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Original Article

Effects of tetramethylpyrazine on cardiomyocyte apoptosis after myocardial ischemia reperfusion in rats

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Abstract: The present study aimed to investigate the effects of tetramethylpyrazine (TMP) on cardiomyocyte apoptosis after myocardial ischemia/reperfusion (I/R) injury in rats. Sprague-Dawley rats were randomly assigned to five groups: sham (normal saline), I/R (normal saline), low-dose TMP (50 mg/kg/day), mid-dose TMP (100 mg/kg/day), and high-dose TMP (200 mg/kg/day) groups. Treatments were delivered by intragastric administration for 2 weeks. After the last administration, myocardial I/R was induced by ligating the left anterior descending artery for 30 minutes followed by 120 minutes of perfusion. TMP decreased the activities of serum lactate dehydrogenase and creatine kinase and levels of reactive oxygen species, malondialdehyde, interleukins 6 and 1β, and tumor necrosis factor-α. However, superoxide dismutase activity was increased after TMP treatment. Moreover, expression of p53, caspases 3 and 9, Bax, and Bcl-2 was significantly decreased in the TMP groups, compared with the I/R group. In conclusion, the protective effects of TMP against I/R may be associated with oxidative stress, inflammation, and apoptosis.

Keywords: Ischemia reperfusion, tetramethylpyrazine, cardiomyocyte apoptosis, oxidative stress, inflammation

Introduction

Cardiovascular disease, particularly ischemic heart disease, is a major cause of death and disability, worldwide [1]. Many organs and tissues undergo reperfusion following ischemia and exhibit cardiomyocyte necrosis and apoptosis, which induce a series of molecular and cellular remodeling processes [2]. In recent years, strategies to protect against myocardial ischemia/reperfusion (I/R) injury have become a hot topic and the role of apoptosis in cardiovascular diseases has gained recognition [3]. In recent years, due to progress in methodologies and knowledge about apoptosis, a close relationship between myocardial I/R injury and apoptosis has been identified. This relationship has been shown to play an important role in the physiological and pathological development of the heart. Recent studies have reported that cardiomyocyte apoptosis is controlled by a series of physiological processes that mediate the signaling pathway, while blocking this signaling can prevent myocardial apoptosis [4, 5]. This has been considered the cytological basis for the development of pathological changes compensatory changes in the heart [6].

Tetramethylpyrazine (TMP) is an important active ingredient of Traditional Chinese Medicine that has been shown to have several molecular-targeting properties [7]. The number of studies on the physiological and pharmacological effects of TMP has increased recently. Several studies have shown that TMP has multiple health benefits, including prevention of ischemic strokes [8], neurovascular disorders [9], oxidation [10], and inflammation [11]. However, at present, the effects of TMP on apoptosis have not been evaluated in a model of myocardial infarction. Therefore, the present study aimed to evaluate the effects of TMP on I/R and potential mechanisms responsible for these effects.

Materials and methods

Chemicals and reagents

Tetramethylpyrazine (purity > 95%) was obtained from the National Institutes for Food and Drug Control (Beijing, China). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) *in situ* apoptosis detection kit was purchased from Nanjing Key Gen

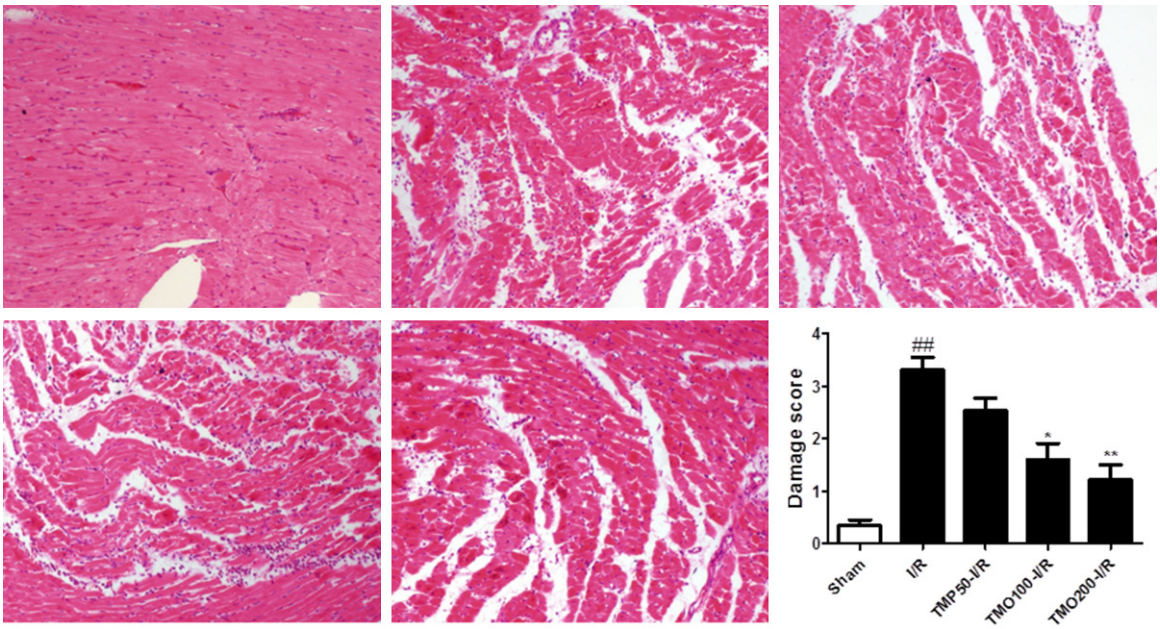

Figure 1. Effects of TMP on cell morphology and hematoxylin and eosin (HE) staining (× 200). Data are shown as mean ± SD. ##P < 0.01 compared with sham group; **P < 0.01 compared with I/R group.

Biotech. CO., LTD. (Nanjing, China). Each of the primary antibodies used in this study, including antibodies for Caspase-9, Caspase-3, Bax, and Bcl-2, were obtained from Cell Signaling Technology (Danvers, MA, USA). IL-6, IL-1β, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were produced by Nanjing KeyGEN Biotech. CO., LTD. (Nanjing, China). CK, LDH, MDA, and SOD kits were provided by Jiancheng Bioengineering Institute (Nanjing, China).

Experimental animals and groups

This study was carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Forty Sprague-Dawley (SD) rats (male, 250-300 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and group-housed in a temperature-controlled room with a 12-hour/12-hour light-dark cycle. All animal treatments in this study were performed humanely and followed Institutional and National Guidelines for Ethical Animal Research.

The rats were randomly assigned to the following four groups (10 rats in each group): sham group (normal saline), ischemic group (normal saline), low dose of TMP (50 mg/kg/day), middle dose of TMP (100 mg/kg/day), and high dose of TMP (200 mg/kg/day). All rats underwent continual intragastric administration for 2 weeks. All rats received the same volume. After 2 weeks, the rats, except for the control group, were made *in vivo* myocardial I/R models.

In vivo myocardial I/R model

The I/R model was induced by ligating the LAD for 30 minutes, followed by 24 hours of reperfusion. Briefly, male SD rats were anesthetized with 10% urethane (5 mg/kg). Following tracheal intubation, 6-0 silk ligature was used to ligate the left coronary artery for a 30-minute ischemic period. After 30 minutes of ischemia treatment, reperfusion was allowed for 24 hours by releasing the ligation of LAD. Sham control group rats were treated with the same surgical procedures, except ligation of the left coronary artery was not performed.

Histological examination of myocardium

The hearts were harvest and fixed in 10% (V/V) neutral buffered formalin solution. They were embedded with paraffin and cut into 4 μm thick sections. The tissues were stained with H&E and observed by light microscopy (Nikon, Tokyo, Japan).

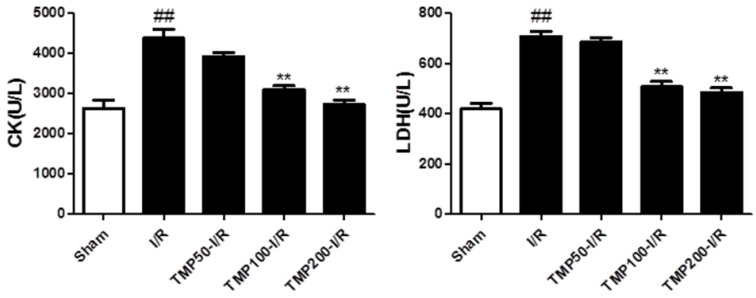

Figure 2. TMP reduces creatine kinase (CK) and lactate dehydrogenase (LDH) activity in serum of I/R induced rats. Data are shown as mean ± SD. ##P < 0.01 compared with sham group; **P < 0.01 compared with I/R group.

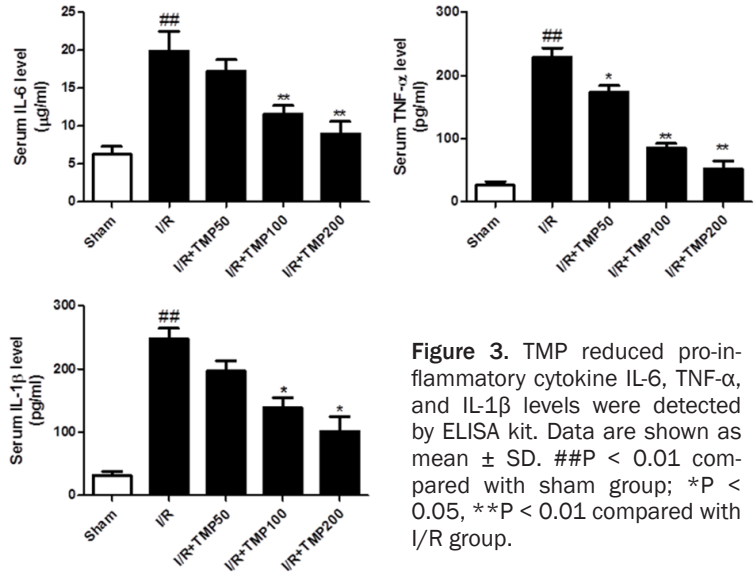

Figure 3. TMP reduced pro-inflammatory cytokine IL-6, TNF-α, and IL-1β levels were detected by ELISA kit. Data are shown as mean ± SD. ##P < 0.01 compared with sham group; *P < 0.05, **P < 0.01 compared with I/R group.

Cardiac marker enzymes detection

Myocardial cellular damage was detected by measuring serum lactate dehydrogenase (LDH) and creatine kinase (CK). Briefly, after the 120-minute reperfusion period, 1 mL of blood was collected through the carotid arteries of live rats. Serum was separated immediately by centrifugation in 3000 r min^{-1} for 10 minutes and stored at -80°C for use. Serum CK and LDH activities were assayed spectrophotometrically using commercially available kits, according to manufacturer instructions.

Determination of inflammatory cytokines in serum

Levels of IL-6, IL-1β, and TNF-α in serum were measured by ELISA kits, according to manufacturer instructions. Concentrations of the cytokines were quantified by standard curves. Absorbency was detected at 450 nm.

Reactive oxygen species (ROS) determination in myocardium

One part of myocardium of the left ventricle was fixed in 10% formalin and flash-frozen in liquid nitrogen, then stored at -80°C for use. Next, this stored tissue was placed on the tissue support with optimum cutting temperature compound (OCT)-embedded. Moreover, 10 μm tissue sections were cut and attached to the slides. For measurement of total ROS, 2',7',-dichlorofluorescein diacetate (DCFH-DA) assay was used. The DCFH-DA itself had no fluorescence and was free to cross the cell membrane. After entering into the cells, the cells can be esterase hydrolysis of 2',7',-dichlorofluorescein (DCFH). DCFH does not penetrate the cell membrane, thus the probe is easily accumulated in the cell. Intracellular reactive oxygen species can oxidize non-fluorescent DCFH to produce fluorescent DCF. Fluorescence intensity was measured at 485 nm for excitation and 525 nm for emission. ROS production was expressed as a percentage in fluorescence relative to the sham group.

Measurement of SOD and MDA

SOD and MDA are both important anti-oxidative enzymes. Myocardial tissue SOD and MDA activities were determined using specific ELISA kits, according to manufacturer instructions.

Western blotting

Total protein was extracted from myocardial tissues and analyzed by Western blotting with specific antibodies. Briefly, myocardial tissues were collected, then flash frozen in liquid nitro-

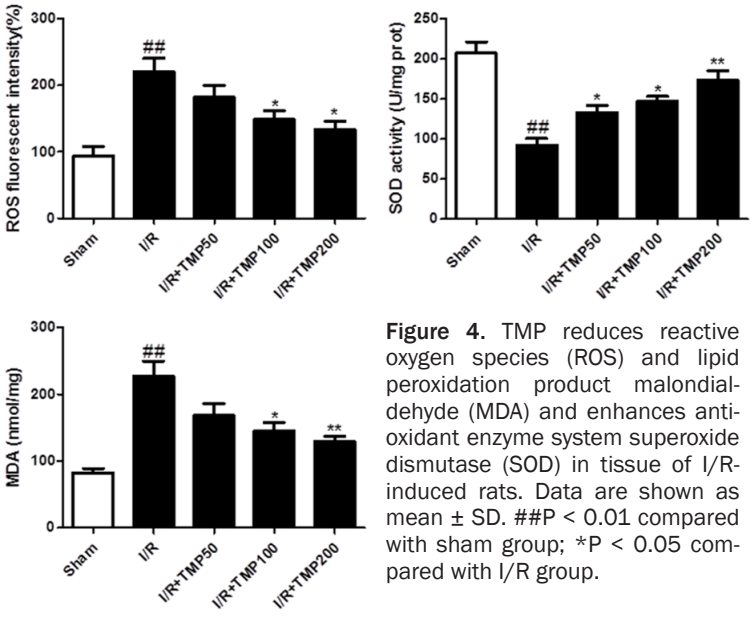

Figure 4. TMP reduces reactive oxygen species (ROS) and lipid peroxidation product malondialdehyde (MDA) and enhances antioxidant enzyme system superoxide dismutase (SOD) in tissue of I/R-induced rats. Data are shown as mean ± SD. ##P < 0.01 compared with sham group; *P < 0.05 compared with I/R group.

gen, immediately. Afterward, heats were homogenized and lysed in extraction buffer for 1 hour on ice. The lysates were centrifuged at 13,000 r for 20 minutes and the supernatant was collected. The protein concentration was determined by BSA assay. Equal amounts of protein were separated by 10% SDS-PAGE and electroblotted onto PVDF membranes. After blocking with 5% non-fat milk for 1.5 hours at 37°C, the membranes were incubated with the primary antibody overnight at 4°C, followed by peroxidase-conjugated secondary antibody for 1 hour at room temperature. Subsequently, immunoreactive protein bands were visualized with the Tanon 5200 Chemiluminescence imaging system.

Apoptosis assay

To detect the cardiomyocyte apoptosis, TUNEL kit was used, according to manufacturer protocol. According to this method, cells were defined as apoptotic cells if the entire nuclear area of the cell was positively labeled.

Statistical analysis

Values are represented as mean ± SD and were analyzed using one-way analysis of variance (ANOVA) and Student's t-tests, using GraphPad Prism Software (GraphPad Inc., La Jolla, CA, USA). P < 0.05 indicates statistical significance.

Results

Effects of TMP on histopathological changes in I/R model

As shown in **Figure 1**, the myocardial structure in the sham group exhibited a regular arrangement, normal cardiac muscle fibers, and no necrosis. Compared with the sham group, myocardial fiber loss and disarray could be observed in the I/R group. The phenomena were reversed by TMP in a dose-dependent manner.

Effects of TMP on serum levels of LDH and CK in I/R model

Leakage of LDH and CK from myocardial tissues to blood is an indicator of acute myocardial infarction. As shown in **Figure 2**, compared with the sham group, LDH and CK in the I/R group both significantly increased (P < 0.01), while TMP pretreatment (especially 200 mg/kg administration) decreased LDH and CK levels.

Effects of TMP on the inflammatory reaction in I/R model

Levels of TNF-α, IL-1β, and IL-6 were significantly increased in the I/R group, compared with the sham group (**Figure 3**). Oral administration of TMP decreased expression of TNF-α, IL-1β, and IL-6, especially at the high dosage. Taken together, those results demonstrate that TMP could inhibit inflammatory reaction in the I/R model.

Effects of TMP on antioxidant reactions in I/R model

As shown in **Figure 4**, ROS production and MDA activity were markedly decreased, compared to the I/R group (P < 0.01, respectively). Compared with the I/R group, ROS and MDA in the treatment of TMP groups all decreased in a dose-dependent manner. Additionally, TMP significantly increased the activity of SOD.

Effects of TMP on myocardial apoptosis in I/R model

TUNEL staining suggested that more brown stained cells were found in the I/R group than

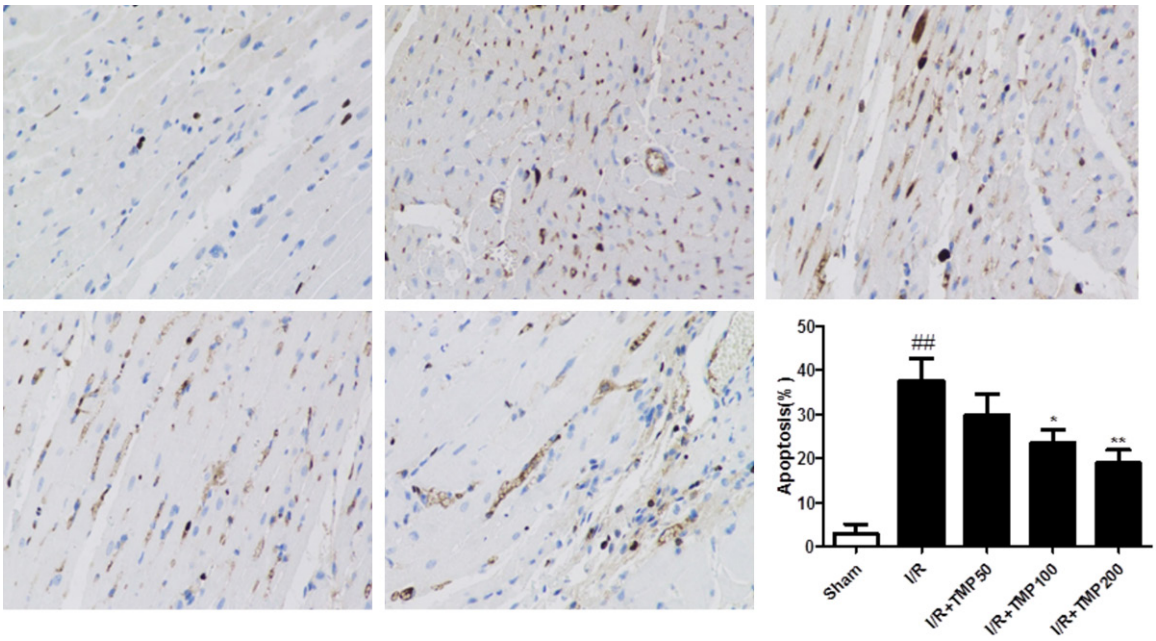

Figure 5. Effects of TMP suppression on cardiomyocyte apoptosis (× 400). Data are shown as mean ± SD. ##P < 0.01 compared with sham group; *P < 0.05, P < 0.01 compared with I/R group.

those in the sham group (P < 0.01). Compared with the I/R group, the low dose of TMP and the high dose of TMP groups decreased the number of apoptotic cells (P < 0.05, P < 0.01, respectively). Effects of TMP treatment for the myocardial I/R model on myocardial apoptosis are shown in **Figure 5**.

Effects of TMP on apoptosis related protein expression in I/R model

Apoptosis related protein (Caspase-9, Caspase-3, Bax, and Bcl-2) expression was determined by Western blot. As shown in **Figure 6**, upregulated levels of Caspase-9, Caspase-3, and Bax and downregulated level of Bcl-2 were observed in the I/R group, compared with the sham group. TMP restored those changes.

Discussion

The underlying pathophysiological mechanisms of I/R injury have not been fully elucidated. However, previous studies have suggested that apoptosis, inflammation, oxidative stress, and calcium overload during the first few minutes of reflow are involved [12]. Apoptosis is a programmed cell death pathway that is necessary to maintain tissue homeostasis, but excessive apoptosis or its dysregulation can lead to various pathological processes, including myocardial I/R injury [13]. Cardiomyocyte apoptosis is involved in the normal growth of myocardial tissue [14]. However, apoptosis of cardiomyocytes that occurs in conjunction with cardiac pathology plays an important role in the deterioration of cardiac function. Cardiomyocyte apoptosis has been shown to decrease the number of working cells in the heart. Extensive fibrosis after cardiomyocyte apoptosis can lead to remodeling and malignant arrhythmia [15]. Apoptosis is a cell suicide process initiated by multiple signaling transduction pathways and is an important mechanism for homeostasis. Myocardial apoptosis accelerates the development of necrosis, which may affect the degree of myocardial injury [16]. However, there are few mechanisms known to reduce myocardial apoptosis. Reperfusion injury is associated with an inflammatory cascade and oxidative stress that perpetuates further damage to cardiac tissue after a period of ischemia [17]. ROS produced from normal cellular metabolism, as well as exposure to external factors, can damage intracellular DNA, bio-membrane lipids, proteins, and other macromolecules [18]. In addition, increasing evidence has revealed that inflammation plays a vital role in occurrence and development of I/R. Suppression of excessive inflammation can ameliorate impaired cardiac function, showing therapeutic potential for

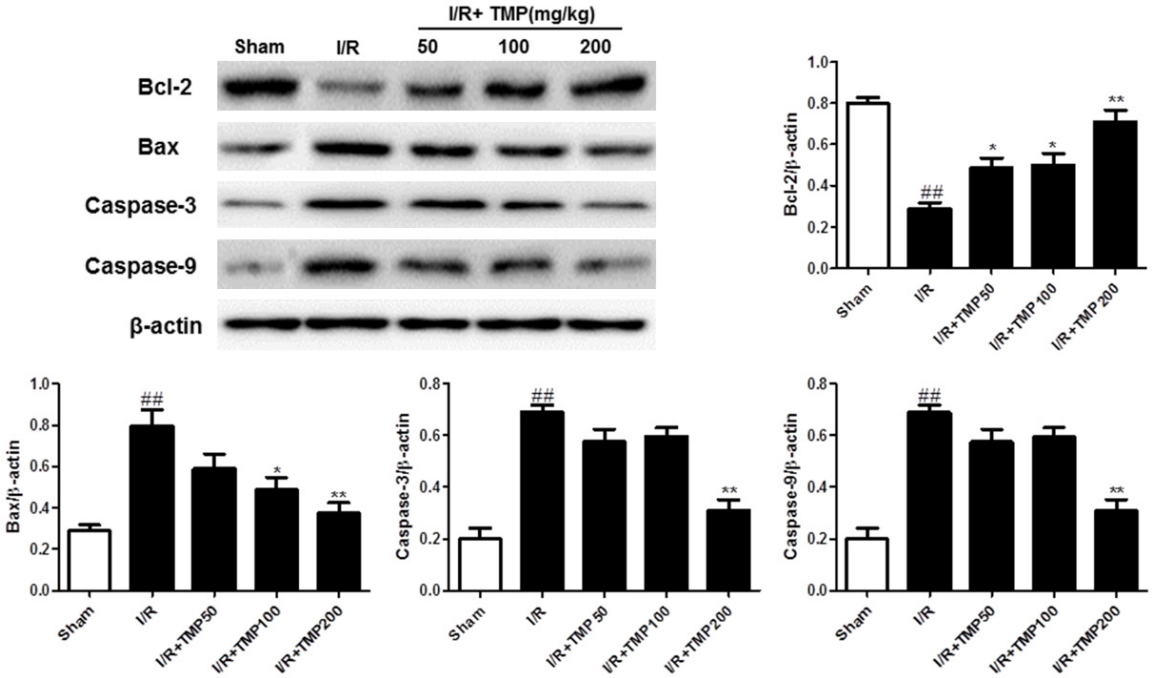

Figure 6. Apoptosis-related proteins Bcl-2, Bax, caspase-3, and caspase-9 were measured by Western blot. ##P < 0.01 compared with sham group; *P < 0.05, **P < 0.01 compared with I/R group.

I/R diseases [19]. Several studies have shown that inflammatory cascades and oxidative stress result in apoptosis during reperfusion injury[20]. In this study, TMP reduced serum levels of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) and oxidative stress (ROS, MDA) during I/R injury, in a dose-dependent manner.

Apoptosis results in tissue damage that is secondary to reperfusion injury after ischemia, a vital pathophysiological mechanism associated with myocardial I/R injury [21]. Cardiomyocyte apoptosis is a characteristic of normal myocardial tissue growth, but occurrence in heart muscle plays an important role in the deterioration of cardiac function. In myocardial I/R injury, excessive apoptosis can aggravate the destruction of myocardial tissue [22]. The exact mechanisms of myocardial apoptosis induced by myocardial I/R have not yet been elucidated. Treatment of cardiomyocyte apoptosis has also been cleanly performed in animal experiments [23]. In the present study, TUNEL assay was performed to examine myocardial apoptosis. In contrast to the sham group, myocardial apoptosis in the I/R group was significantly increased, whereas TMP pretreatment significantly decreased apoptosis in myocardial tissue. Furthermore, recent studies have suggested that apoptosis contributes, in part, to overall myocyte death during the reperfusion period. Bcl-2, an important mitochondrial regulator during myocardial apoptosis, blocks the release of cytochrome C and downregulates caspase activity to exert anti-apoptosis effects. In addition, apoptosis-related proteins, such as Bax, caspase-3, and caspase-9, also play pivotal roles in apoptosis. In this study, pretreatment with TMP significantly decreased myocardial apoptosis and expression of apoptosis-related proteins, such as Bax, caspase-3, and caspase-9, while increasing Bcl-2 levels compared with I/R rats. Results indicate that TMP exerts anti-apoptosis effects by regulating Bax/Bcl and caspase-3/-9.

Apoptosis is a mechanism of regulating cellular life and death. Elucidation of apoptosis can improve the understanding of diseases, including cardiovascular diseases, leading to new medical therapies and deepening the understanding of normal cellular life processes. In conclusion, the present study demonstrated that TMP exhibits significant cardioprotective effects against I/R injury, associated with antioxidant, anti-inflammatory, and antiapoptotic

activities. Thus, TMP deserves additional experimental and clinical research concerning cardiovascular diseases.

Disclosure of conflict of interest

None.

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