mechanism. Methods: This study was carried out in Children's Hospital of Zhejiang University School of Medicine, during 10 June and 10 December 2014. HUVECs were assigned to the normal (n=6), anoxia (reoxygenation followed by a persistent anoxia for 3 h, n=6) and postconditioning (PostC) groups. The PostC groups then divided into 4 subgroups (10 s × 3, 10 s × 6, 30 s × 3, 60 s × 3, n=6 each) respectively. The apoptosis rate of HUVECs was determined by flow cytometry, levels of lactate dehydrogenase (LDH) in supernatants were detected and the protein expression of p-Akt was measured by Western blotting. Results: Compared with the anoxia group, the PostC groups (especially 10 s × 6 group) showed significant reduction of both the early and late apoptosis rate. The elevation of LDH levels were also markedly diminished, while the expression of p-Akt was up-regulated in HUVECs after PostC (10 s × 6). Conclusion: IPostC showed significant protective effects against A/R injury on HUVECs. Repetitive short A/R period and more cycles trended to be more effective and the potential mechanism may be associated with the activation of p-Akt expression of RISK pathway.

Keywords: Ischemic postconditioning, anoxia/reoxygenation, p-Akt, HUVECs, ischemia reperfusion injury

Introduction

Ischemia/reperfusion (I/R) can lead to local and systemic injury, which involved in variety of clinical conditions, such as myocardial infarction, stroke and organ transplantation, etc [1, 2]. As the crucial site, endothelial cells are frequently affected by I/R injury [3, 4]. Data have demonstrated that under I/R, endothelial cells present pro-inflammatory activity including the induction of leukocyte adhesion molecules, vasoconstrictive agents, and procoagulant factors [5], which finally lead to the dysfunction of energy metabolism. Thus, therapeutic strategies for preserving endothelial function are of great clinical values.

Ischemic preconditioning (IPC) has been confirmed to increase endothelial resistance to

ischemic injury [4, 6]. However, the widely application of IPC has been limited due to the unpredictability of clinical ischemic events and ethical problems. Compared to the ischemic episodes, the onset of reperfusion is more predictable. Thus, a novel promising intervention for clinical reperfusion type of injury named ischemic postconditioning (IPostC) was first described by Zhao et al [7]. It consists of several short periods of reperfusion/ischemia cycles, and proved the cardioprotection in reperfusion injury. Similarly organ protections were latterly confirmed in animal model of heart [8], kidney [9], brain [10], testis [11], intestine [12] and liver [13] I/R injury.

However, the protective effects varied from different species and researches, which may be explained by the lack of optimal algorithm and

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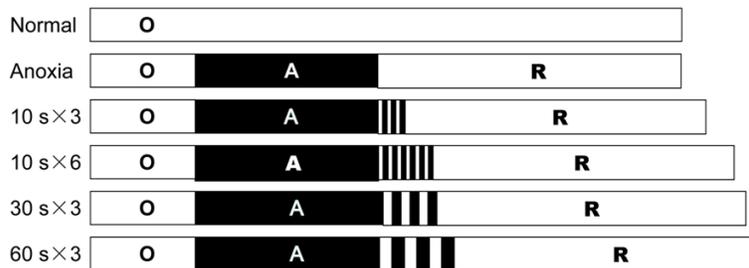


Figure 1. Anoxia postconditioning (PostC) protocol and related groups. HUVECs were assigned to the Normal, Anoxia (reoxygenation followed by a persistent anoxia for 3 h) and PostC groups. The PostC groups then divided into 4 subgroups (10 s × 3, 10 s × 6, 30 s × 3, 60 s × 3, n=6 each) respectively. PostC was initiated at the end of anoxia for different time intervals, according to the protocols. Note: O stands for normal oxygen culture (95% O₂ and 5% CO₂), A stands for anoxia (95% N₂ and 5% CO₂) culture, R stands for reoxygenation (95% O₂ and 5% CO₂) culture.

the unclarified mechanism of IPostC. The same protocol as Zhao et al [7] used in dog, showed no protection in another two studies in pigs [14, 15]. However, change the number of cycles or duration of ischemia and reperfusion intervals, protection could be obtained [15, 16]. Besides, Ashish et al discovered the intravenous use of p-Akt inhibitor 10 min before ischemia can reduce the protective effects of IPostC [17]. Another recent animal study proved the up-regulating of p-Akt may be involved in the mechanism of IPostC [18]. The present study establishes an anoxia/reoxygenation (A/R) model to imitate I/R injury, and aims to determine the protective effects of IPostC on human umbilical vein endothelial cells (HUVECs) under A/R condition, further to investigate the optimal algorithm and the potential mechanism of IPostC.

Materials and methods

This study was carried out in Children's Hospital of Zhejiang University School of Medicine, during 10 June and 10 December 2014. All procedures used in this study were approved by the Ethics Committee of Zhejiang University (China).

Cell culture

HUVECs were acquired from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Chinese Academy of sciences) and were cultivated according to the instructions. HUVECs were used less than sixth passages. We had two systems of cell culture: one is normal cell culture system which consists 95% O₂ and 5% CO₂ while the anoxia system consists of 95% N₂

and 5% CO₂. HUVECs were incubated at 37°C humidified environment containing 5% CO₂ and 95% N₂ (Anoxia condition), while reoxygenation condition was incubated at 37°C humidified environment containing 5% CO₂ and 95% O₂. We achieved A/R by rapidly change cells between two independent systems.

Experimental design and groups

HUVECs were assigned to six groups: normal (6 h for normal oxygen incubation, n=6),

anoxia (3 h anoxia followed by 3 h persistent oxygen, n=6) and PostC groups (3 h anoxia, then PostC, finally followed 3 h reoxygenation, n=24). The PostC groups then divided into 4 subgroups according to different period and cycles of reoxygenation (10 s × 3, 10 s × 6, 30 s × 3, 60 s × 3, n=6 each) respectively. PostC was initiated at the end of anoxia for different time intervals and cycles, according to the protocols (**Figure 1**). Take PostC (10 s × 6) for example, HUVECs were taken out from normal oxygen culture condition after 3 h incubation, then HUVECs were immediately put into 10 s anoxia culture followed 10 s normal oxygen culture for 6 cycles (PostC 10 s × 6). Finally, the cells were put into reoxygenation culture system for another 3 h.

Sample collection

3 h after the reoxygenation, supernatants were collected for LDH levels; HUVECs were collected for apoptosis analysis by flow cytometry staining. The protein of HUVECs was extracted for western blot.

LDH detection

Supernatants samples were centrifuged at 1,000 × g for 10 min at 4°C for LDH analysis according to the instruction of LDH assay kit (Nanjing Jiancheng Bioengineering Institute, China).

Flow cytometry staining for apoptosis

To analysis the apoptosis rate, HUVECs were incubated with Annexin V: FITC Apoptosis Dete-

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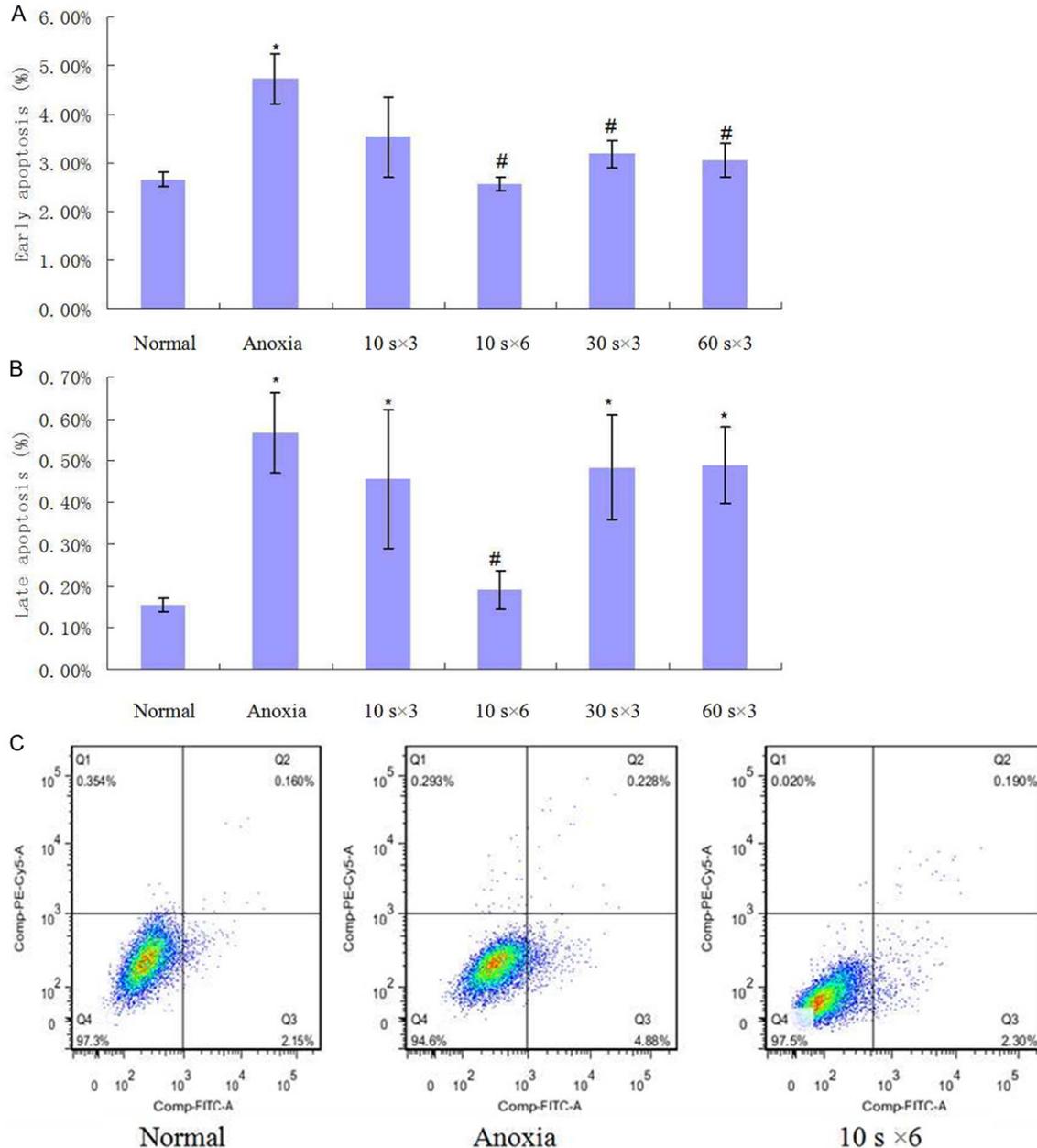


Figure 2. The apoptosis level of each group. A. Quantified early apoptosis of each group. Compared with the Normal group, all the other groups increased the rate of early apoptosis (vs. Anoxia $P=0.002$, vs. $10\text{ s} \times 3$ $P=0.173$, vs. $10\text{ s} \times 6$ $P=0.899$, vs. $30\text{ s} \times 3$ $P=0.414$, vs. $60\text{ s} \times 3$ $P=0.536$); PostC diminished the elevation of early apoptosis, especially the $10\text{ s} \times 6$ group ($P=0.002$) when compared with Anoxia group at 3 h of reoxygenation (vs. $10\text{ s} \times 3$ $P=0.066$, vs. $30\text{ s} \times 3$ $P=0.019$, vs. $60\text{ s} \times 3$ $P=0.012$). Among the four PostC groups, the $10\text{ s} \times 6$ PostC group showed stronger protective effects ($n=6$ in each group. Values are mean (SD) * $P<0.05$ versus with Normal, # $P<0.05$ versus with Anoxia group). B. Quantified late apoptosis of each group. Compared with the Normal group, all the other groups increased the rate of late apoptosis (vs. Anoxia $P=0.008$, vs. $10\text{ s} \times 3$ $P=0.046$, vs. $10\text{ s} \times 6$ $P=0.803$, vs. $30\text{ s} \times 3$ $P=0.031$, vs. $60\text{ s} \times 3$ $P=0.028$); PostC diminished the elevation of late apoptosis, especially the $10\text{ s} \times 6$ group ($P=0.015$) when compared with Anoxia group at 3 h of reoxygenation (vs. $10\text{ s} \times 3$ $P=0.454$, vs. $30\text{ s} \times 3$ $P=0.569$, vs. $60\text{ s} \times 3$ $P=0.601$). Among the four PostC groups, the $10\text{ s} \times 6$ PostC group showed stronger protective effects. ($n=6$ in each group. Values are mean (SD) * $P<0.05$ versus with Normal, # $P<0.05$ versus with Anoxia group). C. Initial flow cytometry scatter plot from Flowjo software of Normal, Anoxia and PostC $10\text{ s} \times 6$. The right low panel stands for the early apoptosis and the right up panel stands for late apoptosis as defined in the Flow cytometry staining for apoptosis part. A and B. Were quantified from these initial flow cytometry scatter plot ($n=6$ in each group). Note: Y axis was propidium iodide while X axis was Annexin V.

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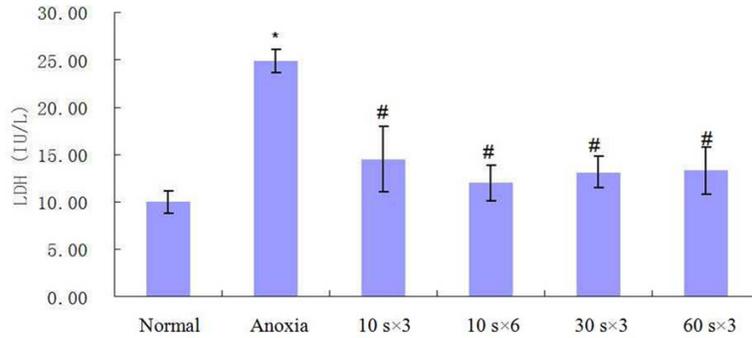


Figure 3. Supernatant LDH levels of each group. Compared with the Normal group, levels of LDH were increased in all the other groups (vs. Anoxia $P < 0.001$, vs. $10 \text{ s} \times 3$ $P = 0.144$, vs. $10 \text{ s} \times 6$ $P = 0.510$, vs. $30 \text{ s} \times 3$ $P = 0.300$, vs. $60 \text{ s} \times 3$ $P = 0.275$); PostC diminished the elevation of LDH level, especially the $10 \text{ s} \times 6$ group ($P < 0.001$) when compared with Anoxia group at 3 h of reoxygenation (vs. $10 \text{ s} \times 3$ $P = 0.002$, vs. $30 \text{ s} \times 3$ $P = 0.001$, vs. $60 \text{ s} \times 3$ $P = 0.001$). Among the four PostC groups, the $10 \text{ s} \times 6$ PostC group showed stronger protective effects. ($n = 6$ in each group. Values are mean (SD) * $P < 0.05$ versus with Normal, # $P < 0.05$ versus with Anoxia group).

ction Kit II (BD Bioscience, CA) for 15 minutes at 4°C in the dark. HUVECs were running on multichannel flow cytometry (Beckman, USA) and analyzed using Flowjo 7.6.5 software. The early apoptosis was defined as Annexin V positive and propidium iodide negative while late apoptosis was Annexin V and propidium iodide both positive.

Measurement of p-Akt expression

Proteins were extracted from HUVECs as previously described [19], quantified using Bradford assay according to the instruction. Then, proteins were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were incubated with rabbit polyclonal anti-p-Akt antibody (Ser473, 1:1000; Signalway antibody, USA) or mouse monoclonal anti- β -actin antibody (1:1000; Dawen bioscience, China) over night at 4°C after blocking with 5% fetal bovine serum in tris-buffered saline with tween (TBST, pH 7.2, 0.05% Tween 20). Membranes were washed three times with TPST (15 min each) and incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (1:2000; Dawen bioscience, China) for 2 h. Western blots were developed with the enhanced chemiluminescence system (ECL kit; Pierce Biotechnology, Rockford, IL, USA) and captured on light-sensitive imaging film. The lane density was converted into chart by image J software.

Statistical analysis

All results are presented as mean \pm SD. Data were calculated with one-way analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparisons. Statistical significance was defined as $P < 0.05$.

Results

Postconditioning diminished the elevation of early and late apoptosis

Compared to the normal group, the anoxia group increased the rate of early apoptosis significantly ($P = 0.002$);

PostC diminished the elevation of early apoptosis, especially the $10 \text{ s} \times 6$ group ($P = 0.002$) when compared with anoxia group at 3 h of reoxygenation (vs. $10 \text{ s} \times 3$ $P = 0.066$, vs. $30 \text{ s} \times 3$ $P = 0.019$, vs. $60 \text{ s} \times 3$ $P = 0.012$) (Figure 2A). Similarly, the late apoptosis rate were also increased in anoxia groups compared to the normal group ($P = 0.008$); PostC reduced the elevation of late apoptosis, especially the $10 \text{ s} \times 6$ group ($P = 0.015$) when compared with anoxia group at 3 h of reoxygenation (Figure 2B). Among the four PostC groups, the $10 \text{ s} \times 6$ PostC group showed stronger protective effects in both early and late apoptosis (Figure 2C).

Postconditioning attenuated the elevation of LDH level

Supernatants levels of LDH were significantly increased in the anoxia group compared to the normal group ($P < 0.001$); PostC attenuated the elevation of LDH level, especially the $10 \text{ s} \times 6$ group ($P < 0.001$) when compared with anoxia group at 3 h of reoxygenation (vs. $10 \text{ s} \times 3$ $P = 0.002$, vs. $30 \text{ s} \times 3$ $P = 0.001$, vs. $60 \text{ s} \times 3$ $P = 0.001$). Among the four PostC groups, the $10 \text{ s} \times 6$ PostC group showed stronger protective effects (Figure 3).

Postconditioning up-regulated the p-Akt protein expressions

The expression levels of p-Akt protein were detected by western blot. The data showed that

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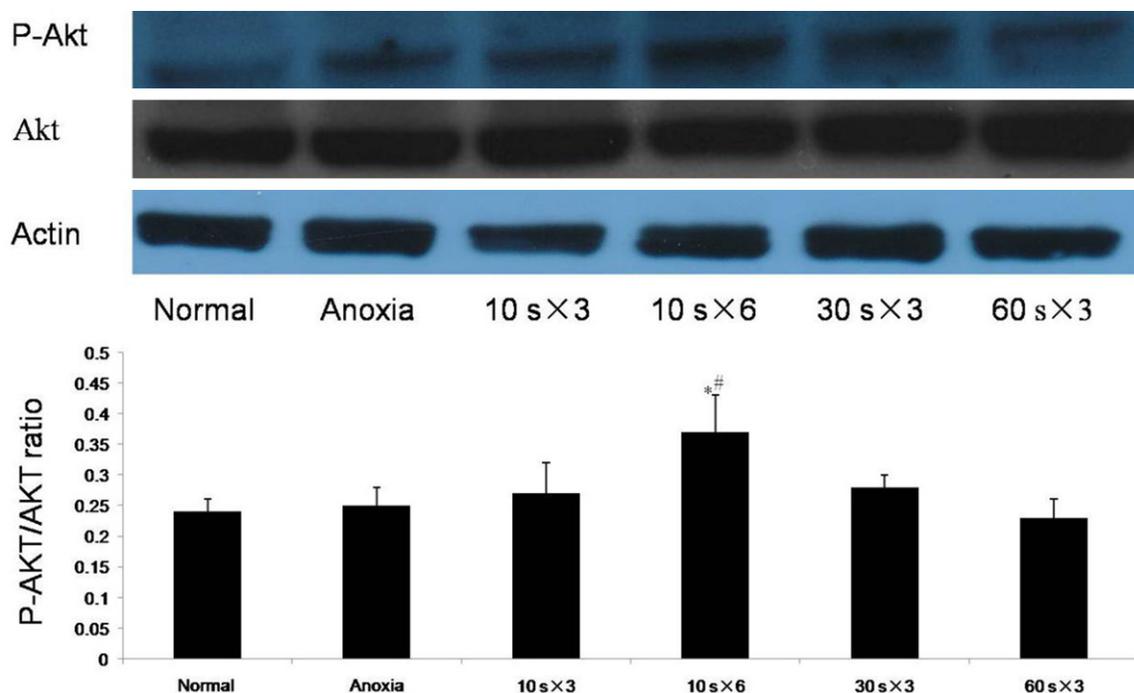


Figure 4. p-Akt/AKT expression of each group. Compared with the Normal group, all the other groups increase the expression of p-Akt, especially the 10 s × 6 group. The lane density was converted into chart by image J software. (n=6 in each group. Values are mean (SD) *P<0.05 versus with Normal, #P<0.05 versus with Anoxia group).

the expression levels of p-Akt in HUVECs were markedly increased in the PostC group (10 s × 6) compared with normal (P=0.047) or anoxia group (P=0.045) (Figure 4).

Discussion

This experiment optimized the algorithm and investigated the underlying protective mechanism of IPostC with an A/R injury model of HUVECs. Our data revealed that IPostC could effectively protect HUVECs from A/R injury, and repetitive short A/R period and more cycles trended to be more effective. The protective effects of IPostC were related to the reduction of apoptosis rate and LDH level. It also up-regulated the expression of p-Akt, which suggested that the potential protective mechanism, at least partially was associated with the activation of p-Akt pathway.

Although IPostC showed confirmatory protection in both animal and human, the degree of protection varied from species and researches. In Zhao's study, dogs' hearts were treated with 60 minutes regional ischemia followed by 3 cycles of 30 s ischemia/30 s reperfusion, showed a promising cardioprotection [7]. While

similar protocol used in pig showed no protection, but 8 cycles 30 s ischemia/30 s reperfusion did protect [15]. Vinten-Johansen et al proposed that smaller rodent hearts required shorter cycles of PostC than larger ones [20]. An open-chest rabbit model revealed that 3 cycles of 10 s ischemia/10 s reperfusion did not protect, while 3 cycles of 20 s ischemia/20 s reperfusion were protective [21]. In addition, the duration of index ischemia is also important in IPostC, the infarct sizes of rats were largely diminished after the IPostC with an index ischemia of 30 or 45 min [22]. However, too brief index ischemia showed no reduction in infarct size [23]. Therefore, the optimal algorithm of IPostC is critical to the degree of protection. Three factors should be considered in optimizing IPostC algorithm: the duration of index ischemia, the period of ischemia and reperfusion, and the number of cycles [24]. In present study, we designed different PostC groups according to different cycles and duration of each ischemia/reperfusion. All the PostC groups showed protective effects on HUVECs against A/R injury, as measured by cell function and apoptosis rate. IpostC significantly attenuated the elevation of LDH level and apoptosis.

Among the PostC groups, the 10 s × 6 group showed stronger protective effects than the other three groups, although the differences were not significant. This is consistent with the previous study focused on the optimum PostC algorithm for I/R injury in myocardium [22]. We speculate that repetitive short stimulus with more cycles is more protective in IPostC and optimizing the algorithm of IPostC may be associated with the efficiency of protection. More researches are needed to optimizing the algorithm of IPostC for various clinical conditions.

The protective mechanisms of IPostC against I/R injury are complex and still not clarified yet. Signal pathways in postconditioning were widely studied these years, two major pathways are RISK pathway (PI3K-Akt and p42/p44 ERKs) [25, 26] and SAFE pathway (JAK/STAT3) [27] respectively. Previous study revealed that, the RISK pathway could inhibit mitochondrial permeability transition pore (MPTP) open at reperfusion, via the downstream components including eNOS/NO and inhibition of GSK3 β [24]. Evidence showed the combination of TNF-α and TNFR2 could activate the JAK/STAT3 pathway, STAT3 controls the transcription of factors that confer cardioprotection after translocation to the nucleus [27]. Recent study showed mitochondrial STAT3 was also related with the inhibition of MPTP open [28]. The formation of the MPTP would lead to mitochondrial rupture and finally result in necrosis of cells [29]. Thus, both the RISK and SAFE pathways appear to converge on mitochondria which may be the end effect or for the protection. Additionally, PI3K-Akt/eNOS pathway was proved to play an important role against the reperfusion injury by regulating cellular apoptosis, activation and inflammatory responses [26]. Akt is an initiator of downstream pathways that inhibit the apoptotic routes by inhibiting the expression of proapoptotic Bax [30]. In present study, the elevation of early and late apoptosis was significantly diminished in PostC groups, especially the 10 s × 6 group. Meanwhile the PostC group (10 s × 6) significantly enhanced Akt phosphorylation (Ser 473) at 3 hours after reperfusion in HUVECs compared with anoxia group. These data were consistent with prior studies, we can conclude that IPostC protected the HUVECs from A/R injury via activating p-Akt pathway, however further studies are still needed for the detail roles of p-Akt in I/R injury.

In conclusion, IPostC diminished apoptosis and LDH level of HUVECs after A/R injury. Repetitive short A/R period and more cycles trended to be more effective and the underlying mechanism may be associated with the activation of p-Akt pathway.

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Disclosure of conflict of interest

None.

Authors' contribution

(1) Conceived and designed the study (Jun-Fen Fu, Jun-Jun Jia); collected the data (Xue-Lian Zhou, Ken Chen, Pei Qian); analyzed and interpreted the data (Xue-Lian Zhou, Jun-Jun Jia); (2) wrote the manuscript or provided critical revisions important for intellectual content (Xue-Lian Zhou, Jun-Jun Jia); and (3) approved the final version of the manuscript (Jun-Fen Fu).

Abbreviations

I/R, Ischemia/reperfusion; A/R, Anoxia/reoxygenation; IPC, Ischemic preconditioning; IPostC, Ischemic postconditioning; HUVECs, Human umbilical vein endothelial cells; LDH, Lactate dehydrogenase.

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