Original Paper

Effects of Trimetazidine on PDCD4/
NF- κ B/TNF- α Pathway in Coronary
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Key Words

Coronary microembolization • Programmed cell death factor 4 • NF- κ B • Trimetazidine • Inflammation

Abstract

Background/Aims: The local inflammatory response caused by coronary microembolization (CME) is the primary cause of progressive cardiac dysfunction. The PDCD4/NF- κ B/TNF- α signaling pathway plays a significant role in CME-induced myocardial inflammation. Trimetazidine (TMZ) reduces myocardial injury, caused by percutaneous coronary intervention, through relieving the CME-induced myocardial systolic dysfunction. Therefore, the present study investigated the role of TMZ pre-treatment in the protection of myocardium after CME and PDCD4/NF- κ B/TNF- α in mini pigs. **Methods:** 20 Bama mini pigs were randomized into sham operation (sham), microembolization (CME), TMZ, and siRNA-PDCD4 groups (n = 5). The CME model was established by injecting polyethylene microspheres via microcatheter into the left anterior descending coronary artery. The TMZ group was injected 2.5 mg/kg drug via ear vein 30 min before CME; whereas, the siRNA-PDCD4 group was transfected with PDCD4 siRNA at the left anterior descending coronary artery via microcatheter 72h before CME. Cardiac function indexes were measured using cardiac echocardiography. The mRNA expression of PDCD4 and TNF- α in the myocardium was detected by quantitative fluorescence PCR, and the protein expression of PDCD4, NF- κ B (p65), and TNF- α by Western blot. **Results:** Echocardiographic parameters showed lower cardiac function and higher serum cTnI level in the CME group than sham, which was manifested as reduced left ventricular ejection fraction (LVEF), left ventricular fractional shortening (FS), cardiac output (CO), and increased left ventricular diastolic diameter (LVEDd). Compared to the CME group, the CME-induced cardiac function injury was reduced, and the serum cTnI level was decreased in the TMZ and siRNA-PDCD4 groups. The expressions of PDCD4, NF- κ B (p65), and TNF- α were significantly increased in the CME than the sham groups ($P < 0.05$), and significantly decreased in the TMZ and siRNA-PDCD4 groups than the CME group ($P < 0.05$). **Conclusion:** TMZ pretreatment effectively reduced the myocardial damage caused by CME via inhibiting the PDCD4/NF- κ B/TNF- α pathway in cardiomyocytes.

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Introduction

Coronary microembolization (CME) is the microvascular obstruction induced even by slight embolism in distal coronary small blood vessels. It occurs during the spontaneous rupture of a coronary atherosclerotic plaque or during the process of percutaneous coronary intervention (PCI) [1], which affects the myocardial blood supply, results in malignant arrhythmia, and decreases the cardiac function, leading to the occurrence of cardiovascular adverse events [2]. In the current study, we found that the CME-induced myocardial local inflammatory response was the primary cause of progressive cardiac dysfunction, while the programmed cell death 4 (PDCD4)/NF- κ B/TNF- α signaling pathway played a major role in the CME-induced inflammatory response. Hence, the inhibition of this pathway would effectively attenuate the myocardial damage after CME [3, 4].

Trimetazidine (TMZ), also known as 1-(2, 3, and 4-trimethylbenzazole)-dihydrochloric acid piperazine, is a piperazine derivative that selectively inhibits fatty acid oxidation and stimulates glucose oxidation; this phenomenon directly modifies the energy substrate utilized by heart [5]. In recent years, an increasing number of studies have found that patients with acute coronary syndrome (ACS), treated with TMZ before PCI, presented reduced perioperative myocardial injury and improved cardiac function. These features might be associated with the inflammatory effects of TMZ [6, 7]. CME is one of the critical causes of perioperative myocardial injury in PCI, and therefore, we hypothesized that TMZ might play a role in myocardial protection by inhibiting the CME-induced myocardial inflammation. In this study, we investigated the effect of pretreatment by TMZ on the cardiac function and the PDCD4/NF- κ B/TNF- α signaling pathway in mini pigs post-CME. In addition, our study also provided an insight into the molecular machinery underlying the preventive effect of TMZ on CME-induced cardiac injury, clinical prevention, and treatment of CME.

Materials and Methods

Materials

42 μ m embolization microspheres (Biosphere Medical Inc., USA); TRNzol-A + Total RNA extraction reagent (KeyGEN, China); primers for TNF- α mRNA, PDCD4 mRNA, and β -actin mRNA (Sagene, China); goat anti-pig PDCD4 polyclonal antibody (LifeSpan BioSciences, USA); rabbit anti-pig NF- κ B (p65) and rabbit anti-pig TNF- α polyclonal antibodies (Abcam, USA); rabbit anti-GAPDH polyclonal IgG antibody (Proteintech, USA).

Experimental animals

20 male or female healthy Bama miniature pigs, weighing 25–30 kg, were provided by the Animal Science and Technology College, Guangxi University. This experiment was carried out in accordance with the Code of Practice for Animal Test and approved by the Ethics Committee of Guangxi Medical University.

Establishment of CME model and experimental grouping

The minipigs were anesthetized by intramuscular injection of ketamine hydrochloride (5–10 mg/kg). The experimental pigs were washed, routinely sterilized, and draped following 3–5 min after anesthesia injection. A 5½-gauge scalp needle was used to puncture the pigs' auricular vein, followed by intravenous injection of diazepam at a dosage of 0.5 mg/kg/h to maintain the anesthetized state. The one-sided femoral artery was mobilized and punctured for the implantation of a 6F arterial catheter to inject 200 U/kg, followed by 100 U/kg/h heparin. During coronary angiography, a microcatheter was passed along a guide wire to the distal end of the first diagonal branch of the left anterior descending coronary artery. 100,000 embolization microspheres (suspended in 1.5 mL physiological saline) were injected via the microcatheter to establish a porcine CME model. [8] Similarly, 1.5 mL normal saline was administered to the mini pigs in the sham group. 20 Bama miniature pigs were equally assigned to the sham, CME, TMZ, and siRNA-PDCD4 groups based on the random number method ($n = 5$ pigs/group). In the TMZ group, the pigs were injected TMZ (2.5 mg/kg) intravenously via the auricular vein 30 min before the surgery. In the siRNA-PDCD4 group,

the pigs were administered 400 μ L PDCD4 siRNA transfection complex into the left anterior descending coronary artery via the microcatheter 72 h before CME.

Preparation of siRNA-PDCD4 transfection complex

The complex was prepared as described previously [9]. The siRNA was transfected using the animal transfection reagent (Entranster™ *in vivo*), according to the manufacturer's instructions. The siRNA-PDCD4 transfection complex was prepared according to the instructions of the kit: 100 μ g PDCD4 siRNA was solubilized in 100 μ L RNase-free water. Then, the siRNA and 100 μ L Entranster™-*in vivo* were diluted in a final glucose concentration of 5%, respectively. Finally, the diluted Entranster™ *in vivo* was mixed with PDCD4 siRNA and stabilized at room temperature for 15 min before intracoronary injection.

Evaluation of mini pigs' cardiac function

In the current early study, we observed that the cardiac function was poor at 9 h after CME. Therefore, this time-point was selected to determine the left ventricular ejection fraction (LVEF), left ventricular diastolic diameter (LVEDd), left ventricular fractional shortening (FS), and cardiac output (CO) of the mini pigs in each group at a probe frequency of 12 MHz. All the values were determined based on the average of 3 cardiac cycles [10], and all echocardiographic examinations were performed by an experienced physician.

Measurement of serum cTnI level

The level of serum c-troponin I (cTnI) was measured by immunoluminescence assay using Bayer's ACS180 automatic chemiluminescence immunoassay system and the double antibody sandwich cTnI assay kit. Briefly, 100 μ L plasma sample was collected and added to the reaction cup, which was followed by the incubation with 100 μ L acridine ester-labeled anti-cTnI antibody at 37°C for 2.5 min. Subsequently, 200 μ L of the total cTnI solid phase reagent (monoclonal anti-cTnI antibody, covalently bound to the magnetic particles), was added to the above complex and incubated for 5 min, followed by washes with deionized water to separate the reaction phase. Finally, the photochemical reaction was initiated by the addition of 300 μ L reagent 1 and 2 (chemiluminescent oxidants) and estimated.

Detection of PDCD4 and TNF- α mRNA expression by quantitative fluorescence polymerase chain reaction (qf-PCR)

The total RNA was extracted from left ventricular myocardium according to the manufacturer's instructions using TRIzol (Invitrogen, USA). The concentration of RNA was determined by NanoDrop (Thermo Fisher Scientific, USA). A reverse transcription kit (TaKaRa, Japan) was utilized for the synthesis of cDNA. The PCR products were detected by SYBR Green I fluorescent labeling, and the gene specific primers were as follows:

PDCD4 primers: upstream: 5'-GCT ACG GTG CTC CTG AGT AT-3', downstream: 5'-GGC AAT GTT CAG CTT CCG AT-3';

TNF- α primers: upstream: 5'-GGC CCA AGG ACT CAG ATC AT-3', downstream: 5'-GCA TAC CCA CTC TGC CAT TG-3'; and

β -*actin* primers: upstream: 5'-CAC CTT CTA CAA CGA GCT GC-3', downstream: 5'-TCA TCT TCT CAC GGT TGG CT-3'

The reaction system and parameters were designed according to the instructions of the kit. Each sample was detected using multiple wells, and negative control wells were also included in each reaction. The relative expression of the gene was determined using the $2^{-\Delta\Delta CT}$ method, and the PCR products were assessed by sequencing.

Expression of PDCD4, NF- κ B (p65) and TNF- α protein in the myocardium by Western blot

The concentration of the total protein in the myocardium, extracted by tissue lysis buffer, was determined by the Lowry method. An equivalent of 50 μ g protein was subjected to 10% SDS-PAGE electrophoresis and transferred to PVDF membrane. The membrane was blocked for 1 h, followed by probing with goat anti-pig PDCD4, rabbit anti-pig NF- κ B (p65) polyclonal antibody, or rabbit anti-pig TNF- α polyclonal antibody for 2 h at room temperature. Consecutively, rabbit anti-GAPDH polyclonal IgG antibody served as an internal reference. The corresponding secondary antibodies were added for 1 h. The immunoreactivity complexes were detected by enhanced chemiluminescence on X-ray films. The integrated absorbance (IA = average

absorbance × area) of the target band was analyzed with the specific software of Bio-Rad Gel Documentation System Gel Doc 2000. After normalization against GAPDH, the IA values of PDCD4/GAPDH, NF-κB (p65)/GAPDH, and TNF-α/GAPDH ratios represented the relative expression of PDCD4, NF-κB (p65), and TNF-α, respectively.

Statistical analysis

The SPSS 23.0 statistical software (IBM, Chicago, USA) was used for data analysis. The measurement data were presented as the mean ± standard deviation ($\bar{x} \pm s$). The between-group comparison was performed using the group t-test, whereas, that of the multiple groups was performed using one-way analysis of variance (ANOVA) with post-hoc tests. The pair-wise comparisons among multiple groups were performed using the LSD test. $P < 0.05$ indicated that the difference was statistically significant.

Results

Changes in the cardiac function of mini pigs

The cardiac function of mini pigs was evaluated with respect to LVEF, FS, CO, and LVEDd values obtained by echocardiographic examinations. Table 1 represents the results after 9 h of operation. (1) Compared to the sham group, the cardiac function of CME group was significantly decreased as indicated by myocardial systolic dysfunction and left ventricular dilatation: decreased LVEF, FS, CO ($P < 0.05$) and increased LVEDd ($P < 0.05$). (2) Compared to the CME group, the TMZ and siRNA-PDCD4 groups showed improved CME-induced cardiac injury, manifested as increased LVEF, FS, and CO ($P < 0.05$) and decreased LVEDd ($P < 0.05$).

Detection of myocardial injury marker cTnI

(1) The serum cTnI of the CME group was significantly higher than that of the sham group (0.199 ± 0.017 vs. 0.039 ± 0.004 ng/mL, $P < 0.05$) at 9 h post-operation. (2) Compared to the CME group, the concentration of serum cTnI in the TMZ group was significantly decreased (0.065 ± 0.011 vs. 0.199 ± 0.017 ng/mL, $P < 0.05$). (3) The concentration of cTnI in the siRNA-PDCD4 group was significantly lower than that of the CME group (0.071 ± 0.012 vs. 0.199 ± 0.017 ng/mL, $P < 0.05$).

Comparison of PDCD4 and TNF-α mRNA expression in each group (Fig. 1 and 2)

(1) Compared to the sham group, the expressions of PDCD4 and TNF-α mRNA in mini pigs' cardiomyocytes of CME group were significantly increased ($P < 0.05$ for both). (2) Compared to the CME group, the expression of PDCD4 and TNF-α mRNA in the TMZ and the siRNA-PDCD4 groups was significantly decreased ($P < 0.05$).

Table 1. Changes in cardiac function ($\bar{x} \pm s$). LVEF: left ventricle ejection fraction; CO: cardiac output; LVEDd: left ventricular end-diastolic diameter; LVFS: left ventricle fractional shortening. * $P < 0.05$ compared to the sham group; # $P < 0.05$ compared to the CME group

Group	n	LVEF (%)	LVFS (%)	CO (L/min)	LVEDd (mm)
Sham	5	69.41±2.65	41.86±5.83	3.91±0.74	3.44±0.36
CME	5	51.37±3.42*	27.63±7.89*	2.98±0.85*	4.91±0.53*
Trimetazidine	5	66.53±2.82#	41.33±5.67#	3.82±0.84#	3.61±0.39#
siRNA-PDCD4	5	64.72±2.66#	40.96±5.61#	3.74±0.79#	3.78±0.40#

Fig. 1. Evaluation of PDCD4 mRNA expression in each group by fluorescent quantitative PCR. (a) Sham group; (b) CME group; (c) Trimetazidine group; (d) siRNA-PDCD4 group. * $P < 0.05$ compared to the sham group; # $P < 0.05$ compared to the CME group.

Fig. 2. Evaluation of TNF- α mRNA expression in each group by fluorescent quantitative PCR. (a) Sham group; (b) CME group; (c) Trimetazidine group; (d) siRNA-PDCD4 group. * $P < 0.05$ compared to the sham group; # $P < 0.05$ compared to the CME group.

Fig. 3. Evaluation of PDCD4 protein expression in each group by Western blot. (a) Sham group; (b) CME group; (c) Trimetazidine group; (d) siRNA-PDCD4 group. * $P < 0.05$ compared to the sham group; # $P < 0.05$ compared to the CME group.

Fig. 4. Evaluation of TNF- α protein expression in each group by Western blot. (a) Sham group; (b) CME group; (c) Trimetazidine group; (d) siRNA-PDCD4 group. * $P < 0.05$ compared to the sham group; # $P < 0.05$ compared to the CME group.

Protein expression of PDCD4, NF- κ B (p65), and TNF- α in each group assessed by Western blot (Fig. 3, 4, 5)

(1) The relative protein expressions of PDCD4, NF- κ B (p65), and TNF- α in the cardiomyocytes of CME group was significantly increased as compared to the sham group ($P < 0.05$). (2) The relative protein expressions of PDCD4, NF- κ B (p65), and TNF- α in the TMZ and siRNA-PDCD4 groups was significantly decreased as compared to the CME group ($P < 0.05$).

Discussion

CME is a common complication of plaque rupture and intervention treatment of bridging vessels in acute coronary syndromes. Unlike the epicardial proximal vascular occlusion, CME-induced reductions in left ventricular function are not closely related to the myocardial

Fig. 5. Evaluation of NF- κ B (p65) protein expression in each group by Western blot. (a) Sham group; (b), CME group; (c) Trimetazidine group; (d) siRNA-PDCD4 group. * $P < 0.05$ compared to the sham group; # $P < 0.05$ compared to the CME group.

range of perfusion defects [11] and cannot be attributed to local myocardial perfusion volume and small infarct area. Dorge et al. [12, 13] confirmed that the microinfarct occurred at local myocardium during the acute period after CME, together with extensive infiltration of inflammatory cells around the microinfarct, which was accompanied by a progressive decline in the cardiac function. Furthermore, our preliminary study demonstrated that PDCD4 was significantly elevated at 9 h after CME and extensively involved in the local inflammatory response of the myocardium. The stimulation of PDCD4 induced the universal activation of NF- κ B, as well as, the release of TNF- α and other inflammatory mediators that played a crucial role in the initiation and progression of CME-induced cardiac dysfunction. In addition, the increase in the level of TNF- α expression was one of the essential physiological responses of post-CME myocardial injury [14–17].

In this study, we demonstrated that the PDCD4/NF- κ B/TNF- α signaling pathway was significantly activated after CME, and it played a critical role in the CME-induced myocardial injury. The transfection of PDCD4 siRNA at 72 h before CME inhibited the expression of PDCD4 in the myocardium. It was also shown to interfere with the activation of NF- κ B activation, a significant reduction of TNF- α expression and serum cTnI, as well as, the improvement of cardiac function. These manifestations indicated that the inhibition of PDCD4/NF- κ B/TNF- α pathway efficiently improved the CME-induced myocardial injury, and suggested that the post-CME inflammatory response may be diminished in order to reduce the myocardial injury caused by the drug-induced inhibition of PDCD4 expression.

TMZ is a metabolic cytoprotectant that maintains the functions of the cell membrane including phospholipid synthesis and inhibition of calcium overload. This drug exhibits anti-inflammatory and anti-oxidative stress effects, whereas the cardioprotective effects are independent of coronary hemodynamics [18, 19]. A large number of clinical studies have shown that the pretreatment with TMZ significantly reduced the post-surgery level of cTnI and myocardial injury, and improved the left ventricular function. The mechanism could be attributed to the reduced local ischemic myocardium neutrophil aggregation and the local inflammatory response elicited by TMZ [20, 21]. Fragasso et al. [22] found that TMZ improved the myocardial systolic dysfunction of the ischemic heart disease, thereby improving the prognosis in patients with ischemic heart failure. In the previous studies, we found that TMZ enhanced the post-CME myocardial systolic function by inhibiting the apoptosis of cardiomyocytes [23]. However, the effect of TMZ on post-CME inflammation and its mechanism is yet unclear.

In the present study, we revealed that the pretreatment with TMZ before CME significantly improved the heart function, reduced the serum cTnI levels, and decreased the CME-induced myocardial injury in mini pigs. Furthermore, the PDCD4/NF- κ B/TNF- α signaling pathway was significantly inhibited by TMZ similar to that observed in the siRNA-PDCD4 group. This phenomenon indicated that TMZ reduced the CME-induced myocardial injury by inhibiting the PDCD4-mediated inflammatory response, which might be one of the vital mechanisms underlying the protective effect of TMZ on CME-induced myocardial injury.

Nevertheless, this study presented some limitations. Polymer microspheres constituted the microemboli agent used to induce the CME model, which differed from the embolic materials enriched with platelets, red blood cells, and other biologically active substances formed during the clinical atherosclerotic plaque rupture. Hence, the pathophysiological changes in the CME model differed from those of the actual CME caused by plaque rupture. Therefore, in future studies, we aspire to utilize the autologous thrombosis microembolization agent for further improvement in the CME model. In addition, this study was based on the animal experiments with small sample size, and hence, the findings necessitate further substantiation by large randomized controlled trials.

In summary, the pretreatment with TMZ effectively improved the CME-induced myocardial injury, which was primarily achieved by inhibiting the PDCD4/NF- κ B/TNF- α signaling pathway and reducing the inflammation in cardiomyocytes. This might be a pivotal mechanism underlying the positive effects of TMZ pre-PCI treatment on patient prognosis.

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Disclosure Statement

This paper has not been published elsewhere in whole or in part. All authors have read and approved the content and agree to submit for consideration for publication. There are no any ethical/legal conflicts involved in the article.

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