

Cardioprotective effects of co-administration of thymoquinone and ischemic postconditioning in diabetic rats

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ABSTRACT

Objective(s): Ischemia/reperfusion (I/R) is a leading cause of myocardial infarction (MI) injury, contributing to excess injury to cardiac tissues involved in inflammation, apoptosis, and oxidative stress. The present study was conducted to examine the effects of combined thymoquinone (TQ) with ischemic postconditioning (IPostC) therapy on apoptosis and inflammation due to I/R injury in diabetic rat hearts.

Materials and Methods: A single dose injection of streptozotocin (STZ; 60 mg/kg) was administered to thirty-two Wistar male rats to induce diabetes. Hearts were fixed on a Langendorff setting and exposed to a 30 min regional ischemia subsequently to 60 min reperfusion. IPostC was induced at the onset of reperfusion by 3 cycles of 30 sec R/I. ELISA, Western blotting assay, and TUNEL staining were applied to assess the cardioprotective effect of IPostC and TQ against I/R injury in diabetic and non-diabetic rats.

Results: Administration of TQ alone in non-diabetic isolated hearts significantly diminished CK-MB, TNF- α , IL-1 β , and apoptosis and enhanced p-GSK-3 β and Bcl-2 ($P < 0.05$). Following administration of TQ, the cardioprotective effects of IPostC by elevating p-GSK-3 β and Bcl-2 and alleviating apoptosis and inflammation were reestablished compared with non-IPostC diabetic hearts.

Conclusion: These results provide substantial evidence that co-administration of TQ plus IPostC can exert cardioprotective effects on diabetic myocardium during I/R damage by attenuating the inflammatory response and apoptosis.

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Introduction

The incidence of cardiovascular disorder in diabetics is considerably more prominent than in the non-diabetic population. In this regard, ischemic heart disease (IHD) is the most prevalent leading factor of disability and fatalities in diabetic patients worldwide (1). The recovery of blood supply is the most essential therapy in IHD. At present, timely reperfusion is a major therapeutic strategy to treat myocardial ischemia, however, reperfusion itself can result in lethal cardiac damage (2). The prominent clinical manifestations of this damage include left ventricular extracellular matrix remodeling, persistent ventricular systolic dysfunction, ventricular arrhythmias, irreversible cardiomyocyte injury, microvascular dysfunction, progressive cell death, and finally, heart failure (3). Furthermore, oxidative stress and cell apoptosis describe pivotal factors in the occurrence and extension of myocardial ischemia/reperfusion (I/R) injury. Therefore, it is important to further investigate the mechanism underlying I/R injury from various perspectives and how to effectively minimize this damage through intervention to protect the heart tissue (4).

Ischemic postconditioning (IPostC) can be defined as a manner of cardioprotection targeting of I/R injury, which is conducted after reopening of the occluded artery and before final restoration (5). Indeed, IPostC

stimulates the endogenous mechanisms that decrease the multiple interactions of reperfusion damage (6). In this regard, the inflammatory response has a pivotal role in I/R damage. Inflammatory signaling pathways produced during reperfusion damage activate NF- κ B and lead to up-regulation of a broad range of crucial pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1), IL-6, IL-8, IL-12 which result in initiating inflammatory responses in the heart. In diabetic patients, the inflammatory response to I/R injury (IRI) is intensified because of the stimulatory role of diabetes in the activation of adhesion molecules and cytokines and accumulation of leukocytes (7).

Besides, mitochondria play a pivotal role in cardioprotection (8). Damage to the outer membrane of mitochondria (OMOM) is concomitant with the actuation of the B-cell lymphoma-2 (Bcl-2) which contributes to permeabilization, caspase activation, and cell apoptosis. Also, immense oxidative stress can contribute to an abruptly enhanced inner mitochondrial membrane permeability due to the opening of mitochondrial permeability transition pore (mPTP) (9). IPostC inhibits mPTP through overexpression of phosphoinositide-3-kinase/protein kinase B (PI3K/PKB) pathway and whereby inactivation of glycogen synthase kinase-3 beta (GSK-3 β) in the myocardium. Activation of the PI3K/Akt/GSK-3 β pathway effectively alleviates cardiac

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cell apoptosis and IRI (10). Studies revealed that chronic diabetes predispose the cardiac cells to IRI. In a sense, IPostC alone could not exert considerable cardioprotective effects on diabetic myocardium from reperfusion (11).

Recently, natural products have drawn extensive attention due to their high efficiency and low side-effects, which is a rational mechanism in augmenting the reaction of cells to endure I/R injury (12). Among these, thymoquinone (TQ) is the crucial component of the essential oil obtained from *Nigella sativa L.* seeds (black cumin) (13). TQ is an active quinone derivative, whose beneficial therapeutic effects are due to its anti-ischemic, anti-myocardial and perivascular fibrosis, anti-hypertensive, anti-hypercholesterolemia, antihistamine, anti-apoptotic, hypolipidemic, hypoglycemic, anti-inflammatory, anti-cancer, and immunity-boosting effects (14-18). Recent studies have demonstrated that TQ could reduce I/R injury in several organs such as the brain, liver, spinal cord, heart, and renal tissue (19, 20). Bearing all this in mind, the underlying molecular mechanism of TQ in the heart tissue is still under investigation.

Indeed, the risk of ischemic conditions including myocardial infarction (MI) increases in diabetic patients and they may mount an exaggerated response to I/R. Hence, the present study was designed to first examine whether TQ and IPostC ameliorate myocardium IRI in type 1 diabetic state and secondly illuminate the cardioprotective roles of TQ and IPostC in inhibiting apoptosis and inflammation mediators in these pathways.

Materials and Methods

Experimental preparation

Fifty-six twelve-weeks-old Wistar male rats (weighing 260±40 g) were supplied from the animal center facility of the research center and maintained under standard animal room conditions [constant 12 hr light/dark cycle, temperature (25±2 °C), and relative humidity (60±5%)]. For the present study, experimental procedures were conducted according to the National Institutes of Health (NIH Publication No. 85-23, revised in 1996) Guidelines for the Care and Use of Laboratory Animals. Initially, animals were randomly separated into two main classifications of diabetic and control (non-diabetic) groups.

Induction of diabetes

After overnight fasting, animals received a single dose intraperitoneal (IP) injection of streptozotocin (STZ, Sigma-Aldrich, MO, USA, 60 mg/kg, IP, dissolved in 0.1 mol/l cold citrate buffer, pH 4.5). Diabetes was confirmed 72 hr after STZ injection using a glucometer and the blood samples acquired from scratching rat tails. Rats with fasting plasma glucose levels of lower than 250 mg/dl (13.8 mmol/l) were excluded from the experiment, whilst more than 13.8 mmol/l was considered diabetic. Eight weeks post-STZ diabetes induction, all animals were sacrificed and experiments were conducted on isolated perfused beating hearts in a Langendorff apparatus.

Induction of regional I/R and IPostC on Langendorff setting

56 rats were heparinized (500 IU/kg, IP) and

anesthetized intraperitoneally with chloral hydrate (0.9 ml/100 g, IP). After median sternotomy, the hearts were rapidly isolated and immersed in Krebs-Henseleit solution (K-H). Subsequently, the hearts were cannulated through the aorta and perfused with K-H solution. A mixture of 5 % CO₂ and 95 % O₂ was bubbled via the perfusate, and pH was kept between 7.35 and 7.45. The hearts were perfused at a constant mean pressure of 75 mmHg throughout the investigation. The thermostatically controlled water circulator (Satchwell Sunvic, UK) maintained the perfusate and bath temperatures at 37 °C. After a 20 min stabilization period, the left anterior descending (LAD) coronary artery was ligated by passing a 5-0 silk suture placed around LAD close to its origin. Exposure to 30 min of ischemia induced by disruption of the aortic supply and 60 min of full reperfusion followed. A critical drop (down to 30-40 % of its baseline value) in coronary flow at the onset of index ischemia and the consequent recovery of the coronary flow in the reperfusion period showed success in inducing the I/R cycle. IPostC was performed by three intermittent cycles of 30 sec R/I (3 cycles of 30 sec).

Experimental design

The experiments were conducted as follows (n=56; 7/per group); the hearts with weak contractions or with arrhythmias were excluded from the experiment and replaced with another one. The exclusion (and replacement) rate for groups was as follows: (I) Control group (C) – non-diabetic rats with no TQ and IPostC treatment=0; (II) Control with TQ group (C+TQ) – non-diabetic rats receiving TQ= 1; (III) Control with IPostC (C+IPostC) – non-diabetic rats with IPostC=0; (IV) Control with IPostC plus TQ (C+IPostC+TQ) – non-diabetic rats with IPostC plus TQ; (V) Diabetic group (D) – diabetic rats with no TQ and IPostC= 2; (VI) Diabetic with IPostC group (D+IPostC) – diabetic rats with IPostC=0; (VII) Diabetic with TQ group (D+TQ) – diabetic rats receiving TQ=0; (VIII) Diabetic with IPostC plus TQ group (D+IPostC+TQ) – diabetic rats with IPostC plus TQ=1. The arrhythmias and weak contraction or may be related to the failure in surgical procedure. In TQ-receiving groups, 5 min before the onset of reperfusion up to 10 min after reperfusion, the hearts were perfused with a K-H solution containing 50 µM TQ.

Assessment of cardiac enzyme activity

The coronary effluent was collected 10 min after the beginning of reperfusion. Ischemic injury was measured using a creatine kinase (CK-MB) assay kit. The CK-MB activity was determined spectrophotometrically by using commercial kits bought from Roche Diagnostic (Mannheim, Germany). Finally, total CK-MB was presented as the average in Unit/l.

Western blot

Protein concentration was determined by the Bradford technique. 40 µg protein samples were electrophoresed and then transferred to polyvinylidene fluoride (PVDF, Millipore, USA) membranes. Next, 5% skim milk dissolved in Tris-buffered saline containing 0.1% Tween (TBST) was applied to block the membranes for 2 hr at room temperature. The membranes were

incubated for 24 hr with primary antibodies against GSK3 β (1:500, Cell Signaling Technology, USA), phosphor (Ser 9) GSK-3 β (1:2000, Cell Signaling Technology), Bcl-2 (1:1500, Cell Signaling Technology), and β -actin (1:4000, Cell Signaling Technology). Upon extensive washing, the blots were probed with a goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (1:2500, Cell Signaling) for 2 hr at room temperature. Then, membranes were visualized by an ECL chemiluminescent system (Bio-Rad Laboratories, Hercules, California).

Preparation of tissue homogenates

The ischemic zone was separated and immediately frozen in liquid nitrogen and stored at -80 °C. About 0.5 g of ventricular tissue was cut into pieces in about 5 ml of ice-cold lysis buffer containing: 1.0 KH₂PO₄ mM/ml, 1.0 KCL mM/ml, 1.0 EDTA, 50 Tris-HCl pH 7.4, 1.0 NaF mM/ml, 1.0 Na₃VO₄ mM/ml, and 1% Triton 100X and protease inhibitor cocktail (Sigma-Aldrich, USA) and then homogenized with a Polytron PT-10/ST homogenizer. The homogenate was undergoing centrifugation at 12,000 \times g for 15 min at 4 °C. The Bradford technique was applied for the revealing of the concentration of proteins levels and cytokine activity in supernatants.

Assessment of IL-1 β and TNF- α levels

The levels of serum TNF- α and IL-1 β were estimated using enzyme-linked immunosorbent assay (ELISA) rat specific kit following the instructions of the kit manufacturer (eBioscience, Austria).

Hematoxylin and eosin (H&E) staining

Myocardial apoptosis was monitored by H&E staining. LV sections with 5 μ m thickness were obtained using a microtome. The sections were then stained with H&E (Beijing Solarbio Science & Technology Co., China) and scrutinized under a light microscope (Leica Microsystems, Germany).

Statistical analysis

All values were assayed in triplicate independent repeated tests and expressed as mean \pm standard error of the mean (SEM). A comparison between the two groups was performed by independent Student's t-test. Comparisons of parameters between different groups were conducted by one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post-hoc* test. Two-way ANOVA was applied for the comparison of parameters within groups. A value of $P < 0.05$ was considered statistically significant.

Table 1. General characteristics: Blood glucose (mmol/l), body weight (g), heart weight (g), and ratio of heart weight to body weight (%) in control and diabetic rats

Groups	Blood glucose (mmol/l)	BW (g)	HW (g)	HW/BW (%)
Controls	6.23 \pm 0.51	271 \pm 12	1.21 \pm 0.09	0.44
Diabetics	31.65 \pm 6.1	209 \pm 13*	1.08 \pm 0.08	0.51*

N=30/group, values are mean \pm SEM. T-test was applied for comparison between two groups; * $P < 0.01$ as compared with the control (non-diabetics) group. BW: Bodyweight; HW: Heart weight

Results

Basic characteristics of the animals

The general and basic characteristic data of diabetic and non-diabetic (control) rats after eight weeks of diabetes induction are indicated in Table 1. Independent t-test revealed dramatic hyperglycemia ($F_{(1,58)} = 31.65$, $P < 0.001$) as well as diminished bodyweight ($F_{(1,58)} = 209$, $P < 0.001$) and enhanced ratio of heart weight to body weight ($F_{(1,58)} = 0.51$, $P < 0.001$) in comparison with those of control rats.

The activity of CK in control and diabetic rats

CK-MB release level (Unit/l) in experimental I/R hearts has been displayed in Figure 1. The two-way ANOVA indicated dramatic therapeutic effect on myocardial CK-MB levels ($F_{(3,60)} = 8.54$, $P < 0.001$). The main effect of diabetes on CK-MB levels was insignificant ($F_{(2,68)} = 1.05$, $P = 0.18$), whilst the interaction effect of treatment \times diabetes was dramatically significant ($F_{(3,50)} = 3.92$, $P = 0.021$). There was an insignificant difference in CK-MB levels between control and diabetic groups, as revealed by one-way ANOVA and Tukey *post hoc*. Use of IPostC at the beginning of reperfusion in the control group markedly decreased myocardial CK-MB release vs control I/R group (13.4 \pm 1.95 vs 18.1 \pm 2.4, $P = 0.03$). Treatment with TQ at the end of ischemia had a similar to IPostC outcome (10.1 \pm 0.9, $P = 0.02$). Besides, administration of TQ plus IPostC enhanced CK-MB reduction even to a greater extent (8.7 \pm 1.1 vs 18.1 \pm 2.4, $P = 0.01$). In the diabetic sham group, only the effect of combination therapy (TQ plus IPostC) was significant compared with the diabetic I/R group (76 \pm 1.1 vs 17.9 \pm 2.3, $P = 0.01$).

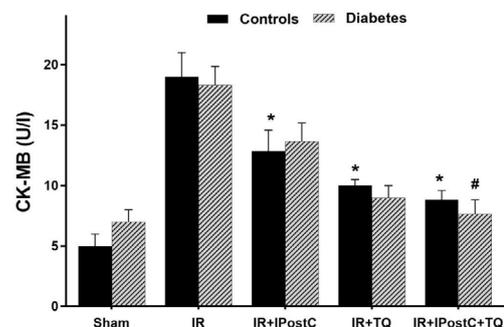


Figure 1. CK-MB release level (U/l) in experimental I/R hearts. * $P < 0.05$ in comparison with the control (non-diabetic) I/R group; # $P < 0.05$ in comparison with the diabetic I/R group. Mean \pm SEM. n=7/group. TQ: thymoquinone; I/R: ischemic/reperused; IPostC: ischemic postconditioning

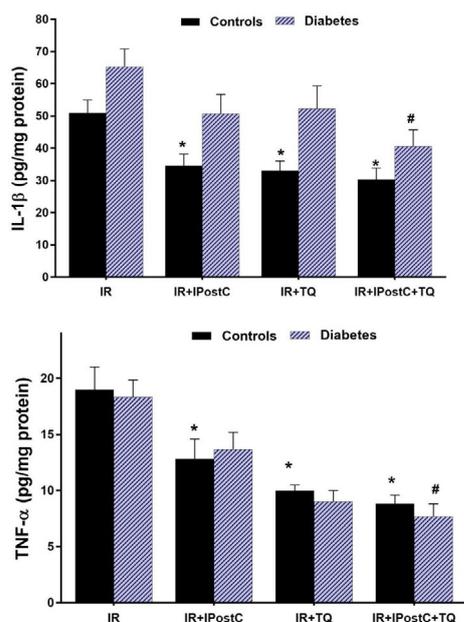


Figure 2. (A) IL-1 β , and (B) TNF- α levels (in pg/mg of sample protein) in experimental I/R hearts. * $P < 0.05$ in comparison with the control I/R group; ** $P < 0.01$ in comparison with the control I/R group; # $P < 0.05$ in comparison with the diabetic I/R group; ## $P < 0.01$ in comparison with the diabetic I/R group. Mean \pm SEM. $n = 7$
TQ: thymoquinone; I/R: ischemic/reperfused; IPostC: ischemic postconditioning

Inflammatory cytokines assays

The main effects of diabetes for IL-1 β were dramatic ($F_{(2, 48)} = 28.08$, $P < 0.01$; $F_{(2, 48)} = 18.91$, $P < 0.01$, respectively). The significant main treatments effects were found for IL-1 β ($F_{(4, 55)} = 25.19$, $P < 0.01$; and $F_{(4, 68)} = 10.03$, $P = 0.03$, respectively). One-way ANOVA demonstrated that administration of TQ in the control I/R group markedly alleviated the levels of IL-1 β , pg/mg

(32.41 ± 4.49 , $P = 0.03$ by TQ vs 50.8 ± 5.48), as compared with untreated control I/R hearts. Nevertheless, in diabetic groups, the positive effects of IPostC ($P = 0.41$) or TQ ($P = 0.07$) were not detected (Figure 2A). Moreover, the combination of TQ and IPostC, both in healthy and diabetic hearts, dramatically reduced IL-1 β levels in treated hearts in comparison with untreated I/R hearts and this effect was robust than every alone therapy along within both diabetic and control groups. IL-1 β levels were 28.5 ± 2.95 vs 50.8 ± 5.76 pg/mg in control I/R hearts, $P = 0.01$ and 40.5 ± 5.67 vs 64.42 ± 7.43 pg/mg in diabetic I/R hearts, $P = 0.03$. Also, the main effects of diabetes and co-treatments for TNF- α were statically significant ($F_{(2, 48)} = 11.59$, $P = 0.02$ and $F_{(4, 55)} = 4.21$, $P = 0.04$, respectively). One-way ANOVA demonstrated that in control hearts, similar to the special effects of combination therapy ($P = 0.03$), treatment with TQ alone ($P = 0.03$) could significantly decrease the myocardial TNF- α levels in comparison with the control I/R group. In diabetic groups, application of TQ alone (9.6 ± 1.5 pg/mg of sample protein, $P = 0.03$) or in combination with IPostC (7.8 ± 0.93 pg/mg of sample protein, $P = 0.01$) diminished TNF- α levels as compared with untreated diabetic I/R groups (17.6 ± 2.9 pg/mg) and co-treatment had an effect on reduction of TNF- α levels (Figure 2B).

Phosphorylation (at Ser 9) of myocardial GSK3 β

In the present study, total and Ser 9-phosphorylated forms of GSK-3 β , Bcl-2, and β -actin were detected using the Western blotting assay (Figures 3B, C). The total forms of GSK-3 β in control and diabetic hearts were similar and there were insignificant differences between all groups. Two-way ANOVA revealed remarkable effects of diabetes ($F_{(2, 48)} = 8.42$, $P = 0.02$), treatments ($F_{(4, 55)} = 14.91$, $P < 0.01$) and a diabetes treatments interaction ($F_{(4, 55)} = 3.63$, $P = 0.05$) on the levels of phosphorylated to the total form of GSK-3 β . In non-diabetic groups, application of TQ (2.02 ± 0.09 , $P = 0.033$)

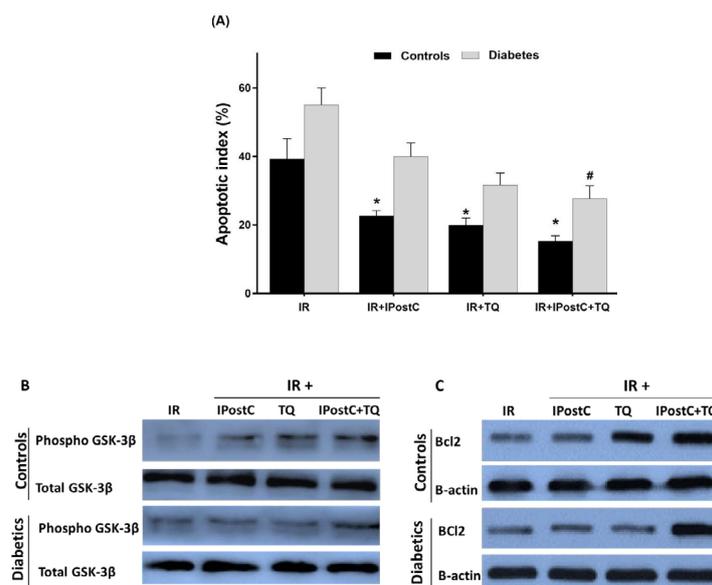


Figure 3. (A) Apoptotic index, (B) Phosphorylation of myocardial GSK-3 β , and (C) Expression of myocardial Bcl-2 protein in experimental I/R hearts. * $P < 0.05$ in comparison with the control I/R group; # $P < 0.05$ in comparison with the diabetic I/R group. Mean \pm SEM. $n = 7$
Bcl-2: B-cell lymphoma 2; I/R: ischemic/reperfused; TQ: thymoquinone; IPostC: ischemic postconditioning; GSK-3 β : Glycogen synthase kinase 3 beta

or their combination (1.5 ± 0.16 , $P=0.02$) significantly enhanced the levels of phosphorylated to the total form of GSK-3 β (in arbitrary unit) in comparison with the corresponding I/R heart group (0.5 ± 0.09). In diabetic groups, notwithstanding, the dramatic enhancement in the GSK-3 β phosphorylation was recognized only under co-treatment (2.1 ± 0.1 vs 0.77 ± 0.08 , $P=0.02$); the single effects of TQ or IPostC in diabetic hearts were not dramatic in comparison with those of diabetic I/R groups (Figure 3B).

Bcl-2 protein expression levels

Bcl-2 levels normalized by β -actin bands in control and diabetic hearts are demonstrated in Figure 5C. Two-way ANOVA analysis results revealed remarkable main effect of therapy ($F_{(4, 55)}=10.94$, $P<0.001$) and marked interaction effect among treatment and diabetes on Bcl-2 expression levels ($F_{(4, 55)}=6.07$, $P=0.02$). The main effect of diabetes on Bcl-2 expression levels was not dramatic ($F_{(2, 48)}=2.31$). Furthermore, in non-diabetic groups, IPostC alone ($P<0.04$) or in combination ($P=0.003$), dramatically elevated the relative expression of Bcl-2 protein in comparison with the I/R control group. In a sense, only significant enhancement in the Bcl-2 protein level was achieved after combining both IPostC and TQ protocols as compared with the diabetic group ($P=0.032$). Treatment of diabetic hearts with IPostC ($P=0.69$) or TQ ($P=0.57$) could not dramatically modify the expression levels of Bcl-2 (Figure 3C).

Myocardial apoptosis after I/R

Apoptosis rate in non-treatment I/R groups was far higher than the other treatment groups. There was a moderate number of apoptotic cells in the I/R+TQ group of control hearts (Figure 4). IPostC or TQ could not decrease the apoptotic cells in diabetic hearts (Figure 5). In a sense, a few apoptotic cells were detected in I/R with IPostC plus TQ groups both in control and diabetic rats (Figures 4 and 5). Furthermore, TQ alone ($P=0.02$) or in combination with IPostC plus TQ ($P<0.01$) significantly decreased AI in control hearts compared with the corresponding I/R group (Figure 3A). The effect of combination treatment was greater than those of single treatments, and only the combination treatment with TQ and IPostC reduced AI in diabetic hearts as compared with diabetic I/R hearts ($P=0.01$).

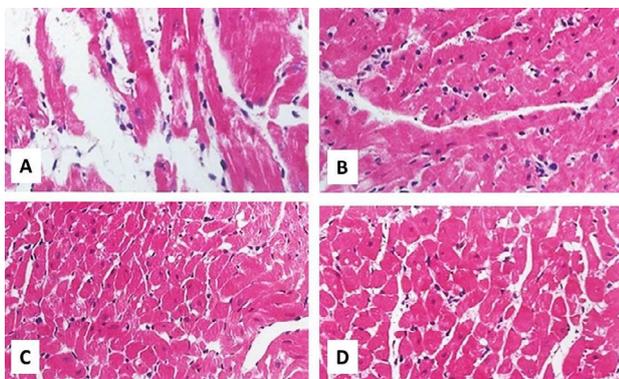


Figure 4. Micrographs of cardiomyocyte representing the process of apoptosis in experimental I/R hearts in control hearts. Myocardial apoptosis was identified via TUNEL staining

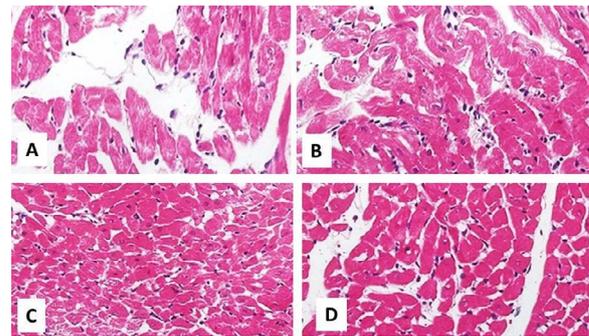


Figure 5. Micrographs of cardiomyocyte demonstrating the process of apoptosis in experimental I/R diabetic hearts. Myocardial apoptosis was identified using TUNEL staining

Discussion

The global prevalence of diabetes has grown sharply, which has become one of the most serious health problems universally. Diabetes is correlated with a higher risk of cardiovascular complications including IHD, coronary artery disease (CAD), congestive heart failure (CHF), and acute myocardial infarction (AMI) (21). Hyperglycemia considerably impaired mitochondrial morphology and function, causing electron leakage and O_2^- generation, which conditions are key contributors to enhanced myocardial vulnerability to IRI (22). Diabetes destroys the intracellular signaling pathways and mediators accountable for resistance against cell death.

Our results revealed that there are several major findings in the present study. Firstly, TQ could protect the myocardium against reperfusion injury in non-diabetic animals by dramatically enhancing GSK-3 β phosphorylation and Bcl-2 activation and whereby decreasing apoptosis and inflammatory responses. Secondly, TQ or IPostC couldn't significantly protect the myocardium in type 1 diabetes during IRI. Notwithstanding, co-treatment by IPostC plus TQ in the diabetic animals alleviated apoptosis and inflammatory response markedly and had more potent cardioprotective effects.

During reperfusion, blood flow may not return consistently to all portions of the previously ischemic organ; this circumstance has been characterized as the "no-reflow" phenomenon (23). IRI occurs in the first minutes of reperfusion, and therefore this period shows an appreciated "window of chance" for myocardial protection. The most effective approaches, such as IPostC, are performed in the first minutes of reperfusion (24). Indeed, IPostC could restrict the generation of reactive oxygen species (ROS), which was obtained from the mitochondrial electron transport chain during early reperfusion (25). In this regard, *N. Sativa* (known as black cummin seed) is one of the most promising medicinal plants, the extraordinary biological activity of *N. Sativa* is ascribed to its oil component, TQ (26). Several previous studies have demonstrated the cardioprotective effect of Quinine and phenolic compounds on myocardial IRI models (27, 28). Regarding the cardioprotective effects of TQ against IRI, Gonca and Kurt (29) revealed that pre-treatment with TQ reduced arrhythmia scores during the reperfusion period. Lu and colleagues demonstrated that TQ is efficient in decreasing myocardial I/R

injury via stimulation of the SIRT1 pathway which can decrease mitochondrial oxidative stress injury and apoptosis (13). Another study revealed that TQ can effectively develop the cardiac function and exert its anti-oxidative and anti-apoptotic activities involving the modulation of autophagy (30). Furthermore, Liu and *et al.* demonstrated that TQ treatment significantly enhanced insulin levels and body weight, and decreased blood glucose and heart rate levels in diabetic animals (31).

Protecting mitochondria from oxidative injury is a rational treatment method to decrease IRI (13). mPTP has been recognized as a crucial modulator of cardiac IRI. It has been well documented that mPTP remains closed throughout the ischemia, whilst it becomes open in the early minutes of reperfusion. mPTP opening is generally induced through the accumulation of ROS, enhanced cytosolic and mitochondrial matrix Ca_2^+ levels, and oxidative stress. Inhibiting the opening of mPTP with its specific blocker including cyclosporin A (CsA) pre/post-ischemia may attenuate myocardial IRI in these patients. Hence, mPTP is a pivotal therapeutic objective for preventing IRI (6, 32). Moreover, apoptosis plays a key role in the pathophysiology of IRI both in diabetic and non-diabetic conditions (33). Apoptosis is divided into two distinct pathways including extrinsic (receptor-dependent) and intrinsic (mitochondria-dependent). Cell apoptosis via the mitochondria pathway is mediated by Bcl-2 family proteins, which is categorized into two groups: anti-apoptotic members, including Bcl-2 and Bcl-xL, and pro-apoptotic members, including Bak, Bad, Bax, and Bid (4). The Bcl-2/Bax balance is vital for sustaining cell homeostasis. mPTP opening may be controlled by Bcl-2 and Bax (32). Bax may mediate mPTP opening through binding of adenine nucleotide translocase (ANT) or voltage-dependent anion channel (VDAC), whilst Bcl-2 may directly impede the interaction between Bax and VDAC/ANT to obstruct the mPTP opening (34). STZ-induced type 1 diabetes resulting in myocardial apoptosis was significantly increased compared with the control group, which is due to cytochrome c release leading to caspase-3 activation and then cell death (10). Our results revealed that STZ-induced chronic diabetes in rats markedly elevated apoptosis (evaluated through comparing TUNEL-positive cells) in IRI in comparison with those of controls.

Also, elevated levels of ROS during reperfusion can induce inflammatory response and overproduction of pro-inflammatory cytokines following I/R insult (35). Important cytokines like TNF- α , IL-1, and IL-6 are the beginning promoters of the inflammation during IRI (36). TNF- α is mainly produced by macrophages, which is involved in the formation and progression of myocardial IRI and promotes myocardial cell apoptosis, thus increasing myocardial damage (37). Inhibition of inflammatory response is considered a cardioprotective mechanism, which is IPostC reduced reperfusion injury. Herein, we revealed that the administration of TQ in non-diabetic hearts diminished apoptosis and inflammatory cytokines in isolated I/R hearts; besides, co-treatment with TQ plus IPostC had a very potent favorable effect on those parameters in control groups. Furthermore, in diabetic groups, TQ or IPostC alone failed to provide

any protection and, in a sense, diabetes abrogated the positive effects of TQ or IPostC on the myocardium. Nevertheless, co-treatment of TQ plus IPostC revealed complete cardioprotection and statically significant diminishing levels of inflammatory cytokines and apoptosis.

Activation of PI3K/Akt signaling is critically involved in protecting the cardiomyocyte against IRI (33). PI3K is recognized as a crucial player in the survival pathway, which has been involved in IPostC protection. Reduction of the amount of phosphorylation and activity of PI3K is one of the causes of diabetes (38). PI3K activation induces phosphorylation of the Ser/Thr kinase Akt which afterward inhibits the formation of Bcl-2 family proteins, stimulates endothelial nitric oxide synthase (eNOS), protein kinase C (PKC), and mTOR/p70s6K, and obstructs downstream GSK-3 β (39). GSK-3 β is a pro-apoptotic kinase, which is a pivotal player in diabetic cardiomyopathy and myocardial IRI (40). GSK-3 β is actuating in normal and non-phosphorylated state and can open the mPTP, contributing to the release of cytochrome c into the cytosol and starting the apoptosis and oxidative reactions (41). On the other hand, mPTP closure is attained by phosphorylation and repression of GSK-3 β at Ser 9. IPostC may inhibit the activity of GSK-3 β by its phosphorylation, and hence, enhance the cell tolerance to oxidative damage (42). Herein, the phosphorylation of GSK-3 β in diabetic rats was markedly fewer than the control group. The suppression of phosphorylation of GSK-3 β in diabetic rats was correlated with enhanced cardiac damage and apoptosis as compared with healthy rat hearts. The amount of GSK-3 β was the same in diabetic and non-diabetic groups of our study and this indicates that chronic diabetes diminishes the power of IPostC to phosphorylate GSK-3 β , keeping this detrimental protein kinase in its active form. Therefore, we hypothesized that type 1 diabetes mellitus might have a negative effect on these crucial pathways involved in IPostC. Indeed, the PI3K/Akt/GSK3 β pathway is upstream of mPTP signal transduction, and was not phosphorylated efficiently by IPostC in diabetic groups as compared with the healthy control animals.

Conclusion

Given together, the results of the present study demonstrate that administration of TQ could exert a cardioprotective effect against reperfusion injury in non-diabetic animals by significantly enhancing GSK3 β phosphorylation and Bcl-2 activation, hence, decreasing apoptosis and inflammatory responses. Also, co-administration of TQ with IPostC can protect the diabetic myocardium during IRI by attenuating the apoptosis and inflammatory response. Bearing all this in mind, TQ improves the potency of IPostC on activating the survival protein kinases and mitigation of the mPTP opening, eventually resulting in cardioprotection during IRI induction in diabetic rats.

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Conflicts of Interest

None.

References

- Suchal K, Malik S, Khan SI, Malhotra RK, Goyal SN, Bhatia J, et al. Protective effect of mangiferin on myocardial ischemia-reperfusion injury in streptozotocin-induced diabetic rats: role of AGE-RAGE/MAPK pathways. *Sci Rep* 2017; 7:42027-42038.
- Yu L, Gong B, Duan W, Fan C, Zhang J, Li Z, et al. Melatonin ameliorates myocardial ischemia/reperfusion injury in type 1 diabetic rats by preserving mitochondrial function: role of AMPK-PGC-1 α -SIRT3 signaling. *Sci Rep* 2017; 7:41337-41350.
- Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Ischemia/reperfusion. *Compr Physiol* 2011; 7:113-170.
- Jian J, Xuan F, Qin F, Huang R. The antioxidant, anti-inflammatory and anti-apoptotic activities of the bauhinia championii flavone are connected with protection against myocardial ischemia/reperfusion injury. *Cell Physiol Biochem* 2016; 38:1365-1375.
- Giustino G, Dangas GD. Ischemia-reperfusion injury and ischemic post-conditioning in acute myocardial infarction: Lost in translation. *Catheter Cardiovasc Interv* 2017; 90:1068-1069.
- Badalzadeh R, Azimi A, Alihemmati A, Yousefi B. Chronic type-I diabetes could not impede the anti-inflammatory and anti-apoptotic effects of combined postconditioning with ischemia and cyclosporine A in myocardial reperfusion injury. *J Physiol Biochem* 2017; 73:111-120.
- Najafi M, Noroozi E, Javadi A, Badalzadeh R. Anti-arrhythmogenic and anti-inflammatory effects of troxerutin in ischemia/reperfusion injury of diabetic myocardium. *Biomed Pharmacother* 2018; 102:385-391.
- Pagliari P, Femminò S, Popara J, Penna C. Mitochondria in cardiac postconditioning. *Front Physiol* 2018;9:287-295.
- Forini F, Nicolini G, Iervasi G. Mitochondria as key targets of cardioprotection in cardiac ischemic disease: role of thyroid hormone triiodothyronine. *Int j mol sci* 2015; 16:6312-6336.
- Badalzadeh R, Mokhtari B, Yavari R. Contribution of apoptosis in myocardial reperfusion injury and loss of cardioprotection in diabetes mellitus. *The J Physiol Sci* 2015; 65:201-215.
- Miki T, Itoh T, Sunaga D, Miura T. Effects of diabetes on myocardial infarct size and cardioprotection by preconditioning and postconditioning. *Cardiovasc Diabetol* 2012; 11:67-80.
- Hassan MQ, Akhtar M, Ahmed S, Ahmad A, Najmi AK. *Nigella sativa* protects against isoproterenol-induced myocardial infarction by alleviating oxidative stress, biochemical alterations and histological damage. *Asian Pac J Trop Biomed* 2017; 7:294-299.
- Lu Y, Feng Y, Liu D, Zhang Z, Gao K, Zhang W, et al. Thymoquinone attenuates myocardial ischemia/reperfusion injury through activation of SIRT1 signaling. *Cell Physiol Biochem* 2018; 47:1193-1206.
- Majdalawieh AF, Fayyad MW. Immunomodulatory and anti-inflammatory action of *Nigella sativa* and thymoquinone: a comprehensive review. *Int Immunopharmacol* 2015; 28:295-304.
- Asgharzadeh F, Bargi R, Beheshti F, Hosseini M, Farzadnia M, Khazaei M. Thymoquinone prevents myocardial and perivascular fibrosis induced by chronic lipopolysaccharide exposure in male rats: - thymoquinone and cardiac fibrosis. *J Pharmacopunct* 2018; 21:284-293.
- Oskouei Z, Akaberi M, Hosseinzadeh H. A glance at black cumin (*Nigella sativa*) and its active constituent, thymoquinone, in ischemia: a review. *Iran J Basic Med Sci* 2018; 21:1200-1209.
- Bamosa AO, Ali BA, al-Hawsawi ZA. The effect of thymoquinone on blood lipids in rats. *Indian J Physiol Pharmacol* 2002; 46:195-201.
- Xu J, Zhu L, Liu H, Li M, Liu Y, Yang F, et al. Thymoquinone reduces cardiac damage caused by hypercholesterolemia in apolipoprotein E-deficient mice. *Lipids Health Dis* 2018; 17:173-182.
- Sezen Ş C, Kucuk A, Özer A, Kılıç Y, Mardin B, Alkan M, et al. Assessment of the effects of levosimendan and thymoquinone on lung injury after myocardial ischemia reperfusion in rats. *Drug Des Devel Ther* 2018; 12:1347-1352.
- Gulsen I, Ak H, Colcimen N, Alp HH, Akyol ME, Demir I, et al. Neuroprotective effects of thymoquinone on the hippocampus in a rat model of traumatic brain injury. *World Neurosurg* 2016; 86:243-249.
- Gregg EW, Sattar N, Ali MK. The changing face of diabetes complications. *Lancet Diabetes Endocrinol* 2016; 4:537-547.
- Yamagishi SI, Nakamura N, Matsui T. Glycation and cardiovascular disease in diabetes: A perspective on the concept of metabolic memory. *J Diabetes* 2017; 9:141-148.
- Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword?. *J Clin Invest* 1985; 76:1713-1719.
- Hu L, Wang J, Zhu H, Wu X, Zhou L, Song Y, et al. Ischemic postconditioning protects the heart against ischemia-reperfusion injury via neuronal nitric oxide synthase in the sarcoplasmic reticulum and mitochondria. *Cell Death Dis* 2016; 7:2222-2234.
- Sun HY, Wang NP, Kerendi F, Halkos M, Kin H, Guyton RA, et al. Hypoxic postconditioning reduces cardiomyocyte loss by inhibiting ROS generation and intracellular Ca²⁺ overload. *Am J Physiol Heart Circ Physiol* 2005; 288:1900-1908.
- Gupta B, Ghosh KK, Gupta RC. Chapter 39 - Thymoquinone. In: Gupta RC, editor. *Nutraceuticals*. Boston: Academic Press; 2016. p.541-550.
- Lee YM, Cheng PY, Chen SY, Chung MT, Sheu JR. Wogonin suppresses arrhythmias, inflammatory responses, and apoptosis induced by myocardial ischemia/reperfusion in rats. *J Cardiovasc Pharmacol* 2011; 58:133-142.
- Badalzadeh R, Yousefi B, Tajaddini A, Ahmadian N. Diosgenin-induced protection against myocardial ischaemia-reperfusion injury is mediated by mitochondrial KATP channels in a rat model. *Perfusion* 2015; 30:565-571.
- Gonca E, Kurt Ç. Cardioprotective effect of thymoquinone: A constituent of *Nigella sativa L.*, against myocardial ischemia/reperfusion injury and ventricular arrhythmias in anaesthetized rats. *Pak J Pharm Sci* 2015; 28:1267-1273.
- Xiao J, Ke ZP, Shi Y, Zeng Q, Cao Z. The cardioprotective effect of thymoquinone on ischemia-reperfusion injury in isolated rat heart via regulation of apoptosis and autophagy. *J Cell Biochem* 2018; 119:7212-7217.
- Liu H, Liu HY, Jiang YN, Li N. Protective effect of thymoquinone improves cardiovascular function, and attenuates oxidative stress, inflammation and apoptosis by mediating the PI3K/Akt pathway in diabetic rats. *Mol Med Rep* 2016; 13:2836-2842.
- Liu S, Wu N, Miao J, Huang Z, Li X, Jia P, et al. Protective effect of morin on myocardial ischemia-reperfusion injury in rats. *Int J Mol Med* 2018; 42:1379-1390.
- Mokhtari B, Badalzadeh R, Alihemmati A, Mohammadi M. Phosphorylation of GSK-3 β and reduction of apoptosis as targets of troxerutin effect on reperfusion injury of diabetic myocardium. *Euro J Pharmacol* 2015; 765:316-321.
- Dejean LM, Martinez-Caballero S, Guo L, Hughes C, Teijido O, Ducret T, et al. Oligomeric Bax is a component of the putative cytochrome c release channel MAC, mitochondrial apoptosis-induced channel. *Mol Biol Cell* 2005; 16:2424-2432.
- Marzilli M, Huqi A. Cardioprotective therapy in reperfusion

- injury: lessons from the european myocardial infarction project—free radicals (EMIP-FR). *Heart Metab* 2010; 46:35-37.
36. Ebrahimi H, Badalzadeh R, Mohammadi M, Yousefi B. Diosgenin attenuates inflammatory response induced by myocardial reperfusion injury: role of mitochondrial ATP-sensitive potassium channels. *J Physiol Biochem* 2014; 70:425-432.
37. Zhang WP, Zong QF, Gao Q, Yu Y, Gu XY, Wang Y, *et al.* Effects of endomorphin-1 postconditioning on myocardial ischemia/reperfusion injury and myocardial cell apoptosis in a rat model. *Mol Med Rep* 2016; 14:3992-3998.
38. Drenger B, Ostrovsky IA, Barak M, Nechemia-Arbely Y, Ziv E, Axelrod JH. Diabetes blockade of sevoflurane postconditioning is not restored by insulin in the rat heart phosphorylated signal transducer and activator of transcription 3—and phosphatidylinositol 3-Kinase-mediated inhibition. *Anesthesiology* 2011; 114:1364-1372.
39. Bopassa JC, Ferrera R, Gateau-Roesch O, Couture-Lepetit E, Ovize M. PI 3-kinase regulates the mitochondrial transition pore in controlled reperfusion and postconditioning. *Cardiovasc Res* 2006; 69:178-185.
40. Fu H, Xu H, Chen H, Li Y, Li W, Zhu Q, *et al.* Inhibition of glycogen synthase kinase 3 ameliorates liver ischemia/reperfusion injury via an energy-dependent mitochondrial mechanism. *J Hepatol* 2014; 61:816-824.
41. Juhaszova M, Zorov DB, Yaniv Y, Nuss HB, Wang S, Sollott SJ. Role of glycogen synthase kinase-3 β in cardioprotection. *Circ res* 2009; 104:1240-1252.
42. Badalzadeh R, Mohammadi M, Yousefi B, Farajnia S, Najafi M, Mohammadi S. Involvement of glycogen synthase kinase-3 β and oxidation status in the loss of cardioprotection by postconditioning in chronic diabetic male rats. *Adv Pharm Bull* 2015; 5:321-327.