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Original Article

Short-term pretreatment with atorvastatin attenuates left ventricular dysfunction, reduces infarct size and apoptosis in acute myocardial infarction rats

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Abstract: Background: Atorvastatin showed a number of cardiovascular benefits, however, the role and underlying molecular mechanisms of short-term atorvastatin-mediated protection remain unclear. Methods: 30 rats were randomly divided into 3 groups: sham group, acute myocardial infarction model group and atorvastatin group. The rats of acute myocardial infarction model were established by ligation of the left anterior descending of coronary arteries. Before surgery, rats in the atorvastatin group received 20 mg/kg/d atorvastatin for 7 days in atorvastatin group. After 4 hours of model established, changes in hemodynamics parameters were recorded and myocardial infarct size was achieved by Evans blue-TTC staining. Myocardium apoptosis was evaluated by TUNEL. The expression of FAS, FAS-L, Bcl-2, Bax, p-BAD, Caspase-8 and Caspase-3 in myocardium were examined by Western blot. Results: In the atorvastatin group, left ventricular function was elevated and infarct size was decreased compared with the model group. Moreover, in the atorvastatin group, the cell apoptosis index was reduced in response to myocardial infarction. The expressions of Bcl-2 were increased and Bax, p-BAD, Fas, Fas-L, caspase-8 and caspase-3 in myocardium were decreased in atorvastatin group. Conclusions: Short-term atorvastatin pretreatment restored left ventricular function and limited infarct size in acute myocardial infarction, which were associated with reduction of the apoptosis in myocardium through Bcl-2 and Fas pathway.

Keywords: Atorvastatin, myocardial infarction, left ventricular function, apoptosis, Bcl-2, Fas

Introduction

Myocardial infarction is now the leading cause of death worldwide. Cardiomyocytes death have limited capacity for regeneration and is a hallmark characteristic of various cardiac diseases. Although many therapeutic advances in myocardial infarction, irreversible loss of cardiomyocytes remains the most difficult problem to resolve. When subject to acute myocardial infarction (AMI), cardiomyocytes apoptosis increased both in animal models and in human [1, 2]. Looking for a method to prevent cardiomyocytes from apoptosis when subject to acute ischemia is a useful thread to resolve the problem.

Statins, an HMG-CoA reductase inhibitor, with the most clinical evidence drugs, has been demonstrated to benefit patients with ischemic heart disease. It is reported that statins can stabilize atherosclerotic plaques through lowering plasma cholesterol levels and have been associated with reduced morbidity and mortality in patients with coronary heart disease [3, 4]. However, the beneficial effects of statins cannot be explained only by its cholesterol-lowering effects, especially in early stage [5]. Now, more and more evidence suggest that statins may exhibit beneficial function related to its pleiotropic effects, such as antioxidant effects, anti-inflammation reaction, and stabilization of atherosclerotic plaques [6-8]. Evidence-based clinical trials focus on short-term application of statin to reduce cardiovascular event. In clinic, short-term and 'high'-dose statins are usually prescribed for acute coronary syndrome. Recent study demonstrated that the initial

statin and higher dose treatment during MI are key elements for their main mechanisms of benefit [7]. Atorvastatin is the most typical statin with large evidence-base randomised controlled trials. Pretreatment with atorvastatin 24 hours prior to percutaneous coronary intervention reduced periprocedural myocardial infarction [9]. Previous reports hinted atorvastatin decreased the apoptosis of myocardial cells and alleviated post-myocardial infarction heart failure through the blockade of caspase-12 and endoplasmic reticulum stress pathway [10]. However, the impact and underlying mechanism of short-term atorvastatin therapy on AMI is not well known, as previous studies have produced inconsistent results [11-13].

Therefore, the purpose of this study was to determine whether short-term pretreatment with atorvastatin during the peri-infarct period improve heart function, reduce infarct size and attenuates apoptosis. The apoptotic protein of Bcl-2, Bax, phosphorylation of Bcl-xL/Bcl-2-associated death promoter (p-BAD), Fas, Fas ligand (Fas-L), caspase-8 and caspase-3 were detected to clarify the underlying mechanism.

Materials and methods

Establishment of the rat AMI model and protocol

Sprague-Dawley rats (male) weighing 180 to 220 g were obtained from the Center for Laboratory Animals, Zhejiang Academy of Medical Sciences (Hangzhou, China). Animals were randomly divided into AMI group (model group, n=12), a AMI model +atorvastatin treatment group (atorvastatin or atorva group, n=12) and a sham opration group (sham group, n=8).

To establish the AMI model, anesthetized rats were fixed on the operating table. The thoracic cavity was opened to expose the heart, and the left anterior descending of coronary arteries (LADs) were ligated [14]. In the sham group, the sutures were passed under the LADs without ligation. The thoracic cavity was closed immediately after the heart was returned. Before surgery, rats in the atorvastatin group received 20 mg/kg/d atorvastatin (Pfizer Inc.,) for 7 days. All experimental procedures were approved by the Animal Ethics Committee of Zhejiang University of Traditional Chinese Medicine.

Hemodynamic examination

After 4 hours of operation, rats were anesthetized and catheters were inserted into the right carotid artery [15]. The Changes of hemodynamics parameters, including the left ventricular end-diastolic pressure (LVEDP), the left ventricular systolic pressure (LVSP), the maximal rate of rise in blood pressure in the ventricular chamber (+dP/dt max) and the maximal rate of decline in blood pressure in the ventricular chamber (-dP/dtmax) were recorded by an 8-channel polygraph system (Medlab-U/4CS).

Infarct size determination

Infarct size was determined by Evans blue-TTC staining as previously described [16, 17]. In brief, the heart was quickly excised and sectioned into 1.0 mm portions, and incubated at 37°C in 1% TTC-PBS for 15 minutes. Each stained cardiac section was photographed and analyzed using the computer-based image analyzer SigmaScan Pro 5.0 (SPSS Science, Chicago, IL). TTC-negative staining region was defined as myocardial infarct size.

TUNEL procedure

The cell apoptosis in the myocardium was determined by TUNEL, according to the manufacturer's instructions (Roche Applied Science, USA). Six micrographs were randomly selected and the numbers of healthy or apoptotic cardiomyocytes were counted. The percentage of TUNEL-positive cells in relation to the total number of cells was determined by counting at least 200 cells in three different fields.

Western blot

For detection of protein expression, total protein from myocardium tissues were extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane, blocked and probed sequentially with primary antibodies. Primary antibodies used are as follows: Fas, Fas-L, Bcl-2, Bax, caspase-3 (Santa Cruz Biotechnology, USA), p-BAD and caspase-8 (Cell Signaling Technology), and β-actin (Santa Cruz Biotechnology, USA) as an internal control. After the secondary antibody incubation, the membranes were rinsed and bound antibodies were detected using enhanced chemilumines-

Table 1. Atorvastatin alleviated left ventricular dysfuntion. Hemodynamic index changes in different groups ($\bar{x} \pm s$)

groups	n	LVSP (mmHg)	LVEDP (mmHg)	+dp/dtmax (mmHg)	-dp/dtmax (mmHg)
sham	8	146.6 ± 5.5	6.0 ± 1.9	5894.1 ± 672.2	4839.6 ± 239.8
model	8	76.3 ± 24.3	17.9 ± 2.7	3699.8 ± 390.0	2695.0 ± 182.3
atorva	8	115 ± 13.9	6.9 ± 2.1	5176.4 ± 418.8	4368 ± 293.6

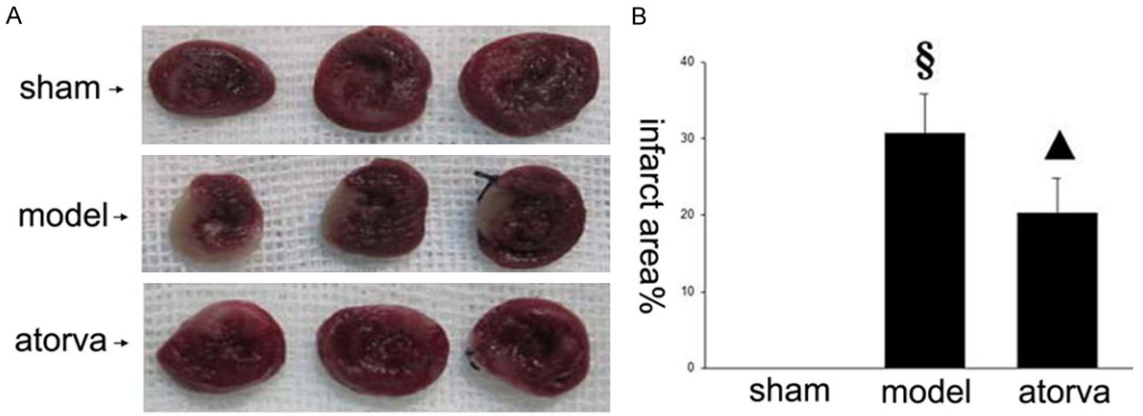

Figure 1. Atorvastatin decreased myocardium infarct area. A. Shown are photograph of infart size of sham, model and atorvastatin group, as defined by TTC analysis. B. Quantification of infarct size of sham, model and atorvastatin group, §P<0.05 *vs* sham group, ▲P<0.05 *vs* model group. Data shown are means ± SE representitive of 8 independent experiments.

cence (ECL, Santa Cruz Biotechnology, USA) followed by autoradiography. Image pro plus 5 software was used to semiquantity protein in every lane.

Statistical analysis

Statistical analyses were performed using SPSS 18.0 software. Data were presented as mean ± standard deviation (SD). The difference between two groups was evaluated by one-way ANOVA followed. The results were considered significant at a value of P<0.05.

Results

A total of 30 rats were initially enrolled in this study. 4 rats in the AMI model group and 4 rats in the atorvastatin group died within the first 4 hours after coronary ligation due to ventricular fibrillation. There were no significant differences in mortality between model group and atorvastatin group.

Atorvastatin alleviated left ventricular dysfunction

Hemodynamic index was compared among every group. As shown in **Table 1**, after LAD ligation, LVSP and ± dP/dt max decreased and LVEDP increased in the model group compared with sham group (§P<0.05 *vs* sham group). However, atorvastatin pretreatment significantly decreased the LVEDP and increased LVSP and ± dP/dt max levels compared with model group (▲P<0.05 *vs* model group). This result suggested that atorvastatin pretreatment protected against Left ventricular dysfuntion in rats after AMI.

Atorvastatin decreased myocardium infarct area and apoptosis

To investigate the roles of atorvastatin in protecting AMI, we examined the myocardium changes of rat heart. As shown in **Figure 1**, after LAD ligation, rat hearts in the model group presented with about 30.6 ± 5.2% infarct area in anterior wall (§P<0.05 *vs* sham group). Compared with the model group, the atorvastatin group showed a significant reduction in the infart area of myocardium by 20.2 ± 4.6% (▲P<0.05 *vs* model group), as defined by TTC analysis.

Cell apoptosis is one of the major outcomes of AMI, which indicates the molecular mechanism of cell loss [18]. So, we observed whether ator-

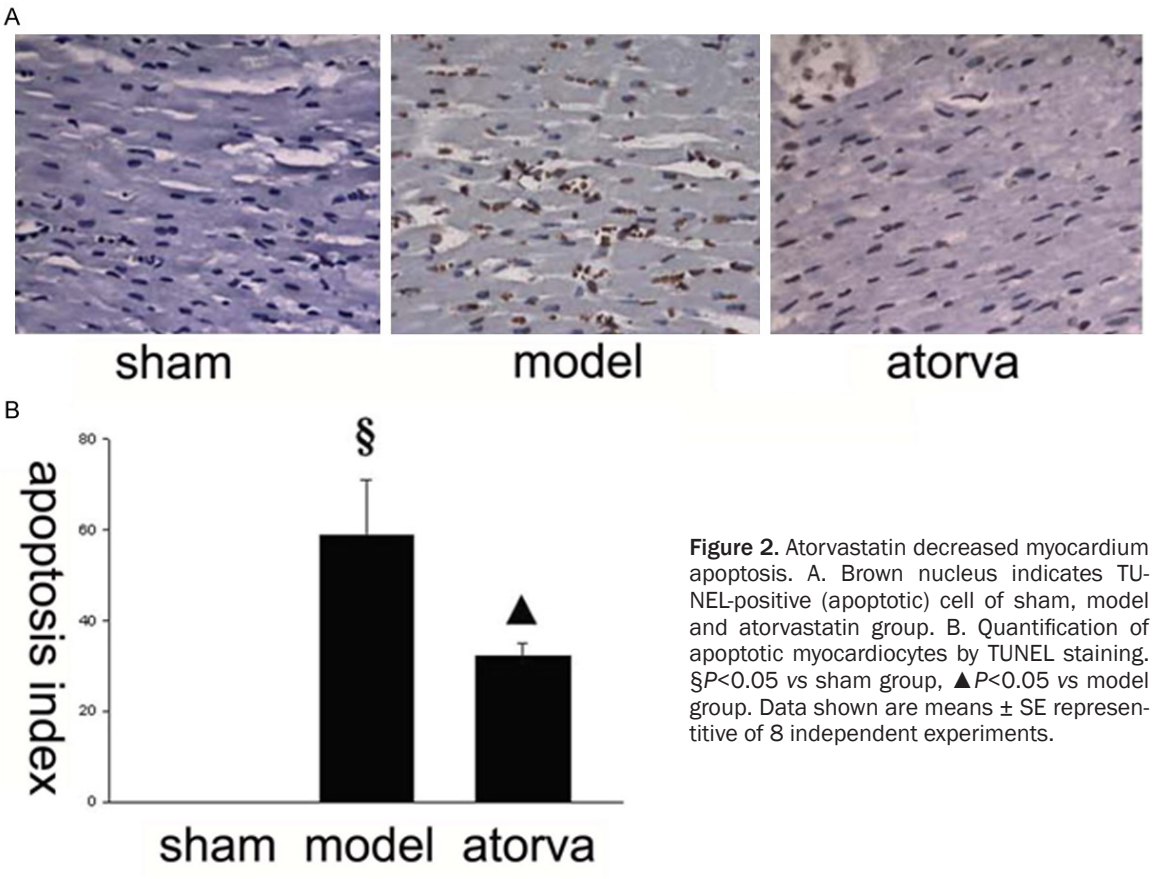

Figure 2. Atorvastatin decreased myocardium apoptosis. A. Brown nucleus indicates TUNEL-positive (apoptotic) cell of sham, model and atorvastatin group. B. Quantification of apoptotic myocardiocytes by TUNEL staining. §P<0.05 *vs* sham group, ▲P<0.05 *vs* model group. Data shown are means ± SE representitive of 8 independent experiments.

vastatin protected myocardial apoptosis from acute ischemia. Using TUNEL assay, we found that the number of apoptotic myocardiocytes increased by 58.9 ± 12.1% induced by LAD ligation (§P<0.05 *vs* sham group). Compared with model group, the atorvastatin pretreatment dramatically decreased cell apoptosis index to 32.3 ± 2.6 (▲P<0.05 *vs* model group) (**Figure 2**).

Effect of atorvastatin on expression of Bcl-2 and Bax in AMI rats

To investigate whether atorvastatin effects apoptosis regulatory proteins induced by LAD ligation, expressions of Bcl-2 and Bax in myocardium were studied by western blot. We first examined the expression of antiapoptotic Bcl-2 protein in myocardium that was exposed to ischemia with or without pretreatment with atorvastatin (**Figure 3**). As shown in **Figure 3A** and **3B**, Bcl-2 expression were downregulated in model group compared with sham groups (0.93 ± 0.16 *vs* 1.80 ± 0.39, §P<0.05 *vs* sham group), whereas atorvastatin pretreatment reversed the downregulation of the protein Bcl-2 compared with the model group (1.60 ± 0.34 *vs* 0.93 ± 0.16, ▲P<0.05 *vs* model group).

We next determined whether atorvastatin had any affects on the levels of Bax. As shown in **Figure 3A** and **3C**, the cardiac protein expression of the proapoptotic protein Bax was significantly increased in model group compared with sham group (0.88 ± 0.21 *vs* 0.23 ± 0.06, §P<0.05 *vs* sham group). However, the expression of Bax in atorvastatin was decreased compared with model group (0.39 ± 0.11 *vs* 0.88 ± 0.21, ▲P<0.05 *vs* model group). This result showed atorvastatin pretreatment increased Bcl-2 expression levels and decreased Bax expression insulted by acute ischemia (**Figure 3**).

Effect of atorvastatin on expression of p-BAD in AMI rats

As shown in **Figure 4A** and **4B**, significantly increased expression of p-BAD in the model group was observed compared with the sham group (1.61 ± 0.35 *vs* 0.77 ± 0.23; §P<0.05 *vs* sham group). However, the upregulation was

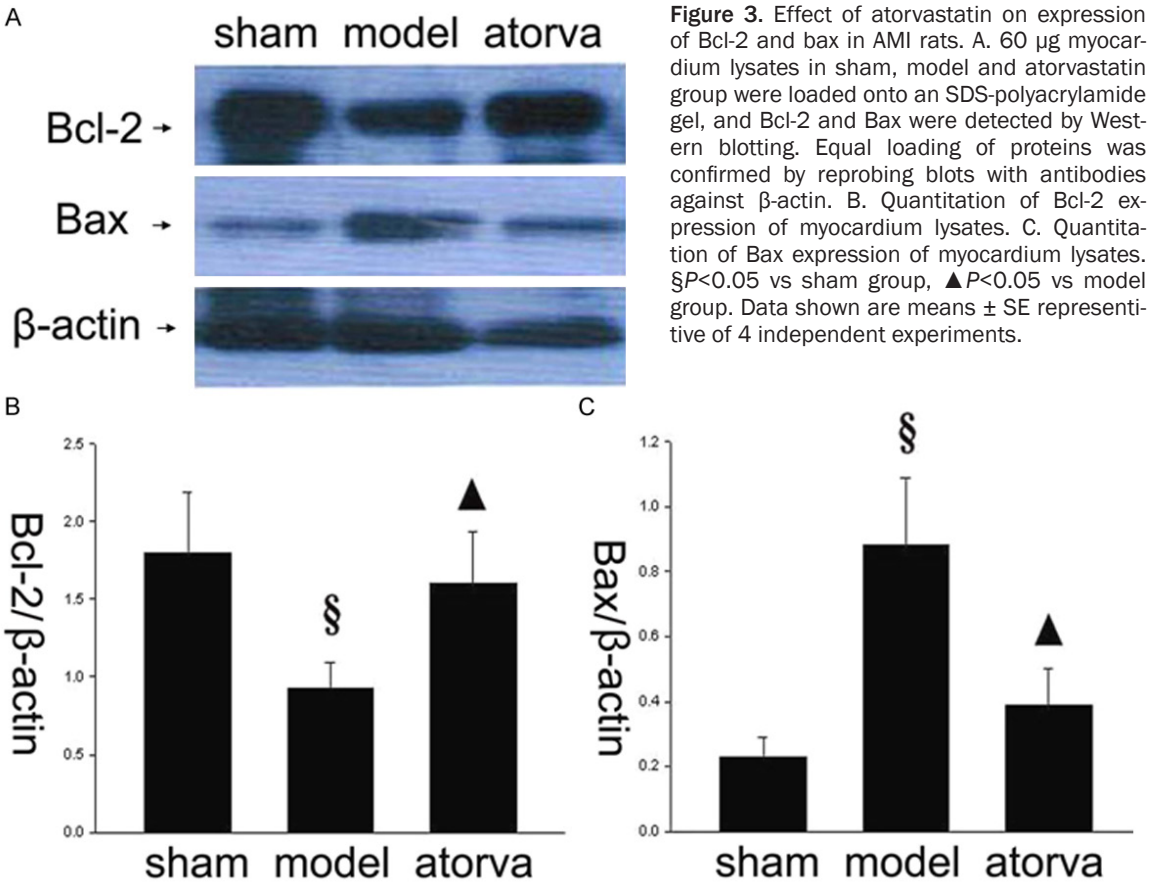

Figure 3. Effect of atorvastatin on expression of Bcl-2 and bax in AMI rats. A. 60 μg myocardium lysates in sham, model and atorvastatin group were loaded onto an SDS-polyacrylamide gel, and Bcl-2 and Bax were detected by Western blotting. Equal loading of proteins was confirmed by reprobing blots with antibodies against β-actin. B. Quantitation of Bcl-2 expression of myocardium lysates. C. Quantitation of Bax expression of myocardium lysates. §P<0.05 vs sham group, ▲P<0.05 vs model group. Data shown are means ± SE representitive of 4 independent experiments.

reversed in the atorvastatin treated group (0.85 ± 0.19 vs 1.61 ± 0.35, ▲P<0.05 vs model group). Meanwhile, western blot analysis revealed that the expression of BAD was contrast to the expression of p-BAD in model group and atorvastatin group.

Effect of atorvastatin on expression of Fas and Fas-L in AMI rats

Next, we investigated the effect of atorvastatin on expression of Fas and Fas-L. As shown in **Figure 5**, western blot analysis demonstrated significantly up-regulated expression of pro-apoptotic protein Fas-L in model group compared with sham group (1.03 ± 0.19 *vs* 0.35 ± 0.08; §P<0.05 *vs* sham group). While, pretreatment with atorvastatin lead to a significant decrease in the Fas-L protein level (0.36 ± 0.12 *vs* 1.03 ± 0.19, ▲P<0.05 vs model group). At the same time, the expression of the proapoptotic protein Fas was significantly increased in model group compared with sham group (0.47 ± 0.11 *vs* 0.19 ± 0.06, §P<0.05 *vs* sham group). However, the expression of Fas was decreased as compared with model group (0.23 ± 0.09 *vs* 0.47 ± 0.11, ▲P<0.05 *vs* model group). This result suggust Fas and Fas-L were involved in atorvastatin-mediated heart pertection in AMI.

Effects of atorvastatin on expression of caspase-8 and caspase-3 in AMI rats

To futher determine whether short-term atorvastatin pretreatment effect on caspase-8 and caspase-3, we tested caspase-8 and caspase-3 activity using an antibody specific to caspase-8 and caspase-3. As shown in **Figure 6**, caspase-8 was increased in myocardium after LAD ligation compared with sham group (0.54 ± 0.13 *vs* 0.26 ± 0.07; §P<0.05 *vs* sham group). Whereas atorvastatin decreased caspase-8 expression compared with model group (0.30 ± 0.04 *vs* 0.54 ± 0.13; ▲P<0.05 *vs* model group). Moreover, caspase-3 in model group was also increased compared with sham group (0.96 ± 0.15 *vs* 0.50 ± 0.09; §P<0.05 *vs* sham group). At the same time, atorvastatin decreased caspase-3 expression compared with model group (0.65 ± 0.15 *vs* 0.96 ± 0.15; ▲P<

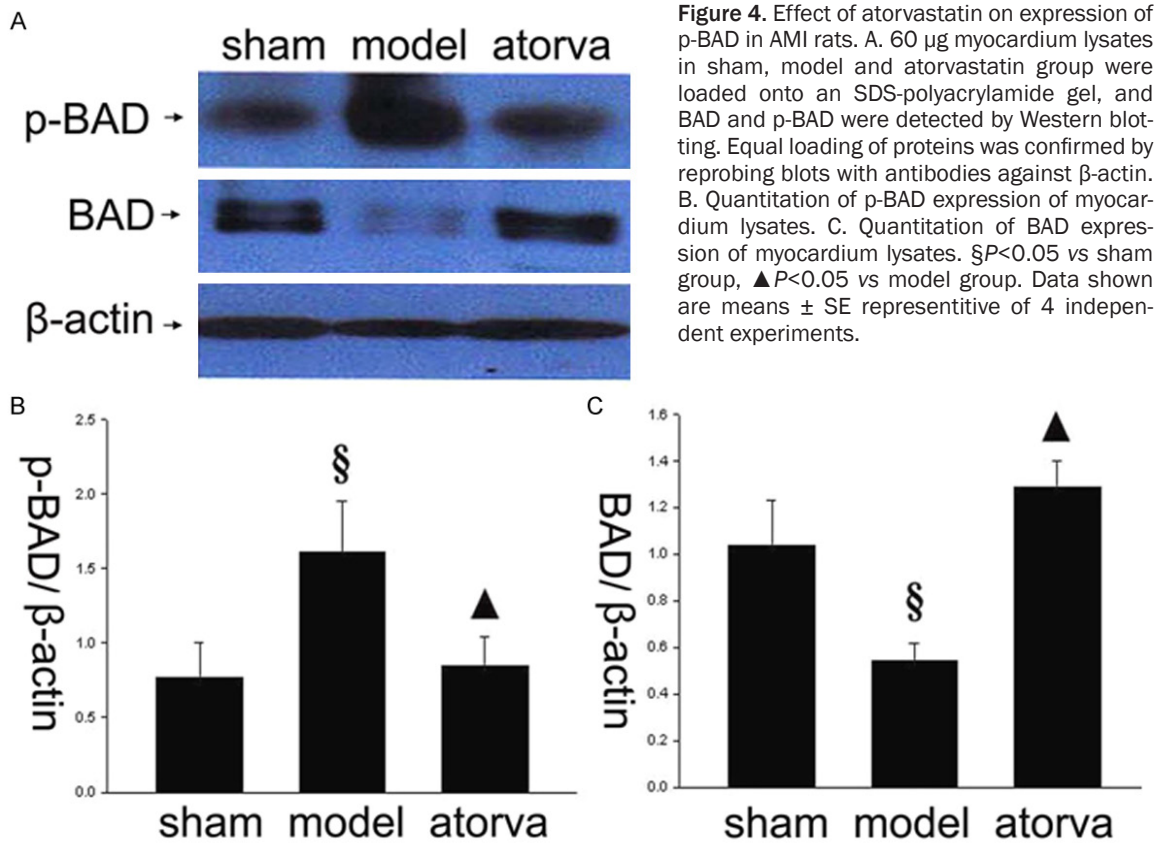

Figure 4. Effect of atorvastatin on expression of p-BAD in AMI rats. A. 60 μg myocardium lysates in sham, model and atorvastatin group were loaded onto an SDS-polyacrylamide gel, and BAD and p-BAD were detected by Western blotting. Equal loading of proteins was confirmed by reprobing blots with antibodies against β-actin. B. Quantitation of p-BAD expression of myocardium lysates. C. Quantitation of BAD expression of myocardium lysates. §P<0.05 *vs* sham group, ▲P<0.05 *vs* model group. Data shown are means ± SE representitive of 4 independent experiments.

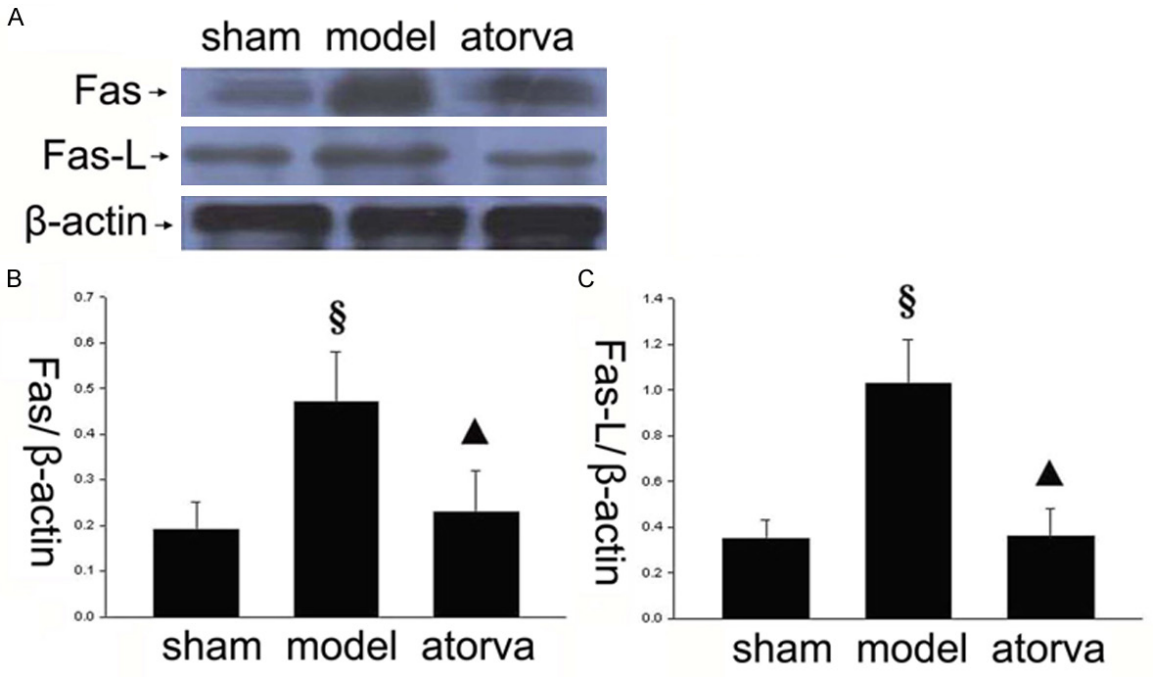

Figure 5. Effect of atorvastatin on expression of Fas and Fas-L in AMI rats. A. 60 μg myocardium lysates in sham, model and atorvastatin group were loaded onto an SDS-polyacrylamide gel, and Fas and Fas-L were detected by Western blotting. Equal loading of proteins was confirmed by reprobing blots with antibodies against β-actin. B. Quantitation of Fas expression of myocardium lysates. C. Quantitation of Fas-L expression of myocardium lysates. §P<0.05 vs sham group, ▲P<0.05 vs model group. Data shown are means ± SE representitive of 4 independent experiments.

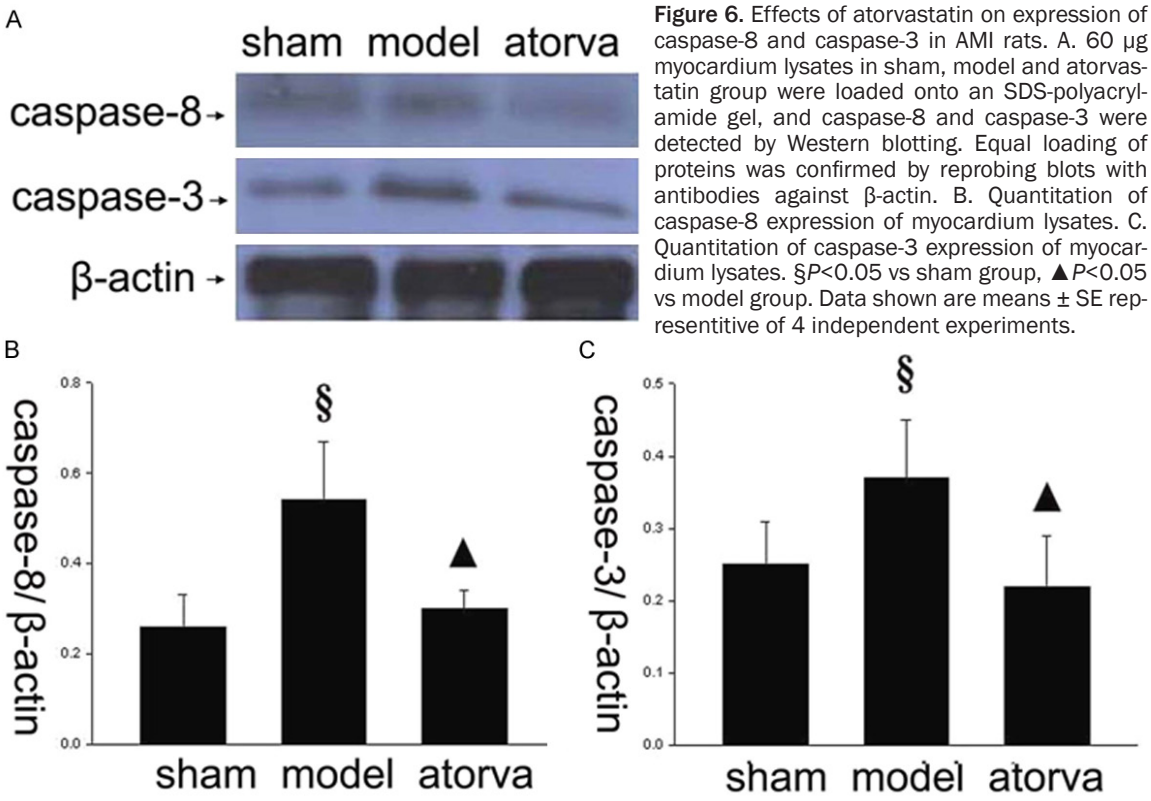

Figure 6. Effects of atorvastatin on expression of caspase-8 and caspase-3 in AMI rats. A. 60 μg myocardium lysates in sham, model and atorvastatin group were loaded onto an SDS-polyacrylamide gel, and caspase-8 and caspase-3 were detected by Western blotting. Equal loading of proteins was confirmed by reprobing blots with antibodies against β-actin. B. Quantitation of caspase-8 expression of myocardium lysates. C. Quantitation of caspase-3 expression of myocardium lysates. §P<0.05 vs sham group, ▲P<0.05 vs model group. Data shown are means ± SE representitive of 4 independent experiments.

0.05 *vs* model group). This data suggested that caspase-8 and caspase-3 were involved in anti-apoptosis of atorvastatin in LAD ligation.

Discussion

Atorvastatin is a useful medicine to effectively decrease blood lipids, which may provide some advantages over other statins [19-21]. Nevertheless, the protective role and underlying mechanism of atorvastatin is still unclear, especially in short-term application [13, 22-24]. Pleiotropic effects but not lipid-lowering may associate with cardiovascular events in short-term statin therapy [25]. The major finding of this study is that short-term pretreatment of atorvastatin could improve left ventricular function and limits infarct size during the AMI period. This effect was associated with a decrease in cardiomyocyte apoptosis and atorvastatin simultaneously modulated of the intrinsic and extrinsic pathways of myocardial apoptosis in AMI.

In this study, we found that 7 days pretreatment with atorvastatin dramatically relieved hemodynamic parameters change in AMI rats. Compared with the model group, atorvastatin administration significantly reduced LVEDP levels, and elevated LVSP and ± dP/dtmax. The precise mechanism which pretreatment of atorvastatin improved left ventricular dysfunction is undetermined. Two factors of atorvastatin that could contribute to the improvement of left ventricular function should be thought: one is that pretreatment of atorvastatin reduced infarct size in our model, as the atorvastatin group showed a significant reduction in the area of myocardium by 20.2 ± 4.6%. Recent study showed atorvastatin significantly reduced myocardial infarct size mediated via the nitric oxide synthase pathway [26]. The second is that atorvastatin reduced apoptosis induced by acute ischemia. We presumed that the reduction in apoptosis in the survival myocardium lead to less loss of contractile elements, thereby contributing to the preservation of left ventricular dysfunction. This result is in accordance with the application of atorvastatin in patients with heart failure to improved left ventricular ejection fraction [27]. Other functions of atorvastatin that could contribute to the improvement of left ventricular function might include that inhibition of inflammation [25], maintaining intracellular calcium homeostasis [28], and downregulation ER stress response [10].

Briefly, there are two major signalling pathways for the regulation of apoptosis. The first pathway is extrinsic pathway, which concerns membrane-bound death receptors, such as Fas/Fas-L [29]. The second is intrinsic pathway, also called 'mitochondrion pathway', which has also been shown to play a critical role in apoptosis [30]. We investigated whether the two apoptosis pathway were involved in the atorvastatin-mediated cardioprotection at the same time. Firstly, to investigate the effects of atorvastatin treatment on mitochondrial protection, we analyzed Bcl-2 and Bax expression. The intrinsic pathway relies on anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins at mitochondria to sense stress, signal and execute apoptosis of the cell [31-34]. The current results showed that pretreatment with atorvastatin reversed the reduction in Bcl-2 expression caused by acute ischemia, suggesting that the Bcl-2 mediated atorvastatin-induced cardioprotection. Meanwhile, a 7 days pretreatment with atorvastatin reduced the activation of Bax. In addition, this study showed that the expression of phosphorylated BAD (ser136) was decreased by atorvastatin pretreatment. Phosphorylation at this site results in loss of the ability of BAD to heterodimerize with the survival protein Bcl-2. Phosphorylated BAD binds to 14-3-3 and is sequestered in the cytoplasm [35, 36]. The phosphorylation of BAD has also been showed to cross talk with the Akt [37]. The current results showed that atorvastatin significantly modulated the expression of p-BAD, Bax and Bcl-2 expression, which suggest atorvastatin decreased myocardial apoptosis through mitochondrial pathway, at least in part.

Another mechanism of apoptosis in AMI model is via signaling by death receptor members, such as Fas and Fas-L [30, 31, 38]. The results showed that the expression of Fas and Fas-L was dramatically upregulated in the infarcted myocardium, the results are in accord with previous study [39]. At the same time, atorvastatin pretreatment significantly reversed the expression Fas and Fas-L. Our results indicated the atorvastatin pretreatment reversed increased expression of Fas and Fas-L induced by acute ischemia. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. In general, the pro-apoptotic members of caspases-8 was looked as the initiators of apoptosis and caspase-3 the executioners of apoptosis [40]. The current Western blot findings show that the expression of caspase 8 and caspase 3 were significantly decreased by atorvastatin pretreatment. caspase 8 is involved in the apoptosis induced by Fas and Bcl-2 family. Bcl-2 and Bax could form a pore that releases pro-apoptotic factors (such as cytochrome c) to form an Apaf1-dependent apoptosome responsible for activation of caspase 3 [41]. Can be seen from above results, atorvastatin simultaneously modulated of the intrinsic and extrinsic pathways of myocardial apoptosis in AMI.

In this study, we found that 7 days of pretreatment with atorvastatin dramatically relieved hemodynamic changes in AMI rats. Moreover, atorvastatin decreased myocardial infarct size and cell apoptosis rate induced by LAD ligation. The myocardial protective effects of atorvastatin pretreatment were achieved by regulating Fas and Bcl-2 family apoptotic signal pathway. The results suggest that the pretreatment with atorvastatin during the infarct period might be useful approach to heart pretection.

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Disclosure of conflict of interest

None.

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