Combined postconditioning with ischemia and cyclosporine-A restore oxidative stress and histopathological changes in reperfusion injury of diabetic myocardium

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Introduction

Diabetes is a main and independent risk factor for coronary artery disease and cardiovascular death, the leading cause of death worldwide (1-3). The mortality rate due to heart ischemic disorders is 2-3 times higher in diabetic subjects compared with non-diabetic ones and the progressive incidence of diabetes increases the burden of cardiovascular disease (4). This warrants the importance of primary prevention against myocardial ischemia/reperfusion (I/R) injury in diabetic patients.

Apoptosis is the most famous form of programmed cell death (PCD) that may occur in multicellular organisms. Myocardial reperfusion injury as well as diabetes are associated with significantly increased cardiomyocyte necrosis and apoptosis (5-8). These are known as examples of pathologic apoptosis (9). Mitochondrial permeability transition pore (mPTP) is crucial to apoptotic pathways; by its opening during the onset of reperfusion or in other circumstances including diabetes, cytochrome C is released from the mitochondria into the cytosol and this, in turn, induces the next phases of cell apoptosis (4, 10, 11). It is well documented that blocking the apoptosis process could prevent the loss of contractile cells, minimize cardiac I/R injury and therefore slow down the occurrence of heart failure (12, 13). CsA is an immunosuppressant which also can selectively inhibit mPTP opening (14). By this mechanism, it poses significant cardioprotective effects and these promising effects have been reported in previous studies on myocardial I/R injury in subjects...
and animal models without any comorbidities (4, 6, 15).

Cardioprotection induced by ischemic preconditioning and postconditioning is related to the inhibition of mPTP via upregulation of the survival signaling pathways including the PI3K/Akt pathway and thereby inactivation of glycogen synthase kinase-3beta (GSK-3β) in cardiac cells (15-17). Activation of the PI3K/Akt/GSK-3β pathway is an effective way to reduce cardiomyocyte necrosis and cardiac I/R injury (6, 12, 18, 19).

Oxidative stress is a typical feature of myocardial I/R injury, and also constitutes a unifying mechanism of injury of many types of disease processes such as diabetes (20-24); it occurs when the antioxidant defense systems in the body are overwhelmed by the extensive generation of reactive oxygen species (25-29). Oxidative stress in the cell is closely linked to the activation of GSK-3β and mPTP opening (4, 6, 28, 30).

It has been reported that chronic diabetes renders the heart more sensitive to I/R injury (4, 15, 31). In addition, the protective ischemic postconditioning (IPostC) strategy could not protect the diabetic myocardium from reperfusion injury (15, 31, 32). The significant interaction of diabetes with cardiovascular disease explains the imperious need to investigate the underlying mechanism responsible for this interaction, and the capabilities of exerting protection effects during I/R injury in diabetic myocardium. Therefore, the aim of this study was to identify whether it is possible to protect diabetic myocardium against I/R injury through IPostC application and/or mPTP inhibition and the role of cardiomyocytes oxidative stress in this regard, if any.

**Materials and Methods**

**Materials**

Streptozocin (STZ) was supplied by Tocris Company (London, UK). All materials of Krebs solution were obtained from Merck Company (Germany). Superoxide dismutase (SOD), glutathione peroxidase (GPX), and total antioxidant capacity (TAC) detection kits were provided by Randox (UK). Finally, lactate dehydrogenase (LDH) detection kit was obtained from Roche Diagnostics (Germany) and 8-isoprostanen acid detection kit was purchased from Cayman Chemicals (USA).

**Animals**

40 adult male Wistar rats (250–350 g) were obtained from animal house of Tabriz University of Medical Sciences (TUMS); 5 per subgroup. All animals received humane treatment in accordance with the guide for the Care and Use of Laboratory Animals (National Institutes of Health, Publication no. 85–23, revised 1996). The experimental procedures were approved by the Local Animal Ethics Committee. At the beginning of the experiment, the animals were randomly divided into two main categories of diabetic and non-diabetic (control) groups. The animals were housed under standard laboratory conditions at 24±2 °C, relative humidity of 55±5%, and 12 hr dark and 12 hr light. All animals were allowed free access to food and water.

**Induction of diabetes**

Diabetes was induced by a single intraperitoneal injection of STZ, 50 mg/kg body weight; STZ was dissolved in 0.1 M citrate buffer, pH 4.5). STZ lead to disruption of pancreatic islet cells and reduced secretion of insulin resulting in type 1 diabetes mellitus. Development of the diabetes was confirmed 72 hr later by measuring blood glucose levels using a glucometer device through the sampling of blood by slightly scratching rat tails. The animals with blood glucose levels higher than 300 mg/dl were considered diabetic, as previously described (15). Blood glucose testing was also repeated at fourth and eighth weeks after STZ injection. After 8 weeks from STZ administration (chronic diabetes (31)), the diabetic animals as well as the controls were sacrificed and all experiments were performed on isolated perfused beating hearts in Langendorff set up.

**Induction of regional ischemia/reperfusion and ischemic postconditioning**

The isolated hearts were retrogradely perfused via the aorta with a Krebs–Henseleit solution containing (in mM/l): NaCl 118; KCl 4.7; CaCl₂ 2.5; MgSO₄ 1.2; NaHCO₃ 25; KH₂PO₄ 1.2; glucose 11.1. The perfusion pressure of the solution was adjusted constantly at 75 mmHg and the solution was gassed with a mixture of 95% O₂, 5% CO₂ at 37 °C and pH 7.4. After a stabilization period of 15 min, all hearts were subjected to regional ischemia for 30 min followed by reperfusion for 45 min. Regional ischemia and reperfusion were induced by occluding and re-opening of left anterior descending (LAD) coronary artery, using a 5-0 silk ligature placed around the LAD, close to its origin, respectively. An immediate fall in coronary flow at the onset of index ischemia (to about 30-40% of its baseline value) and the recovery of the coronary flow upon reperfusion served as evidence of effective coronary occlusion and reperfusion (15, 33). Ischemic postconditioning (IPostC) in corresponding groups (see below) was induced by three cycles of 30 sec reperfusion and ischemia (3 cycles of 30 sec R/I), applied immediately at the onset of reperfusion (15, 34).

**I/R Experimental protocol**

For I/R experiments, the control and diabetic animals were divided into eight subgroups (n=8) as: 1) Control (C); 2) Control with ischemic postconditioning (C+IPostC); 3) Control with cyclosporine-A (C+CsA); 4) Control with ischemic postconditioning plus cyclosporine-A (C+IPostC+CsA); 5) Diabetic (D); 6) Diabetic with ischemic postconditioning (D+IPostC); 7) Diabetic with cyclosporine-A (D+CsA); and 8) Diabetics with ischemic postconditioning plus cyclosporine-A (D+IPostC+CsA). The isolated hearts in all groups were perfused with a
Krebs-Henseleit solution and underwent a 30 min regional ischemia followed by a 45 min reperfusion. In IPostC receiving groups, the hearts received 3 cycles of 30 sec R/I at the onset of reperfusion. Furthermore, in CsA receiving groups, 5 min before the onset of reperfusion up to 10 min of reperfusion, the hearts were perfused with a Krebs-Henseleit solution containing 0.01 mM CsA as an inhibitor of mPTP. The experimental groups that did not receive CsA, were perfused with a pure Krebs-Henseleit solution at the corresponding time.

**LDH release measurement**

Myocardial cellular damage was evaluated by measuring LDH release in coronary effluent. LDH released from ischemic tissue was determined from coronary effluent collected at 10 min after reperfusion. LDH activity was measured spectrophotometrically with a commercially available assay kit (Roche Diagnostics, Germany). The absorbance of the solution for LDH was detected at 492 nm. The results were reported in U/L.

**Lipid peroxidation marker 8-isoprostane (8-isoP)**

After reperfusion for 45 min, myocardial tissue samples were obtained inferior to the site of ligation (ischemic zone). Then, the tissue samples were weighed accurately and homogenated. The homogenate was centrifuged at 10000 g for 10 min at 4 °C. The protein level of supernatants was determined by the Bradford method. Enzyme-linked immunoassay (ELISA) was used to measure myocardial free 8-isoprostane (8-isoP) levels as a marker of lipid peroxidation according to the methods provided by the manufacturer (Cayman Chemical, USA). Fifty-ml standards and samples were added in duplicate to the 96-well plate provided in the kit, followed by addition of 8-isoprostane acetylcholinesterase tracer and antibody. The prepared plates were then incubated overnight at room temperature. The next day, the plates were washed 5 times with the wash buffer, followed by addition of Ellman’s reagent. After optimal development, the plates were read at 405 nm, and the optical density values were transferred to the final concentration based on protein content of each sample and expressed as pg/mg of protein.

**Superoxide dismutase (SOD)**

SOD activity as an antioxidant enzyme was determined using a commercially available kit according to Kakkar (35). In this method, xanthine and xanthine oxidase were used to generate superoxide radicals. Superoxide radicals react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride in the presence of reduced nicotinamide adenine dinucleotide to form a formazan dye. The SOD was measured by the degree of inhibition of this reaction. The intensity of the color is inversely proportional to the activity of the enzyme. SOD activity in the supernatant was measured at 505 nm by a spectrophotometer.

**Glutathione peroxidase (GPX)**

GPX activity as another antioxidant enzyme was determined according to the method of Rotruck (36). GPX in the tissue homogenate oxidizes glutathione by cumene hydroperoxide and simultaneously, H₂O₂ is reduced to water. This reaction is arrested at 10 min using trichloroacetic acid and the remaining glutathione is reacted with DTNB solution to result in a colored compound, which is measured spectrophotometrically. In the presence of glutathione reductase (at a concentration ≥0.5 units/l) and 0.28 mmol/l of NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NAD⁺. The decrease in absorbance at 340 nm was measured using a spectrophotometer.

**Total antioxidant capacity (TAC)**

TAC in homogenates was measured using a commercial kit, according to the manufacturer’s instruments and with reference to the Miller method (37). Briefly, 1 ml chromogen (metmyoglobin 6.1 μmol/l, ABTS 610 μmol/l) was added with 20 μl of samples as well as a calibrator (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 1.79 mmol/l) and then, the samples and calibrator were incubated in 200 μl substrate (hydrogen peroxide, 250 μmol/l) at 37 °C for 3 min. Thereafter, the final absorbance was detected at 600 nm, spectrophotometrically.

**Histopathological studies**

Myocardial tissue obtained from ischemic zone was immediately isolated and placed in formalin 10%. Briefly, after tissue processing steps, several serial sections of blood vessel segments (5 μm thick) were stained with standard hematoxylin–eosin (H&E) and studied using light microscopy by a blind pathologist. The extent of tissue disorganization was noted for each sample. The grading of severity of histopathologic findings was performed as follows: 1. Normal tissue (−), 2. Edema and increased space between cells (mild), 3. Edema plus inflammatory process (moderate), and 4. Edema, inflammatory process and necrosis (extensive) (38).

**Statistical analysis**

All values were expressed as means±SEM. The between-group variables were analyzed using one-way ANOVA followed by Tukey’s post hoc test. Differences were considered statistically significant when P<0.05.

**Results**

**LDH activity**

The levels of LDH release into the coronary effluent of both control and diabetic subgroups were shown in Figure 1. The LDH release into the coronary effluent was significantly decreased by IPostC or CsA alone as well as by a combination of IPostC and CsA in treated-control subgroups in comparison with the
untreated control subgroup (P<0.05). However, in diabetic hearts only the combination therapy, IPostC, and CsA could significantly reduce the LDH level (P<0.05) and neither IPostC alone nor CsA alone significantly influenced LDH levels as compared with the untreated-diabetic subgroup (Figure 1).

**Lipid peroxidation index: 8-isoprostane (8-isoP) level**

The level of 8-isoP was measured as an index of lipid peroxidation. As shown in Figure 2, the 8-isoP levels were significantly reduced by application of IPostC alone, and IPostC plus CsA in non-diabetic hearts compared with corresponding untreated- controls (P<0.05). Co-administration of IPostC and CsA in diabetic animals led to a significant decrease in the 8-isoP level; whereas, the effect of IPostC alone as well as CsA alone was not statistically significant in diabetic animals as compared with those of untreated-diabetic group.

**SOD activity**

The effects of three therapeutic interventions on myocardial SOD activity are shown in Figure 3. IPostC, CsA and IPostC plus CsA didn’t significantly influence the SOD activity neither in control nor in diabetic subgroups and the SOD levels were the same in all groups.

**GPX activity**

Neither of IPostC nor CsA alone significantly increased the myocardial GPX activity in control or diabetic rats. In addition, combination of IPostC and CsA enhanced significantly the GPX activity only in control animals (P<0.05). In diabetic animals, although IPostC plus CsA increased the GSH level compared to the untreated-diabetic group, this change was not statistically significant (Figure 4).

**TAC**

TAC level changes were also different in control and diabetic subgroups. IPostC couldn’t significantly affect on TAC in diabetic hearts. However, administration of CsA alone or in combination with IPostC increased significantly the level of TAC in control and diabetic animals (P<0.05) (Figure 5).
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Table 1. Degree of histopathological changes in myocardial tissues in control (C) and diabetic (D) hearts

| Changes     | Edema | Inflammation-like process | Necrosis |
|-------------|-------|---------------------------|----------|
| Groups      |       |                           |          |
| Control     | ++++  | +++                       | +++      |
| C+IPostC    | ++    | +                         | -        |
| C+CsA       | ++    | +                         | -        |
| C+IPostC+CsA| +     | +                         | -        |
| Diabetic    | ++++  | +++                       | +++      |
| D+IPostC    | ++++  | +++                       | +        |
| D+CsA       | ++++  | +                         | -        |
| D+IPostC+CsA| ++    | +                         | -        |

C: control; D: diabetic; IPostC: ischemic postconditioning; CsA: cyclosporine A

Histopathological findings

Table 1 shows the effect of IPostC and CsA alone and in combination on the degree of histopathological changes in myocardial tissues in nondiabetic control (C) and diabetic (D) hearts. Histopathological findings in nondiabetic and untreated-control group (Figure 6) showed necrotic (infarcted) zone with edema, separation of muscles fibers, and vanishing of the cell nuclei. Treatment with IPostC and CsA alone or in combination in control subgroups showed mild edema, reduced inflammatory process and no area with necrosis.

Figure 4. Myocardial GPX activity in non-diabetic control (top, a) and diabetic (bottom, b) hearts. The data were expressed as mean±SEM. *P<0.05 vs. group C (top). C: control; D: diabetic; IPostC: ischemic postconditioning; CsA: cyclosporine A

Figure 5. Myocardial TAC level in non-diabetic control (top, a) and diabetic (bottom, b) hearts. The data were expressed as mean±SEM. *P<0.05 vs. group C (top) or vs. group D (bottom). C: control; D: diabetic; IPostC: ischemic postconditioning; CsA: cyclosporine A

Figure 6. Standard hematoxylin–eosin (H&E) staining of heart slices in the non-diabetic control (C) subgroups for the evaluation of the histopathological changes. Treatment with IPostC and CsA alone or in combination in non-diabetic control subgroups showed mild edema, reduced inflammatory process and no area with necrosis. C: control; IPostC: ischemic postconditioning; CsA: cyclosporine A

Figure 7. Standard hematoxylin–eosin (H&E) staining of heart slices in the diabetic (D) subgroups for the evaluation of the histopathological changes. Concomitant administration of IPostC and CsA prevented the extension of edema and inflammatory process keeping no necrotic area in diabetic hearts. D: diabetic IPostC: ischemic postconditioning; CsA: cyclosporine A

On the other hand, in the untreated-diabetic group, there was extensive edema, cell swelling, and tissue necrosis (Figure 7). Administration of IPostC or CsA could not reduce considerably the extent of edema, inflammation or necrosis in diabetic subgroups. However, concomitant administration of IPostC and
CsA prevented the extension of edema and inflammatory process keeping no necrotic area in diabetic hearts, approximately comparable with those of the control subgroup receiving both IPostC and CsA.

**Discussion**

In the present study in the rat myocardial I/R injury model, the combined postconditioning with ischemia and CsA exerted a significant protective effect on the diabetic heart similar to nondiabetic ones and potentially reduced the level of LDH release. The sole application of IPostC or CsA could significantly affect the hearts only in nondiabetic control rats; their protective effects were not comprehensively shown in diabetics. There is a controversy over to the role of CsA in oxidative stress; this issue goes back to 1988. Crompton et al. (1988) believe that CsA a potent inhibitor of pore is currently added (39). Brockemeier et al. (1992) believe that CsA can reduce oxidative stress (40). On the contrary Hong et al. (2002) believe that CsA increases oxidative stress (41).

The cardioprotective effects of IPostC protocols and CsA have been often shown in animal species and humans (6, 15, 34, 42, 43). In a previous study, we have reported that IPostC with the strategy of three cycles of 30 sec reperfusion and 30 sec ischemia, or administration of CsA applied at the onset of reperfusion, confer potential cardioprotection in the I/R hearts of nondiabetic rats, not in diabetics (15).

Considering that CsA could inhibit the opening of the mPTP, it has been fundamental in implicating mPTP as a viable target for cardioprotection, which can be modulated at the beginning of myocardial reperfusion (43). Nevertheless, there is still conflicting data on its effectiveness, given that researchers have recently reported that administration of CsA at reperfusion fails to reduce infarct size in the hearts of anesthetized open-chest rats (44). However, the findings of all experimental and clinical studies consistently agree that chronic diabetic or hyperglycemic circumstances diminish or dissipate the cardioprotective effects of IPostC or other protective tools (4, 6, 15, 31, 32, 34, 45), which the results of our study also confirm in this regard. On the other hand, we evaluated the concomitant effects of both protocols, on which there were no previous reports neither in healthy nor in diabetic I/R hearts. We observed that one can overcome the failure of protective protocols in diabetic myocardium with increasing their potency.

Chronic diabetes is a multifactorial metabolic condition with several abnormalities including hyperglycemia, elevation of plasma fatty acid concentration, exacerbation of inflammatory reactions, over production of reactive species and oxidative stress and alteration in the activity of intracellular signaling pathways and mediators which ultimately may lead to the damages of important body organs such as the cardiovascular system (46, 47). Any of these changes in diabetes can increase the sensitivity of the heart to I/R insult and play a role in the failure of cardioprotection. No therapeutic approach has yet been demonstrated clinically effective against cardiac injury in diabetic population. Therefore, fortifying the diabetic heart against I/R injuries would have clinically important outcomes.

Oxidative stress is one of the key features of I/R injury. Cardioprotection is attributed to various factors including the inhibition of cellular elements involved in reactions of oxidative stress and production of free radicals (4, 6, 48). It has been shown that postconditioning effects on myocardial I/R are associated with decrease in lipid peroxidation and increase in the capacity of antioxidant systems in rat hearts exposed to I/R injury (27, 28, 49), which the results of the present study in healthy rats confirm.

One of the critical compartments involved in the production of oxidative stress in the cells is the mitochondria. Studies have revealed that IpostC with activating the reperfusion injury signaling kinase (RISK) or survivor activating factor enhancement (SAFE) signaling pathways ultimately lead to the inhibition of mPTP opening during the first min of reperfusion (50, 51). In our results, through inhibiting mPTP using CsA administration in both control and diabetic groups, we showed the protective effects of CsA in control groups as we saw the same with IPostC alone, while in diabetic groups the CsA effects were feeble.

In the early minutes of reperfusion, opening of mPTP leads to the releasing of cytochrome C by the mitochondria into the cytosol and dissipating of mitochondrial membrane potential which eventually jeopardizes the potency of mitochondria to use the newly presented oxygen; the mitochondria is now converted to the source of free radical production and oxidative stress build up in the cell. The released cytochrome C is thought to activate the pro-apoptotic proteins, thereby the apoptosis process is also initiated in the cell (10, 11, 51). By enforcing the lipid peroxidation reactions, oxidative stress causes demolition of cellular membranes and in that way the cell is brought to necrosis and cell death. These processes are seem to be more severe in diabetic conditions and this feature may play an important role in the interference of diabetes with cardioprotective influences of IPostC or CsA or other protective agents used in previous studies like opioids, anesthetics, and other pharmaceuticals (45, 51, 52).

In the present study, we illustrated that increasing the power of protection by simultaneous administration of two protective approaches, IPostC and CsA, at the onset of reperfusion can protect the diabetic myocardium against I/R injury. More likely, CsA directly and IPostC indirectly have inhibited the mPTP opening more strongly, and this way, they have exerted protective effects in the protection of diabetic hearts;
however, it may not be the exclusive way. It has been found out in animal studies that IpostC can also activate the mitochondrial ATP-sensitive potassium channels during reperfusion (50, 53). The positive influences of these channels have been well-explored in reestablishing the mitochondrial membrane potential and functional integrity of the mitochondria (54, 55). On the other hand, mPTP inhibition would not be the primary mechanism of action for CsA use; CsA is believed to inhibit calcineurin, a calcium activated phosphatase and reduce interleukin release, which is also cardioprotective (43). Therefore, the positive effect of combined postconditioning in control and diabetic hearts may be attributed not only to the mPTP closing at reperfusion but also to the other effects of CsA or IpostC. Thus, it is probably possible to protect the diabetic heart from reperfusion injury through amplification of any protective pathways or mediators’ actions. Further research is needed to clarify the main and more impressive pathways and mechanisms involved in the interaction of diabetes with cardioprotection.

**Conclusion**

The results of the present study showed that by enforcing the protective effects of IpostC and CsA through their combined application at the onset of reperfusion, the I/R injury was reduced in diabetic hearts similar to controls and considerable reduction in the level of oxidative stress was associated with the significant cardioprotection in this regard.

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**Conflict of interest**

There are no conflicts of interest in this study.

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