

*Original Article***Atorvastatin inhibits Fas expression in ischemia-reperfusion induced myocardial cell injury***Laxman Dubey\*, Zeng Hesong\****Abstract**

**BACKGROUND:** Atorvastatin has been shown to be cardioprotective in ischemia-reperfusion (I/R) injury. Inhibition of Fas expression prevents I/R induced apoptosis. However, the influence of atorvastatin on Fas expression in I/R injury was not studied. Therefore, we designed this study to see the influence of atorvastatin on cardiomyocyte apoptosis and Fas expression following acute I/R in vivo.

**METHODS:** Thirty Wistar rats were selected and divided into three groups (n = 10): acute ischemia-reperfusion (I/R) group, acute ischemia-reperfusion and treated with atorvastatin group and sham-operated group. Apoptosis of the cardiomyocytes was observed under electron microscopy and determined by optic microscopy with TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling) staining. To detect the expression of Fas in the cardiomyocytes, immunohistochemistry method was used. Image analysis system was used to quantitatively estimate the positive metric substances of immunohistochemistry through the mean optic density.

**RESULTS:** Numerous apoptotic cardiomyocytes were found in ischemic fields in ischemia-reperfusion groups and weren't observed in the sham-operated group. Fas expression was significantly higher in the ischemia-reperfusion group as compared to sham-operated group, but was decreased significantly in atorvastatin treated group as compared with I/R group.

**CONCLUSION:** Upregulation of Fas expression in myocardial ischemia-reperfusion can induce cardiomyocyte apoptosis, and atorvastatin can significantly inhibit cardiomyocyte apoptosis by inhibiting Fas expression.

**KEY WORDS:** Fas, atorvastatin, ischemia-reperfusion, apoptosis.

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**A**poptosis is a genetically programmed form of cell death that is mediated by the activation of the caspase cascade and results in the cleavage of protein substrates and oligonucleosomal fragmentation of DNA. Apoptosis of the cardiomyocytes has been demonstrated in animal models with coronary artery occlusion <sup>1</sup>, and experimental evidence suggests that myocardial cells are able to undergo apoptosis during ischemia followed by reperfusion <sup>2</sup>. Both ischemic and reperfused rat myocardium can undergo apoptotic cell death, however the myocardium, which is subjected to ischemia followed by reperfusion, under-

goes accelerated apoptosis <sup>3</sup>. Apoptosis is a regular, non-necrotic form of cell death which follows two major pathways: mitochondrial pathway, which is characterized by the release of mitochondrial cytochrome c and subsequent activation of caspase-9; whereas the other, death receptor pathway, involves the binding of a death ligand, such as Fas ligand (FasL), to Fas resulting in activation of caspase-8. Fas/APO-1/CD95, member of the tumor necrosis factor (TNF) receptor superfamily, is a widely expressed cell surface receptor that can initiate apoptosis when activated by its ligand (FasL). It has been shown that the Fas pathway

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is functional in cardiac myocytes and plays a critical role in myocardial death due to ischemia-reperfusion *in vivo* <sup>4</sup>. In *lpr* mice, a naturally occurring mutant deficient in Fas, there is marked reduction in infarct size and abundance of apoptotic cardiac myocytes following ischemia and reperfusion that also signifies the importance of Fas pathway in ischemia-reperfusion injury <sup>5</sup>.

Hypercholesterolemia is a major risk factor in development of cardiovascular disease. HMG-CoA reductase inhibitors (statins) were originally designed to reduce serum cholesterol levels and thus reduce this risk factor. However, recent studies have demonstrated that statins appear to have beneficial effects independent of their cholesterol-lowering properties; these so-called pleiotropic properties include anti-inflammatory effects, plaque stabilization, improved endothelial function and inhibition of vascular smooth muscle cell proliferation. These pleiotropic effects thus have a major role in protecting the myocardium against ischemic injury. In addition, it has been shown that atorvastatin can protect the isolated mouse heart against reperfusion-induced injury <sup>6</sup>. Pretreating the rats with simvastatin 18 hour prior to the induction of ischemia-reperfusion has been shown to reduce cardiac dysfunction and improve coronary flow <sup>7</sup>. It was shown that functional Fas system contributes to apoptotic myocardial cell death in response to ischemia/reperfusion injury <sup>4, 5</sup>. However, the influence of statins on Fas expression and cardiomyocyte apoptosis following ischemia-reperfusion was not studied. Therefore we designed this research to study the effects of atorvastatin on cardiomyocyte apoptosis following acute ischemia and reperfusion *in vivo* and Fas gene expression in order to elucidate the possible mechanism of atorvastatin on inhibition of cardiomyocyte apoptosis.

## Methods

### Animal model and treatment

Thirty healthy adult male Wistar rats (supplied by the Center of Experimental Animals, Tongji

Medical College), weighing 200-250 grams, were equally divided into 3 groups (n = 10):

- 1) Coronary artery ligated and reperfused group (I/R group)
- 2) Coronary artery pseudo ligated group (sham-operated group)
- 3) Coronary artery ligated and reperfused and pretreated with atorvastatin group (atorvastatin treated group)

Atorvastatin treated group received 10 mg/kg of atorvastatin in 2 ml saline for 3 days, administered once daily by a stainless steel oral gavage tube. Ischemia-reperfusion (I/R) and sham-operated groups were given saline alone.

### Surgical preparation

Each rat was anesthetized by ether, hair was cut off and skin was sterilized by routine procedure. Then, an incision was made in the skin on the left side of the chest, and the pectoral muscles were gently retracted to expose the ribs. An incision was made through the third intercostal space, and the ribs were gently spread to expose the heart and the heart was taken out. Left anterior descending (LAD) branch of coronary artery was ligated at the intersection between conus arteriosus and left atrial appendages, and then heart was put back into the chest cavity. Thirty minutes later chest cavity was reopened and heart was taken out with above-mentioned method. Then, ligation of LAD was released and heart was put back into the cavity again. Wound at the incision site was sprayed by penicillin powder to prevent the infection and then was sutured. Performance on the sham-operated group was the same as that on acute I/R group, but LAD was only suspended with a string and the ligation was not done in this group. Three hours after operation all rats were killed by decapitation, heart was removed immediately and washed with cold normal saline (NS). After that, myocardium with ischemia and reperfusion field (corresponding field in sham-operated group), was dissected out immediately from the anterior wall of the left ventricle and the specimens were fixed in fixative (4% paraformaldehyde containing 1% DPEC) followed by paraffin

embedding for 24 hours. Specimens were cut into slices. Slices were placed into the fixing solution (3% glutaraldehyde) for another 1 hour. After repeatedly washing, the specimens were post-fixed in 1% osmium tetroxide and dehydrated in graded series of ethanol. After epoxy resin infiltration and embedding, ultra-thin sections were made. After double staining with uranium acetate and lead citrate, these sections were observed by electron microscopy.

#### **In situ detection of cardiomyocyte apoptosis**

In situ death detection kit-AP was obtained from Boehringer Mannheim Co. Germany. TUNEL staining was done according to the manufacturer's recommendations. In brief, paraffin-embedded sections were dewaxed and incubated for 10 min with 3% H<sub>2</sub>O<sub>2</sub> to neutralize the activity of endogenous catalase, and then washed two times for 5 min with PBS. After digestion with Protease K solution (20 µg/ml) at 37°C for 15 min, each section was incubated with TUNEL reaction mixture at 37°C for 2 h. Each section was incubated with normal goat serum at 37°C for 30 min, and then washed three times for 5 min with PBS. Each section was incubated with Converter-AP solution at 37°C for 40 min, and then washed three times for 5 min with PBS. Each section was stained with DAB/H<sub>2</sub>O<sub>2</sub> at 37°C for 3 min and counterstained with hematoxylin for 30 s. The sections were washed in tap water, and then dehydrated, clarified and mounted. Brown colors of nuclei were taken as the positive staining of apoptotic cardiomyocytes, and apoptotic cardiomyocytes in each group were quantified by counting the mean of positive cells per 100 fields in 200X power lens under light microscopy.

#### **Immunohistochemistry detection**

Immunohistochemistry detection was done following the instructions provided by the manufacturer (Boster Biological Technology Company, Wuhan, China). In brief, paraffin sections were dewaxed by routine method and incubated for 10 min with 3% H<sub>2</sub>O<sub>2</sub>. Each section was incubated with blocking solution (normal goat serum) at room temperature for

15 min and then washed with distilled water and with PBS for 5 min. Each section was added with rabbit polyclonal anti-rat Fas antibody and incubated at 37°C for 1 hour and afterward these sections were washed three times for 3 min with PBS. Each section was incubated with biotinylated goat anti-rabbit IgG at 37°C for 15 min and then washed three times for 3 min with PBS. Each section was stained with DAB and counterstained with hematoxylin. The sections were washed in tap water, dehydrated, clarified and mounted. Fas expression in each group was quantified by counting the mean optic density of positive fields per 10 fields in 400X power lens with image analysis system.

#### **Statistical analysis**

Values are expressed as mean ± SD. SPSS 13.0 software was used (Department of Statistics, Tongji Medical College, Huazhong University of Science and Technology) for statistical analysis. *T*-test was used to compare mean difference of two samples. *P*-values less than 0.05 were considered to be statistically significant.

## **Results**

### **Effect of atorvastatin on cardiomyocyte apoptosis after I/R injury**

Observed under the transmission electron microscope, size and shape of the nucleus of the cardiomyocyte in the sham-operated group were normal and nuclear chromatin was evenly distributed. Only cytoplasm showed some injured changes during operation; swelling of the mitochondria and dissolution of few myofilaments were noted. Significant apoptosis was absent (figure 1).

In the ischemia-reperfusion group, cardiomyocytes showed features characteristic of apoptosis; nucleus was shrunk, cytoplasm was condensed and cellular size was decreased, and the nuclear chromatin was marginated. TUNEL staining showed that brown colors of nuclei (positive staining for apoptosis) were dispersed even in the apoptotic cardiomyocyte (figure 2).

Cardiomyocytes in atorvastatin treated group also showed features of apoptosis, but was less than in pure I/R group (figure 3).

Therefore, the count of apoptotic cells were increased significantly in the cardiomyocytes that suffered from 30 minutes of ischemia by ligation of LAD and 3 hours of reperfusion.

There was significant difference between I/R group and sham-operated group ( $134.45 \pm$

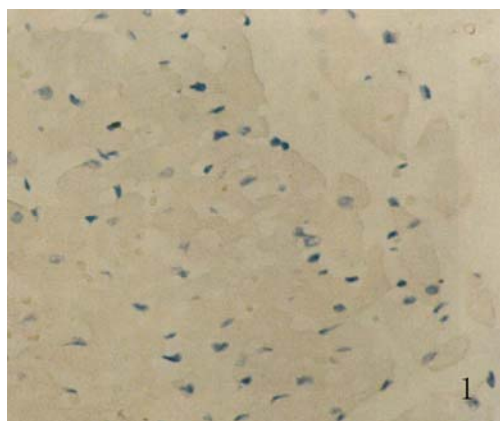
$40.56/\text{field}$  vs.  $0.18 \pm 0.09/\text{field}$ ,  $P < 0.001$ ); it indicated that acute I/R could cause the apoptosis of cardiomyocytes. Apoptotic cardiomyocytes in atorvastatin treated group was significantly less than the pure I/R group ( $90.66 \pm 19.44/\text{field}$  vs.  $134.45 \pm 40.56/\text{field}$ ,  $P < 0.05$ ). This indicated that atorvastatin could significantly inhibit apoptosis of the cardiomyocyte induced by I/R (table 1 and figure 4).

**Table 1.** Apoptotic cells in different groups per field.

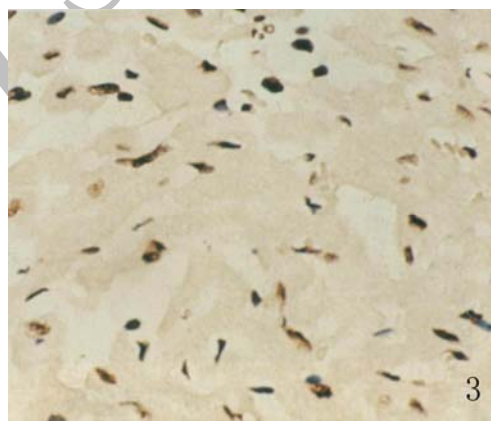
I/R Group	Sham-Operated Group	Atorvastatin Treated Group
$134.45 \pm 40.56^{\wedge}$	$0.18 \pm 0.09$	$90.66 \pm 19.44^*$

$\wedge P < 0.001$  vs. sham-operated group

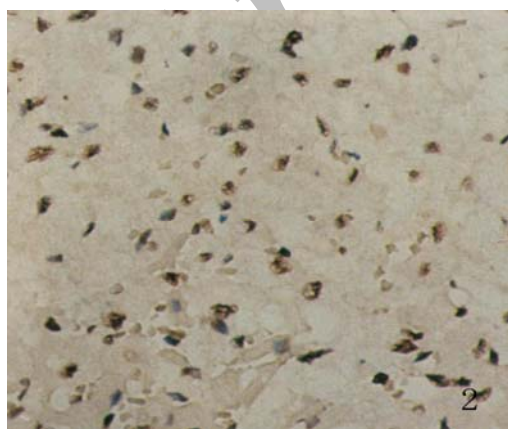
$* P < 0.05$  vs. IR group



**Figure 1.** Absence of the apoptotic cardiomyocytes in sham-operated group, staining with TUNEL X200.



**Figure 3.** Few apoptotic cardiomyocytes in atorvastatin treated group, staining with TUNEL X200.



**Figure 2.** Numerous apoptotic cardiomyocytes in ischemia-reperfusion group, staining with TUNEL X200.

#### Influence of atorvastatin on Fas expression after acute I/R injury

The absorbance value of Fas protein in the cardiomyocyte of I/R group was significantly higher than that in sham-operated group ( $0.13 \pm 0.032$  vs.  $0.06 \pm 0.017$ ,  $P < 0.05$ ). But, the Fas protein absorbance value in the cardiomyocyte of the atorvastatin treated group was significantly less than that in I/R group ( $0.07 \pm 0.016$  vs.  $0.13 \pm 0.032$ ,  $P < 0.05$ ) (table 2 and figure 5). These results indicated that upregulation of Fas protein expression might be the mechanism for cardiomyocyte apoptosis following ischemia and reperfusion. Moreover, atorvas-

tatin treatment could inhibit the expression of Fas protein in the cardiomyocytes undergone

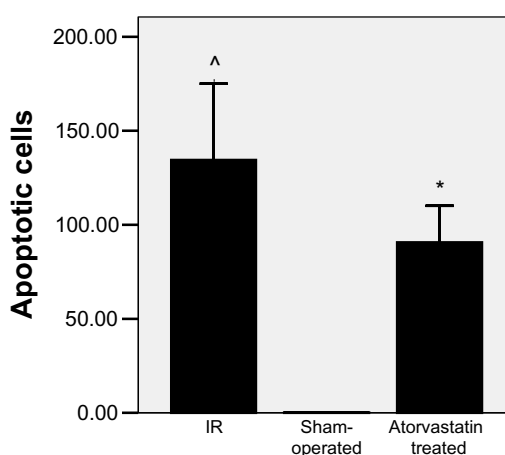
acute ischemia-reperfusion and thus inhibits cardiomyocyte apoptosis.

**Table 2.** Mean optical density of Fas expression.

Target	I/R Group	Sham-Operated Group	Atorvastatin Treated Group
Fas	0.13 ± 0.032*	0.06 ± 0.017	0.07 ± 0.016 <sup>^</sup>

\* P<0.05 vs. sham-operated group

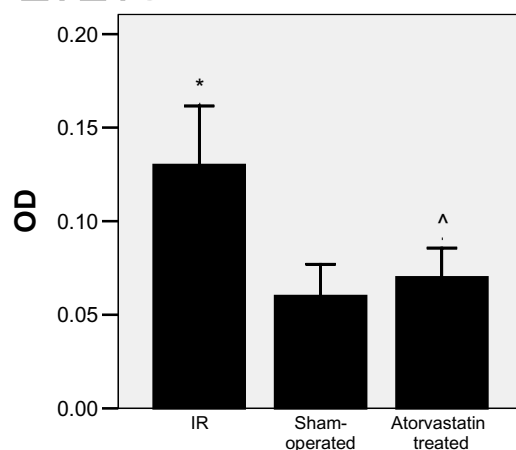
<sup>^</sup> P<0.05 vs. I/R group



<sup>^</sup> P<0.001 vs. sham-operated group

\* P<0.05 vs. IR group

**Figure 4.** Comparison of apoptotic cells in all three groups.



\* P<0.05 vs. sham-operated group

<sup>^</sup> P<0.05 vs. I/R group

**Figure 5.** Comparison of Fas expression on all three groups.

## Discussion

Apoptosis, an autonomous cell death, has become increasingly recognized as the mechanism of cell death during hypoxia, ischemia, and hemodynamic overload and heart failure. Although, prompt and effective reperfusion of the ischemic myocardium plays an important role in minimizing cardiomyocyte injury associated with acute myocardial ischemia, studies have shown that myocardial reperfusion itself results in enhanced myocardial injury<sup>8,9</sup>. It has been demonstrated that myocardium subjected to ischemia followed by reperfusion undergoes accelerated apoptosis<sup>3</sup>. The studies concerning control of coronary heart disease has been focused on the inhibition of cardiomyocyte apoptosis induced by ischemia-reperfusion, reduction of cardiomyocyte loss, protection of cardiac function and lowering the morbidity of complication after myocardial infarction. Two central pathways, death receptor pathway and the mitochondrial pathway mediate the process of apoptosis. Both the mitochondrial and the death receptor pathways have been shown to exist in the heart. In the mitochondrial pathway, diverse stimuli, including nutrient and growth/survival factor deprivation, hypoxia, and oxidative stress, stimulate the translocation of cytochrome c from the mitochondrial intermembrane space and inner membrane to the cytoplasm<sup>10</sup>. In contrast, death receptor pathway involves the binding of soluble or cell membrane-bound ligands to cell surface receptors such as Fas and tumor necrosis factor receptor 1 (TNFR1)<sup>11</sup>. Fas is a widely expressed cell surface receptor that can initiate apoptosis when activated by its ligand (FasL)<sup>12</sup>. Trimeric Fas ligand (FasL), an integral membrane protein, binds to a Fas trimer. This is presumed to induce a conformational change in Fas that enables its cytoplasmic tail to recruit Fas-associated death domain protein (FADD) through interactions involving death domains in both molecules. FADD, in turn, recruits procaspase-8 through homotypic interactions involving death effector motifs. The death-inducing signaling complex-induced proximity of procaspase-8 molecules stimulates

its autoactivation, following which active caspase-8 can then initiate the caspase cascade that leads to the characteristic morphologic changes of apoptosis and phagocytosis via the proteolytic activation of other caspases, including caspases-3, -4, -6, -7, -9 and -10.

Studies have shown that statins reduce plasma cholesterol levels and improve survival in patients with coronary artery disease. However, several studies have indicated that statins appear to have beneficial effects independent to their cholesterol-lowering properties. These so called pleiotropic properties include anti-inflammatory effects, plaque stabilization, improved endothelial function, and inhibition of vascular smooth muscle cell proliferation. Several *in vitro* and *in vivo* studies have demonstrated that treatment of animals with statins prior to the onset of myocardial ischemia reduces ischemia-reperfusion injury. Pretreating the rats with simvastatin 18 hour prior to the induction of ischemia-reperfusion significantly reduced the cardiac dysfunction and improved coronary flow<sup>7</sup>. In addition, Bell RM et al have demonstrated that atorvastatin can protect the isolated mouse heart against reperfusion-induced injury<sup>6</sup>. Statins have been shown to upregulate COX2 expression and studies have shown that COX2 plays important protective roles in the regulation of myocardial damage after ischemia-reperfusion injury. COX2 also produces PGI<sub>2</sub>, which is reported to have protective effects against ischemia-reperfusion injury<sup>13</sup>. It has been indicated that the Fas death pathway is functional in cardiac myocytes and critical for myocardial damage due to ischemia-reperfusion *in vivo*<sup>4</sup>. Yue TL et al have demonstrated that overexpression of Fas in rabbit myocytes plays an important role in the acceleration of cellular damage after ischemic injury and that downregulation of Fas expression may be critically involved in the protection of myocytes against apoptosis<sup>14</sup>. However, influence of statins on Fas expression and cardiomyocyte apoptosis following ischemia and reperfusion was not studied. Fas, a member of TNF receptor family, has been shown to induce apoptosis in myocardial cells in various

diseases. Despite earlier reports of low or undetectable expression of Fas and FasL in the heart, recent reports indicated that Fas and FasL are constitutively expressed in the myocardium<sup>15</sup> and are involved in myocardial cell apoptosis<sup>5</sup>. Zhang X et al have shown that Fas-deficient mice do not exhibit ischemia-reperfusion-induced apoptosis<sup>16</sup>. In our present study we also found that the basal level of Fas in non-ischemic ventricular tissue of sham-operated group was low or below detectable level and Fas expression was markedly upregulated in areas at risk of ventricular tissues of rats subjected to ischemia followed by reperfusion. In atorvastatin treated group, the expression of Fas was reduced significantly. Our data suggest, therefore, that Fas is involved in ischemia-reperfusion induced apoptosis in rat myocytes and downregulation of Fas expression by atorvastatin may be the possible mechanism of protection of myocytes against apoptosis induced by ischemia and reperfusion. However, the exact mechanism of inhibition of Fas overexpression by atorvastatin is not clear.

Statins upregulate the expression and function of endothelial nitric oxide synthase (eNOS) independent of cholesterol levels. It is demonstrated that pretreatment with statins reduces myocardial injury after acute ischemia and reperfusion by increasing the expression of eNOS. Birnbaum Y et al have demonstrated that 3 days atorvastatin treatment significantly attenuates ischemia reperfusion injury<sup>17</sup>. They indicated that the protective effect of statins was completely abolished by non-selective NOS inhibitor (L-NAME), suggesting that this protective effect is mediated via the NOS activity. Studies have shown that the beneficial effects of statins on myocardial cell death are absent in eNOS<sup>-/-</sup> mice, suggesting that eNOS is the mediator of protection. However, eNOS<sup>-/-</sup> mice lacking compensatory increase in iNOS expression have shown larger myocardial infarct size<sup>18</sup> than the mild type mice. In contrast, strain of eNOS<sup>-/-</sup> mice which has a compensatory increase in iNOS mRNA expression had smaller myocardial infarction size than the

wild type. Furthermore, Scalia et al have demonstrated that simvastatin failed to decrease infarct size in iNOS<sup>-/-</sup> mice suggesting that iNOS may also play a role in myocardial protection<sup>19</sup>. Acute simvastatin treatment has shown to protect the myocardium from ischemia-reperfusion injury when given right at the beginning of the reperfusion period by activating PI3K/AKT pathway<sup>20</sup>. It has been shown that atorvastatin can protect the isolated murine heart against reperfusion-induced injury via the activation of the prosurvival PI3K/AKT pathway<sup>6</sup>. It has been demonstrated that activation of ecto-5'-nucleotidase through the activation of PI3K after ischemia is involved in the cardioprotective mechanism of statins in ischemia-reperfusion injury<sup>21</sup>. Cardioprotection against ischemia-reperfusion injury via ecto-5'-nucleotidase activation might be mediated by an increase of adenosine, the main product of ecto-5'-nucleotidase. Notably, the PI3K/AKT signaling pathway is important in maintaining the expression of FLIP, an inhibitor of Fas-mediated apoptosis<sup>22, 23</sup>. In addition, this signaling pathway can mediate activation of eNOS, which induces upregulation of NO release from endothelial cells<sup>24, 25</sup>. Studies have suggested that the anti-apoptotic effect of NO can be mediated through a number of mechanisms such as nitrosylation and inactivation of caspases, up-regulation of p53, and heat shock proteins<sup>26</sup>. Considering recent discovery that NO negatively regulates Fas induced apoptosis through a decreased expression of Fas<sup>27, 28, 29</sup>, it is possible that statins inhibit Fas expression via increasing NO synthesis and thus may inhibit cardiomyocyte apoptosis induced by ischemia and reperfusion.

In conclusion, apoptosis is a programmed cell death, and its molecular mechanism is rather complicated. Upregulation of Fas expression may be responsible for the apoptosis of cardiomyocyte during ischemia followed by reperfusion, and atorvastatin treatment may prevent ischemia-reperfusion-induced apoptosis of cardiomyocytes by inhibiting expression of Fas. However, the exact mechanism of Fas inhibition by statins is still far from clear.

In the future, possible anti-apoptotic mechanism of statins in myocardial cells following

ischemia and reperfusion should further be studied.

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