*Acta Physiologica Sinica*, August 25, 2017, 69(4): 367–377
DOI: 10.13294/j.aps.2017.0018 http://www.actaps.com.cn



**Original Article**

# Activation of caspase-12 at early stage contributes to cardiomyocyte apoptosis in trauma-induced secondary cardiac injury

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**Abstract:** Trauma-induced secondary cardiac injury (TISCI) is associated with increased adverse cardiac events and death. We have previously reported that TISCI results in myocardial apoptosis and secondary cardiac dysfunction. However, the underlying mechanism is unclear. To identify the time course of trauma-induced cardiomyocyte apoptosis and possible apoptotic pathway, traumatic rat models were built with Noble-Collip drum. Meanwhile, normal rat cardiomyocytes were cultured with traumatic plasma (TP) for 48 h. Cardiomyocyte apoptosis, cardiac function and the apoptosis related enzymes, including caspase-3, -8, -9, and -12, were determined. The results showed that there was no direct injury of rat hearts immediately after trauma. However, compared with hearts from the sham rats, hearts isolated from traumatic rats exhibited reduced $+dP/dT_{max}$ and $-dP/dT_{max}$ 24 h after trauma. In traumatic rats, myocardial apoptotic index and caspase-3 activity obviously increased 6 h after trauma, and achieved the maximal value 12 h after trauma. The activity and expression of caspase-12, an endoplasmic reticulum (ER) stress-specific caspase, elevated markedly 3 h after trauma and reached its peak 6 h after trauma. Otherwise, caspase-8 (extrinsic apoptotic pathway) and caspase-9 (intrinsic apoptotic pathway) in the myocardial tissue of traumatic rats were activated 24 h after trauma. Meanwhile, incubation of normal rat cardiomyocytes with TP increased caspase-12 activity at 6 h, caspase-3 activity at 12 h, caspase-8 and -9 activities at 24 h, respectively. TP-induced cardiomyocyte apoptosis was virtually abolished by Z-ATAD-FMK (a caspase-12 specific inhibitor). In addition, there was a significant negative correlation between myocardial caspase-12 activity and trauma-induced secondary cardiac dysfunction. Our present study demonstrated that caspase-12 is firstly activated and plays an important role in TISCI rats. Inhibition of caspase-12 mediated apoptosis may be a novel strategy in ameliorating posttraumatic cardiomyocyte apoptosis and secondary cardiac injury.

**Key words:** trauma; heart injuries; caspase; endoplasmic reticulum stress; apoptosis

# 创伤早期激活的caspase-12引起心肌细胞凋亡及继发性心脏损伤

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Received 2016-12-02 Accepted 2017-02-15

This work was supported by grants from the National Natural Science Foundation of China (No. 81500185, 81341057), the Open Foundation of the Beijing Key Laboratory of Metabolic Disorders Related Cardiovascular Diseases of Capital Medical University (No. 2014DXWL04), the Science and Technology Innovation Fund (No. 057595) and 331 Early Career Researcher Grant of Shanxi Medical University, China (No. 201402).

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**摘 要：**创伤引起的继发性心脏损伤(trauma-induced secondary cardiac injury, TISCI)是创伤致死的重要原因。本研究组前期研究观察到创伤可以导致心肌细胞凋亡和继发性心功能受损，但具体机制不清。本研究旨在观察创伤时心肌细胞凋亡发生的时间规律，并探讨创伤导致心肌细胞凋亡的可能途径。采用Noble-Collip鼓制备创伤大鼠模型；利用创伤后血清培养正常大鼠心肌细胞48 h。在不同时间点检测心肌细胞凋亡、心脏功能及凋亡相关蛋白酶caspase-3、-8、-9和-12。结果显示，继发性心功能受损出现在创伤后24 h，而创伤大鼠心肌凋亡指数和caspase-3活性均在创伤后6 h就显著增加，12 h达峰值。大鼠心肌组织caspase-12 (介导内质网应激反应性凋亡途径)活性和表达在创伤后3 h出现明显升高，6 h达峰值；而心肌组织caspase-8 (介导外源性凋亡途径)和caspase-9 (介导内源性凋亡途径)均于创伤后24 h激活。同时，采用创伤血清培养正常大鼠心肌细胞，结果同样显示，创伤血清可以引起正常大鼠心肌细胞caspase-3活性显著增加，且心肌细胞caspase-12的活化早于caspase-3、-8和-9的激活；给予caspase-12特异性抑制剂Z-ATAD-FMK处理后，创伤血清引起的心肌细胞凋亡显著降低。此外，创伤后6 h心肌组织caspase-12活性的增高与创伤后24 h继发性心功能减低呈现显著的负相关。本研究表明，在TISCI时caspase-12最早激活，且激活的caspase-12是创伤所致心肌细胞凋亡的重要原因，因此抑制caspase-12介导的细胞凋亡有望成为减轻TISCI的新举措。

**关键词：**创伤；心脏损伤；半胱天冬酶；内质网应激；凋亡
**中图分类号：**R331

Cardiac injury following trauma is an important cause of death and is usually unsuspected [1]. It is well known that severe trauma results in direct heart damage, such as cardiac rupture [2] and coronary artery dissection [3]. However, recently published clinical reports have clearly demonstrated that trauma causes cardiac dysfunction [4] or myocardial failure [5] several days [4] or months [5] after trauma even in the absence of direct myocardial injury, which substantiates the existence of trauma-induced secondary cardiac injury (TISCI) [6]. Nevertheless, the mechanisms responsible for TISCI still remain elusive.

Our previous results revealed that mechanical trauma could cause secondary cardiac dysfunction and significantly increase caspase-3 (a final common pathway in caspase-dependent apoptosis) activity. Nonselective caspase inhibitor administration could attenuate posttraumatic cardiac dysfunction [7]. These results demonstrated that traumatic injury led to cardiomyocyte apoptosis, which contributed to posttraumatic secondary cardiac dysfunction. Caspase-dependent apoptosis is a critical cell death pathway for myocardial apoptosis, which possesses multiple caspases and works in a cascade fashion. Considerable evidence from many investigators has since shown that caspase-dependent apoptosis is inextricably associated with caspase activity, in which caspase-3 activation as a final common pathway [8]. Since TISCI in the clinical has higher mortality rates [6], it is necessary to investigate the time course of apoptosis occurrence, which might be beneficial for preventing secondary cardiac injury earlier.

It has been described that there are three major caspase-dependent apoptotic pathways, including the death receptor-mediated (extrinsic) pathway, the mitochondrion-associated (intrinsic) pathway, and the endoplasmic reticulum (ER) stress-specific pathway, which are initiated by caspase-8, caspase-9, and caspase-12, respectively [9]. When activated, these caspases cleave and activate downstream effector caspases, including caspase-3 that in turn executes apoptosis. Previous studies have shown that in many pathological conditions, caspase-3 was activated through different apoptotic pathways [10, 11]. In addition, studies have illustrated that caspase-12-mediated ER apoptotic pathway played a role in rat traumatic brain injury pathology independent of the receptor- or mitochondria-mediated apoptotic pathways [12]. Although existing studies have demonstrated that caspase-3 activity increased, which pathway is involved in caspase-3 activation and the secondary heart injury after mechanical trauma has not yet been identified.

Therefore, the aims of the current study were to: (1) investigate the time course of cardiomyocyte apoptosis after traumatic injury; (2) delineate possible initiated apoptotic pathway involved in posttraumatic cardiomyocyte apoptosis and cardiac dysfunction.

## 1 MATERIALS AND METHODS

### 1.1 *Animals*

The animal-related experimental procedures were performed according to the ‘‘Guiding Principles in the Care and Use of Animals’’ published by the National Institutes of Health, “the Guide for the Care and Use of Laboratory Animals Protocol” published by the People’s Republic of China, and approved by the Insti-

tutional Animal Care and Use Committee of Shanxi Medical University.

1.2 *Induction of traumatic rat model and treatment*

Mechanical trauma model was established as previously described [13, 14]. In brief, adult male Sprague-Dawley rats (200–240 g) were anesthetized with chloral hydrate (400 mg/kg, intraperitoneally). Then the rats were placed in a Noble-Collip drum (WYM-1, Thomas Jefferson University, USA) and subjected to a total of 200 revolutions at rate of 40 r/min. Traumatic rats were injured when the wheel was rotated. Sham rats were exposed to the same revolution, but fixed on the inner wall of drum with tape avoiding traumatic injury. After completion of the procedure, the rats were euthanized at different time points. Rats in trauma+Z-VAD-FMK group received Z-VAD-FMK (a broad-spectrum caspase inhibitor, 4 mg/kg, intravenously; V116, Sigma, USA) [7] immediately after trauma.

1.3 *Cardiomyocyte culture and treatment*

The ventricular cardiomyocytes were isolated using an enzymatic technique as previously reported [15]. Briefly, the normal rats were anesthetized with pentobarbital sodium (100 mg/kg, intraperitoneally). Then the hearts were rapidly removed and retrogradely perfused in a Langendorff system (37 °C, ML870B2, AD Instruments, AU) with a $Ca^{2+}$-free bicarbonate-based buffer (containing 120 mmol/L NaCl, 5.4 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L$NaH_2PO_4$, 5.6 mmol/L glucose, 20 mmol/L $NaHCO_3$, 10 mmol/L 2, 3-butanedione monoxime, and 5 mmol/L taurine), which was continuously gassed with 95% $O_2$ + 5% $CO_2$. The enzymatic digestion was commenced by adding collagenase type II (0.5 mg/mL; 17101-015, Gibco, USA) and protease type XIV (0.02 mg/mL; P5147, Sigma, USA) with 50 μg of $CaCl_2$ to the perfusion buffer. The digested ventricular tissue was cut into chunks and aspirated with a transfer pipette for facilitating the cell dissociation. The cell pellet was resuspended for a three-step $Ca^{2+}$ restoration procedure (i.e. 125, 250, and 500 μmol/L $CaCl_2$). Myocytes were plated at $0.5 \times 10^4$–$1 \times 10^4$ cells/$cm^2$ in culture dishes precoated with laminin. After 1 h of culture in a 5% $CO_2$ incubator at 37 °C, the medium was changed to FBS-free MEM. After 24 h, cardiomyocytes were cultured for 48 h with medium containing 10% plasma (isolated 1.5 h after trauma) obtained from sham or traumatic rats [16]. To delineate the role of caspase-12 in cardiomyocyte apoptosis initiated by traumatic injury, cardiomyocytes were subjected to one of the following treatments: (1) normal plasma (NP); (2) traumatic plasma (TP); (3) TP plus caspase-12 inhibitor Z-ATAD-FMK (50 μmol/L; 1152-1, BioVision, USA). At the end of the experiments, cardiomyocytes were lysed in lysis buffer, and apoptotic rate as well as caspase-3, -8, -9, -12 activities were determined.

1.4 *Quantitative determination of myocardial apoptosis with the terminal deoxyneucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay*

To determine myocardial apoptosis in a quantitative manner, the hearts were perfused first with 0.9% NaCl for 5 min and then 4% paraformaldehyde in PBS (pH 7.4) for 20 min. Four longitudinal sections from the free wall of the left ventricle were cut and further fixed in 4% paraformaldehyde in PBS for 24 h at room temperature. Fixed tissues were embedded in a paraffin block and two slides at 4- to 5-μm thickness were cut from each tissue block. Myocardial apoptosis were detected by using a TUNEL apoptosis detection kit (17-141, Millipore, USA) according to the manufacturer's instructions. An additional staining was performed with monoclonal anti-α-sarcomeric actin (ab1321, Abcam, UK). This staining enables the identification of myocyte and, therefore, a distinction between myocyte nuclei and nuclei of other cells in cardiac tissue. After rinsing with PBS, the slides were coverslipped with mounting medium containing DAPI (D8200, Solarbio, China) to permit total nuclei counting.

With the use of a ×20 objective, tissue slide was digitally photographed with confocal microscope (FV1000, Olympus, JPN). For each slide, 10 fields were randomly chosen, and a total of 100 cells per field were counted. Total nuclei (DAPI staining, blue) and TUNEL positive nuclei (green) in each field were counted, then the index of apoptosis was calculated using the formula: number of TUNEL-positive nuclei/total number of nuclei × 100%. Results from different fields taken from the same animal were averaged and counted as one sample. Assays were performed in a blinded manner.

1.5 *Detection of the cardiac function in isolated perfused rat heart*

At different time points after trauma, the animals were reanesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally) and heparinized with sodium heparin (1 000 U/kg, intraperitoneally). Ten minutes after heparin injection, the heart was rapidly removed out and mounted on Langendorff heart perfusion apparatus

(LE05.200, Panlab, ES) via aorta for retrograde perfusion with Krebs-Henseleit (K-H) buffer at constant pressure (10 kPa) and constant temperature (37 °C). K-H buffer solution was composed of (in mmol/L): 118 NaCl, 4.75 KCl, 1.19 $KH_2PO_4$, 1.19 $MgSO_4 \cdot 7H_2O$, 2.54 $CaCl_2 \cdot 2H_2O$, 25 $NaHCO_3$, 0.5 EDTA, and 11 glucose. The buffer was saturated with 95% $O_2$/5% $CO_2$ (pH 7.4). The hearts were paced at 260 beats/min with a Grass Stimulator and coronary flow (CF) was measured via an in-line flow probe connected to an ultrasonic flow meter.

After the hearts were equilibrated for 30 min, a water-filled latex balloon-tipped catheter was inserted into the left ventricle cavity through the mitral orifice and connected to a pressure transducer for assessing contractile function. The balloon was initially inflated with saline to produce an end-diastolic pressure of 8 to 10 mmHg, which was on the plateau of the Starling curve for this preparation. Left ventricular pressure (LVP) and CF were continually recorded via PowerLab data acquisition system (AD Instruments, AU). The left ventricular (LV) systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), LV developed pressure (LVDP = LVSP – LVEDP), the maximal positive and negative values of the instantaneous first derivative of LVP ($+dP/dT_{max}$ and $-dP/dT_{max}$) were continuously recorded with PowerLab system. The data were analyzed by Chart software (AD Instruments, AU).

1.6 *Measurement of caspase-3, -8, -9, -12 activities in cardiomyocyte*

Caspase-3, -8, -9 and -12 activities detection was performed with caspase fluorometric assay kits (Abcam, UK) according to the manufacturer's instructions. In short, myocardial tissue was homogenized in ice-cold lysis buffer, and then the lysate was centrifuged at 14 000 *g* for 10 min at 4 °C. Supernatants were collected, and protein concentrations were measured by the bicinchoninic acid method (23225, Thermo Scientific, USA). And cardiomyocytes were resuspended and lysed in chilled cell lysis buffer. The substrate DEVD-AFC (ab39383, Abcam, UK) was used to determine caspase-3 activity, and the same was true for the substrates IETD-AFC (ab39534, Abcam, UK) for caspase-8, LEHD-AFC (ab65607, Abcam, UK) for caspase-9 and ATAD-AFC (ab65664, Abcam, UK) for caspase-12. The supernatant or cell suspension was incubated with reaction buffer (containing 10 mmol/L dithiothreitol) and DEVD/IETD/LEHD/ATAD-AFC (final concentration 50 μmol/L) at 37 °C for 1.5 h. Activities of caspase-3, -8, -9 and -12 were determined using a microplate reader (SpectraMax M2/M2e, Molecular Devices, USA) at 400 nm excitation filter and 505 nm emission filter.

1.7 *Determination of caspase-12 and glucose-regulated protein 78 (GRP78) protein expression with Western blot analysis*

Proteins from cardiac tissue were separated on SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes (W00163, Millipore, USA), and Western blotted with primary antibodies against caspase-12 (1∶1 000; ab62484, Abcam, UK) or GRP78 (1∶2 500; ab21685, Abcam, UK) followed by secondary antibody incubation. The blot densities were detected and analyzed with a LI-COR imaging system (9201-01, LI-COR Biosciences, USA).

1.8 *Detection of apoptosis using Annexin V-FITC/propidium iodide (PI)*

The apoptotic cells were detected by using Annexin V-FITC apoptosis detection kits (G003, Nanjing KeyGen Biotech Co., China). The cardiomyocytes were collected, washed twice with PBS, resuspended in binding buffer (10 mmol/L HEPES/NaOH pH 7.4, 140 mmol/L NaCl and 2.5 mmol/L $CaCl_2$) and incubated with Annexin V at room temperature in the dark for 15 min. The cells were then centrifuged, resuspended in binding buffer, and incubated with PI. Subsequently, binding buffer was added, and the apoptotic cells were assessed using a flow cytometer (FACSCalibur, BD Biosciences, USA).

1.9 *Statistical analysis*

All values in the text and figures were presented as means ± SEM. Time and group differences were determined by two-way ANOVA for repeated measures. For nonrepeated measures, data were subjected to ANOVA followed by the Scheffe's correction for *post hoc t*-test comparison. Probabilities ≤ 0.05 were considered to be statistically significant.

## 2 RESULTS

### 2.1 Myocardial apoptosis played a critical role in posttraumatic cardiac dysfunction

In order to prove the contribution of myocardial apoptosis to cardiac dysfunction induced by mechanical trauma, cardiac function and apoptosis were respectively measured by isolated perfused heart assay and

TUNEL. As summarized in Fig. 1*A* and 1*B*, compared with sham group, both $+dP/dT_{max}$ [(3 414 ± 208) mmHg/s *vs* (4 251 ± 168) mmHg/s, $P < 0.01$] and $-dP/dT_{max}$ [(−3 301 ± 458) mmHg/s *vs* ( −5 221 ± 488) mmHg/s, $P < 0.05$] were significantly decreased 24 h after trauma. As illustrated in Fig. 1*C*, there were no obvious TUNEL-positive nuclei detected in samples derived from sham rats. However, 24 h after trauma, TUNEL-positive nuclei were markedly increased in samples from traumatic rats ($P < 0.01$ *vs* sham). Z-VAD-FMK (a broad-spectrum caspase inhibitor, 4 mg/kg) treatment effectively diminished the increased TUNEL-positive nuclei ($P < 0.01$ *vs* trauma group). Meanwhile, administration of Z-VAD-FMK immediately after trauma significantly blocked cardiac dysfunction caused by traumatic injury [$+dP/dT_{max}$: (4 111 ± 189) mmHg/s, $P < 0.01$ *vs* trauma group; $-dP/dT_{max}$: (−4 997 ± 351) mmHg/s, $P < 0.05$ *vs* trauma group], despite that administration of this compound had no visible effect in sham group (data not shown). The results showed that Z-VAD-FMK could markedly improve reduced cardiac function induced by trauma, which suggested that myocardial apoptosis played an important role in posttraumatic cardiac dysfunction.

### 2.2 Myocardial apoptosis occurred earlier than cardiac dysfunction caused by trauma

To clarify the time course of myocardial apoptosis in mechanical trauma, different time points after trauma were chosen. The results showed that myocardial apoptotic index significantly increased 6 h after trauma compared with sham group [(6.71 ± 1.91)%, $P < 0.01$ *vs* sham], and reached the peak 12 h after trauma [(14.42 ± 3.46)%, $P < 0.01$ *vs* sham]. It still remained at high level 24 h after trauma [(8.12 ± 2.54)%, $P <$

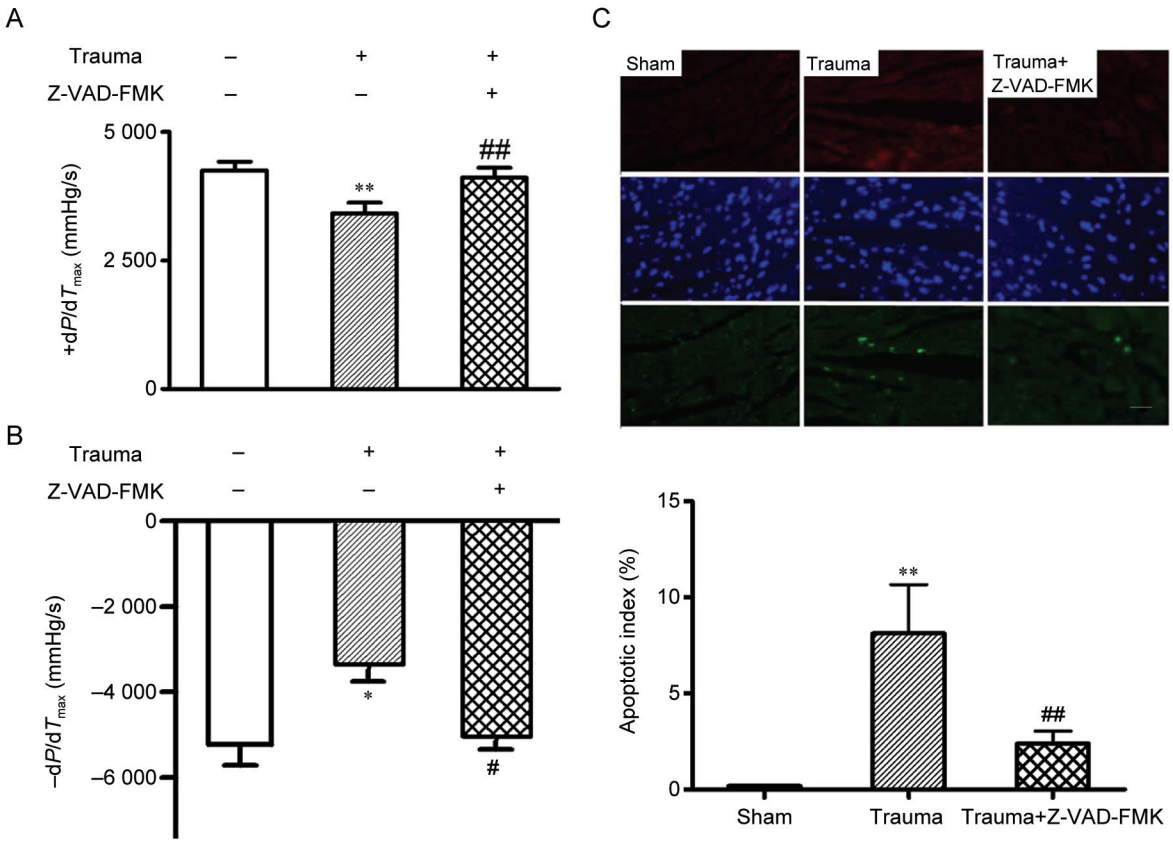


Fig. 1. The effect of Z-VAD-FMK, a nonselective caspase inhibitor, on mechanical trauma induced cardiac dysfunction and myocardial apoptosis. *A*: The maximal positive values of the instantaneous first derivative of left ventricular pressure ($+dP/dT_{max}$) 24 h after trauma. *B*: The maximal negative values of the instantaneous first derivative of left ventricular pressure ($-dP/dT_{max}$) 24 h after trauma. *C*: The terminal deoxyneucleotidyl transferase-mediated dUTP nick-end (TUNEL) labeling 24 h after trauma. Cardiac myocytes were identified by anti-α-actin antibody (red), total nuclei were labeled with DAPI (blue), and apoptotic nuclei were detected by TUNEL staining (green). Scale bar, 20 μm. The results were expressed as an apoptotic index. Mean ± SEM, $n = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* trauma group.

0.01 *vs* sham] (Fig. 2*A*). To obtain more evidence in a specific and quantitative manner, myocardial caspase-3 activity was determined. As shown in Fig. 2*B*, the trend change of caspase-3 activity after trauma was the same as that of apoptotic index. However, there were no significant differences in $+dP/dT_{max}$ and $-dP/dT_{max}$ at 0 h, 3 h, 6 h, and 12 h after trauma between trauma group and sham group (Fig. 2*C* and *D*). Taken together, these results clearly demonstrated that myocardial apoptosis appeared earlier than cardiac dysfunction in mechanical trauma rats.

### 2.3 Caspase-12 was firstly activated in caspase-dependent pathways after traumatic injury

To further explore the possible apoptotic pathways elicited within 24 h after trauma, the activities of caspase-8, -9 and -12 in myocardial tissue were detected. As illustrated in Fig. 3*A* and *B*, myocardial caspase-8 and caspase-9 activities greatly increased 24 h after trauma (caspase-8: 2 312 ± 648 *vs* 1 449 ± 296, $P < 0.01$ *vs* sham; caspase-9: 875 ± 460 *vs* 470 ± 222, $P < 0.01$ *vs* sham). However, caspase-12 activity was significantly elevated 3 h after trauma (6 613 ± 824 *vs* 2 731 ± 108, $P < 0.01$ *vs* sham), reached its maximum 6 h after trauma (8 957 ± 165, $P < 0.01$ *vs* sham), and significantly decreased 12 h after trauma (3 216 ± 379, $P > 0.05$ *vs* sham) (Fig. 3*C*). To obtain more evidence in a quantitative manner, myocardial caspase-12 protein expression was determined. As shown in Fig. 3*D*, cleaved caspase-12 protein expression was markedly upregulated in cardiac tissue 3 h after traumatic injury ($P < 0.05$ *vs* sham) and reached a maximal level 6 h after trauma ($P < 0.01$ *vs* sham). To further determine excessive ER stress caused by mechanical trauma, ER stress pathway marker GRP78 was detected. As illustrated in Fig. 3*E*, the protein expression of myocardial GRP78 was enhanced 3, 6, 12 h after trauma compared with that of sham group ($P < 0.05$, $P < 0.01$, and $P < 0.05$ *vs* sham, respectively).

In addition, the time courses and the activities of the caspase-3, -8, -9 and -12 in the cultured normal rat cardiomyocytes incubated with TP were also analyzed. As can be seen in Fig. 4*A*, the activity of the effector caspases-3 was increased at 12 h ($P < 0.01$ *vs* NP group) and peaked at 24 h ($P < 0.01$ *vs* NP group). The activities of caspase-8 and caspase-9 were similarly increased at 12 h and reached a peak activity at 24 h (Fig. 4*B* and *C*). In contrast, the enzymatic activity of caspase-12 was increased 6 h after treatment with TP ($P < 0.05$ *vs* NP group), and 12 h reached its maximum ($P < 0.01$ *vs* NP group) (Fig. 4*D*). These results demonstrated for the first time that in the early period of trauma, cardiomyocyte apoptosis was induced by activated

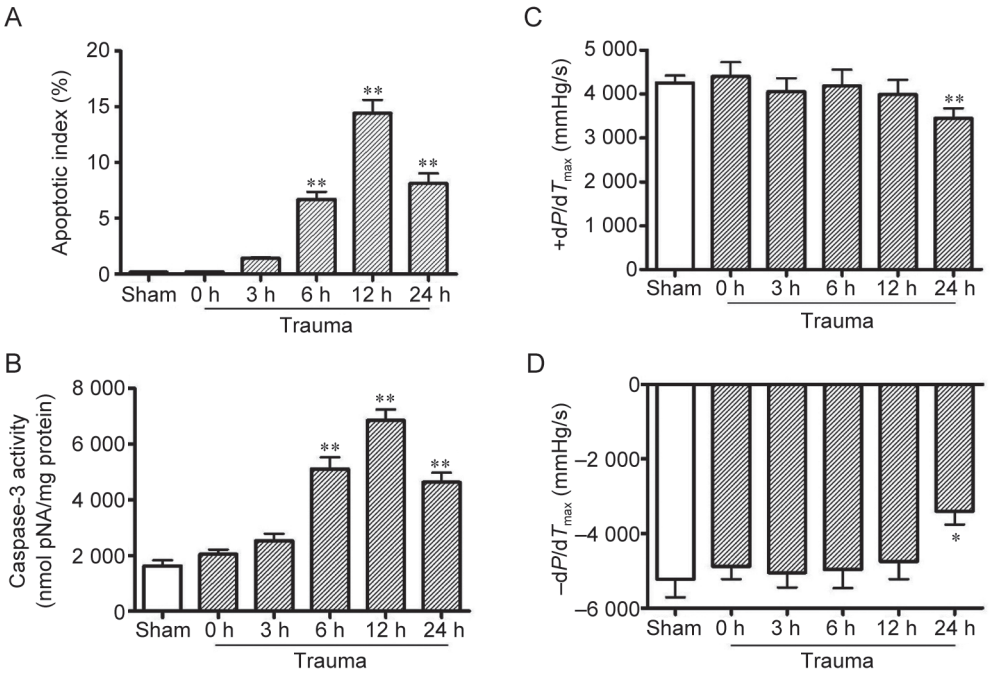


Fig. 2. The time course of myocardial apoptosis and cardiac function within 24 h of trauma. *A*: Apoptotic index by TUNEL labeling. *B*: Caspase-3 activity. *C*: $+dP/dT_{max}$. *D*: $-dP/dT_{max}$. Mean ± SEM, $n = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* sham group.

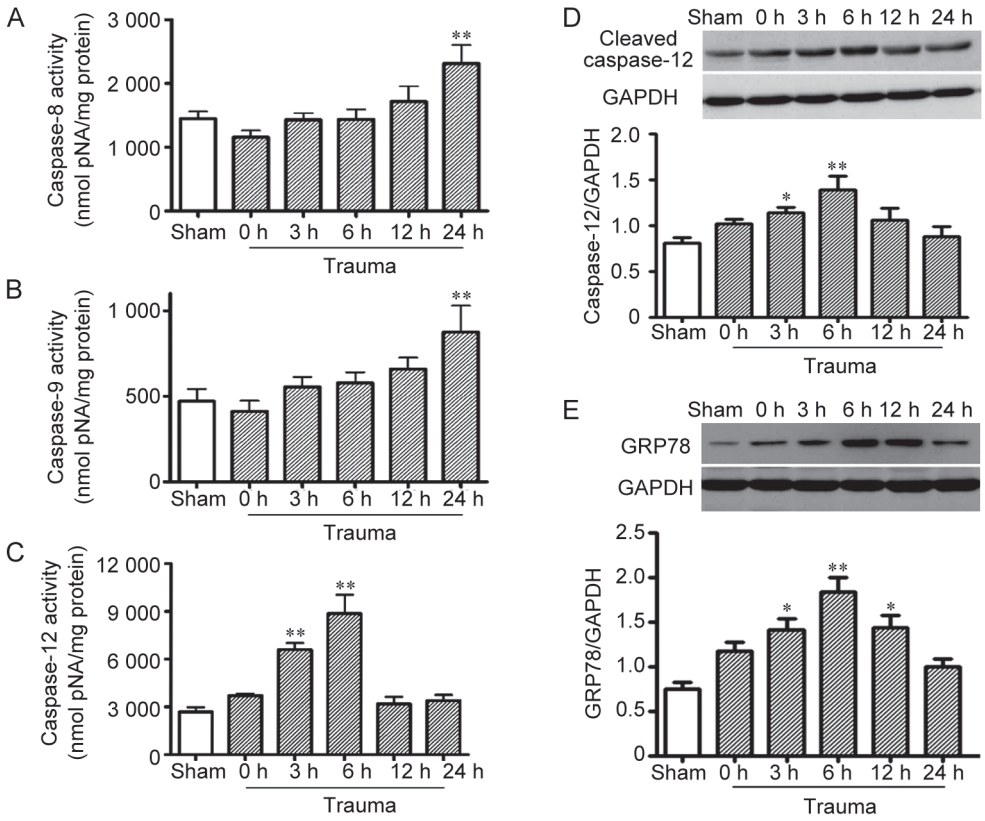


Fig. 3. The activities of caspase-8, -9, -12 and protein expression of ER stress markers (caspase-12, GRP78) in myocardial tissues within 24 h of trauma. *A*: Caspase-8 activity. *B*: Caspase-9 activity. *C*: Caspase-12 activity. *D*: Caspase-12 protein expression. *E*: GRP78 protein expression. Mean ± SEM, $n = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* sham group.

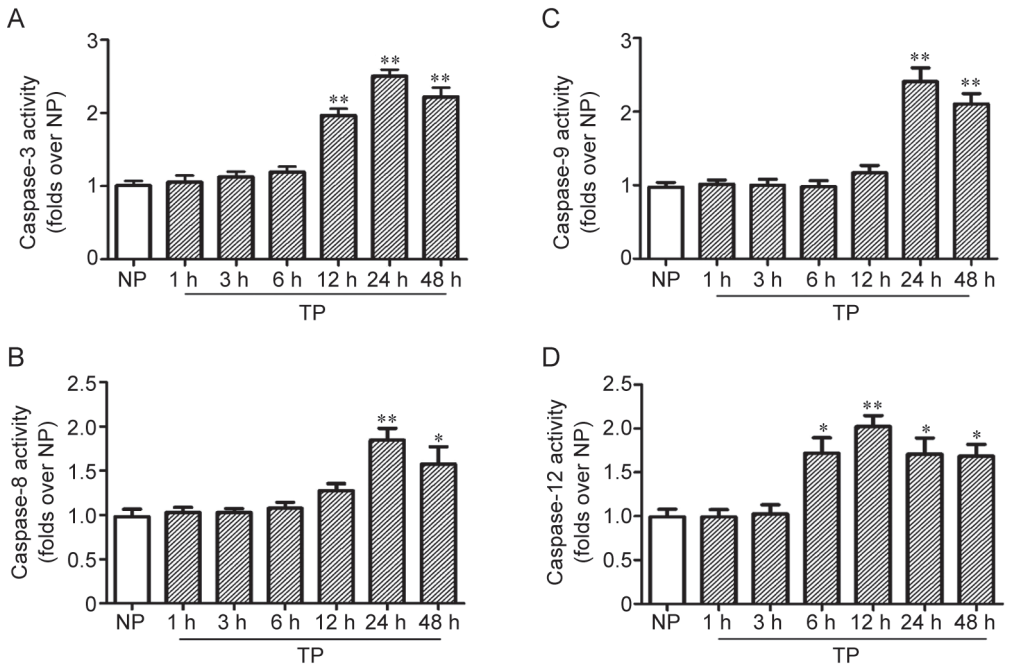


Fig. 4. The activities of caspase-3, -8, -9 and -12 in cultured normal rat cardiomyocytes treated with traumatic plasma (TP). *A*: Caspase-3 activity. *B*: Caspase-8 activity. *C*: Caspase-9 activity. *D*: Caspase-12 activity. Mean ± SEM, $n = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* normal plasma (NP) group.

caspase-12, an ER stress-specific caspase, followed by the activation of caspase-8 (extrinsic pathway) and caspase-9 (intrinsic pathway).

### 2.4 Activated caspase-12 played a causative role in cardiomyocyte apoptosis induced by TP

To dissect the cause-effect relationship between TP-activated caspaes-12 and TP-initiated cardiomyocyte apoptosis, cardiomyocytes were treated with Z-ATAD-FMK (a caspase-12 specific inhibitor). As shown in Fig. 5*A*, pre-treatment with Z-ATAD-FMK markedly lowered the amount of apoptotic cells compared with the cells exposed to TP alone ($P < 0.05$ *vs* TP group). Additionally, treatment with Z-ATAD-FMK significantly reduced TP-induced caspase-3 activation ($P < 0.01$ *vs* TP group) (Fig. 5*B*). The result strongly suggested that early activated caspase-12 was causatively linked with cardiomyocyte apoptosis induced by TP.

### 2.5 Caspase-12 activity was negatively correlated with cardiac function in posttraumatic rats

To further determine the role of elevated caspase-12 activity in post-traumatic myocardial dysfunction, the relationship between caspase-12 activity of myocardial tissue 6 h after trauma and cardiac function 24 h after trauma were analyzed. As summarized in Fig. 6*A*, an obvious negative correlation between myocardial caspase-12 activity and $+dP/dT_{max}$ was observed. Moreover, strong negative correlation was also shown between caspase-12 activity and $-dP/dT_{max}$ (Fig. 6*B*).

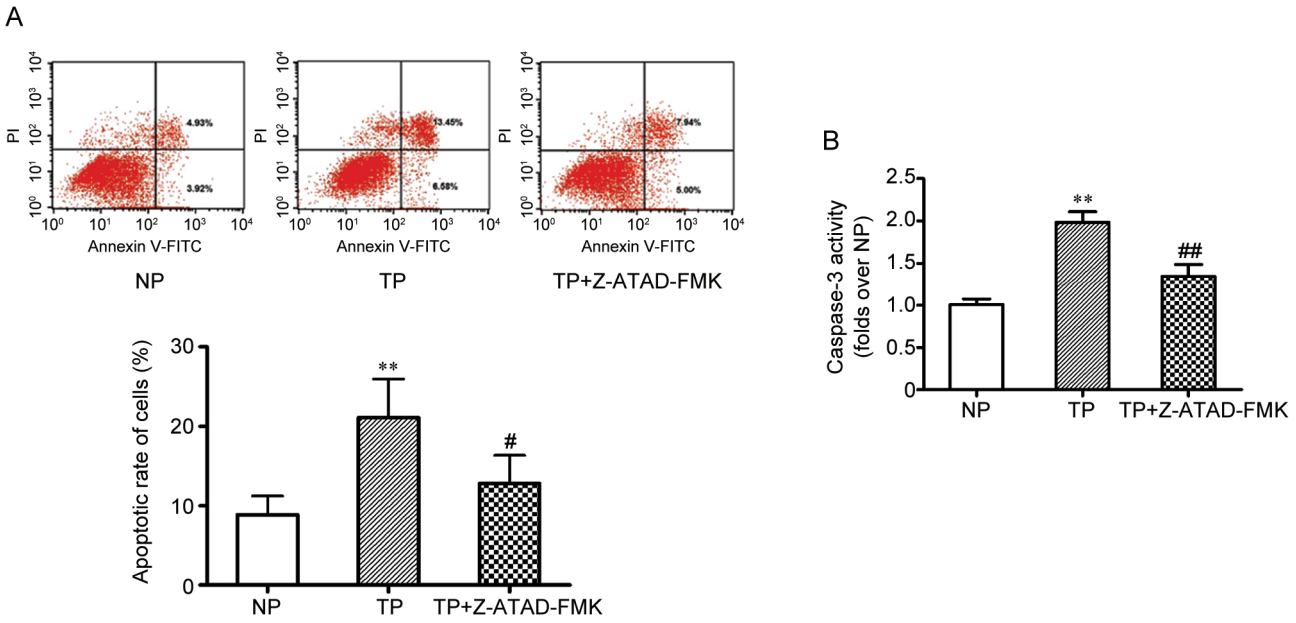


Fig. 5. The effect of caspase-12 inhibitor Z-ATAD-FMK on traumatic plasma (TP)-induced apoptosis in cultured normal rat cardiomyocyte. *A*: Flow cytometric analysis of apoptosis by Annexin V/propidium iodide (PI) staining. *B*: Caspase-3 activity. Mean ± SEM, $n = 10$. $^{**}P < 0.01$ *vs* normal plasma (NP) group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* TP group.

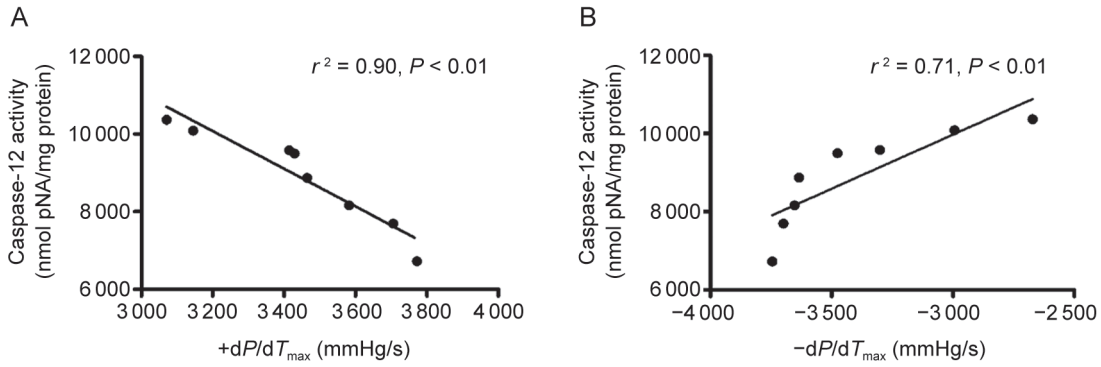


Fig. 6. The relationship between caspase-12 activity 6 h after trauma and cardiac function 24 h after trauma in traumatic hearts.

## 3 DISCUSSION

In our present study we made several novel observations. First, we proved that myocardial apoptosis contributed to posttraumatic cardiac dysfunction. Second, we provided direct evidence that myocardial apoptosis occurred earlier than cardiac dysfunction in mechanical trauma. Finally, we demonstrated for the first time that traumatic injury firstly activated caspase-12, which was causatively linked with cardiomyocyte apoptosis and highly correlated with cardiac dysfunction in the traumatic rats. These results strongly suggested that early activated caspase-12 was likely responsible for the trauma-induced cardiac dysfunction, and that the inhibition of caspase-12-induced apoptosis might be helpful to prevent or attenuate secondary cardiac injury induced by trauma.

Worldwide, trauma is a leading cause of death and long-term disability. Numerous studies have indicated that trauma patients are at risk of developing secondary cardiac injuries [17]. Noble-Collip drum exposure was a well-accepted traumatic model that mimics whole body nonpenetrative mechanical trauma induced secondary heart injury [7, 13, 14, 16]. Previous studies have proved that this mild trauma model was characterized by the 100% survival rate during the first 24 h after trauma for lack of circulatory shock and without direct heart injury [7, 13, 14].

Due to higher mortality rates in clinics, it is indispensable to seek the effective measures to predict and avoid TISCI. Existing studies have demonstrated that the trauma model caused myocardial injury and myocardial apoptosis contributed to TISCI [7]. But the time course of cardiac apoptosis was not clear. In our study, cardiac apoptosis was proved to play a critical role in myocardial dysfunction of posttraumatic rats. Simultaneously, our current results indicated that myocardial apoptosis markedly increased 6 h after trauma, which was much earlier than 24 h after trauma of cardiac dysfunction. These results suggested that inhibition of myocardial apoptosis at the early period of trauma might reduce TISCI.

Caspase-dependent apoptosis plays a vital role in myocardial apoptosis [18]. One of the most widely recognized biochemical features of caspase-dependent apoptosis is the activation of a class of cysteine proteases known as caspases [19]. There are mainly three pathways identified that activate caspases and induce myocardial apoptosis. Extrinsic (the death receptor-mediated) pathway leading to caspase-8 activation is activated through a cell surface signal. Intrinsic pathway (also called mitochondrial apoptotic pathway) is triggered by the release of certain mediators such as cytochrome c (Cyt c), followed by the activation of caspase-9. Finally, the mechanism of ER stress-associated pathway is that $Ca^{2+}$ is released from the ER during ER stress, which in turn activates caspase-12 [20]. Caspase-8, -9, -12 subsequently activates the executioner caspase, caspase-3. Previous studies have shown that both mild [21] and severe [22] traumatic brain injury induced ER stress, and caspase-12-mediated ER apoptotic pathway was rapidly induced and played an important role in traumatic brain injury pathology [12]. Our present results demonstrated that myocardial caspase-3 activity increased 6 h after trauma, and caspase-12 was activated 3 h after trauma. While caspase-8, -9 activities were not induced until 24 h after trauma. Moreover, caspase-12 expression was significantly upregulated 3 h after trauma. Due to the proapoptosis mediators that trigger cardiomyocyte apoptosis in traumatic animals are present in the TP [16], normal rat cardiomyocytes were exposed to TP. The results showed that cardiomyocyte caspase-12 was activated earlier than caspase-8, -9. These results strongly manifested that caspase-12-mediated apoptotic pathway was firstly activated in traumatic rat heart.

The ER has several crucial functions, including protein synthesis, maturation and folding, as well as the maintenance of intracellular $Ca^{2+}$ homeostasis. Accumulation of misfolded proteins and alteration of $Ca^{2+}$ homeostasis initiate an adaptive response in the cell, termed the unfolded protein response (UPR, ER stress response). Excessive or prolonged ER stress triggers apoptosis in various cell types. ER stress-induced apoptosis pathways mainly include: (1) $Ca^{2+}$/calpain/caspase-12; (2) inositol requiring enzyme1 (IRE1)-mediated activation of apoptosis signal-regulating kinase 1/c-Jun N-terminal kinase (ASK1/JNK); (3) PKR-like ER kinase/α subunit of eukaryotic translation initiation factor 2 (PERK/eIF2α) or activating transcription factor 6 (ATF-6) dependent induction of the proapoptotic transcription factor CEBP homologous protein (CHOP) [23]. IRE1, PERK and ATF6 regulate the UPR through their respective signaling pathways by binding to the immunoglobulin-binding protein (BiP) in unstressed cells [24]. BiP is a protein chaperone also known as GRP78. In response to ER stress, unfolded proteins accumulate in the ER and promote GRP78 release from IRE1, PERK and ATF6. It has

been proved that caspase-12 mediates ER-specific apoptosis, since mice deficient in caspase-12 are resistant to ER stress-induced apoptosis [25]. Previous study demonstrated that traumatic brain injury could trigger ER stress, increased expression and processing of caspase-12, and further resulted in cell apoptosis [12]. Our present study demonstrated that ER stress pathway markers, caspase-12 and GRP78, were upregulated by mechanical trauma, and elevated caspase-12 activity played a causative role in trauma-induced cardiomyocyte apoptosis. Furthermore, there was a strong negative correlation between elevated myocardial caspase-12 activity and cardiac dysfunction in mechanical trauma. Although the relationship between ER stress and cardiomyocyte apoptosis in TISCI remained indistinct, the current study was at least suggested that the blockage of caspase-12-mediated apoptosis pathway might become a potential treatment for TISCI.

It is worth mentioning that there is crosstalk between caspase-12-mediated ER stress apoptotic pathway and the other two apoptosis pathways. In the first place, mitochondria are connected to the ER at specific sites called mitochondrion associated membranes (MAMs). MAMs are adjacent to the ER and contain important $Ca^{2+}$ transport-related proteins including inositol1, 4,5-triphosphate receptor ($IP_3R$) [26]. During ER stress, $Ca^{2+}$ released from the ER may activate the permeability transition pore complex (PTPC), and ultimately induce Cyt c release. When Cyt c released at early stages of apoptosis binds to $IP_3R$ at the ER [27], the ER $Ca^{2+}$ release becomes unrestrained. And then increased cytosolic $Ca^{2+}$ can stimulate mitochondrial release of Cyt c and further activate caspase-9[28]. Furthermore, it has been suggested that procaspase-9 is a substrate of caspase-12 and that caspase-9 can be activated in a Cyt c-independent manner [29]. However, there is little known about the role of death receptor pathway in ER stress-induced cell death. It has been reported that ER stress triggers caspase-8 activation in embryonal carcinoma cells [30]. The recent study has found a novel link between ER stress and the death receptor through IRE1α-NF-κB-TNFα-TRAF2 pathway [31]. The last but not the least, the latest studies have reported that Bcl-2 family proteins are involved in the signal crosstalk between ER stress and mitochondrial dysfunction. Bcl-2 and Bcl-xL (antiapoptotic members of Bcl-2 family) can affect $IP_3R$ in a variety of ways, thus reducing cytoplasmic $Ca^{2+}$ and inhibiting $Ca^{2+}$-dependent cell death [32]. In addition, it has been reported that overexpression of X-linked inhibitor of apoptosis protein (XIAP) reduces caspase-12 cleavage in mice [33]. In the present study, caspase-12 was activated earlier than caspase-8, -9 in both myocardial tissue of traumatic rats and cardiomyocytes treated with TP. But whether there is crosstalk between caspase-8, -9 activation and caspase-12-mediated ER stress apoptotic pathway in traumatic models must be confirmed by further experiments. Additionally, decreased cardiac caspase-12 activity 12 h after trauma (*in vivo*) and 24 h after treated with TP (*in vitro*) might be the result of antiapoptotic proteins which requires verification in the future study.

In conclusion, our present study demonstrated for the first time that mechanical trauma resulted in caspase-12 activation and subsequent cardiomyocyte apoptosis. Moreover, activated caspase-12 was interrelated with cardiac dysfunction, which requires further study to determine a definite relationship. Though our study has some limitations, it strongly suggests that TISCI could be minimized by inhibition of caspase-12-incuced apoptosis pathway.

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