

## Effects of livin over-expression on myocardial ischemia reperfusion injury in rats

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**Abstract :** To evaluate the effects of livin over-expression on myocardial ischemia reperfusion injury. Rats were subjected to 30 min of left coronary artery occlusion followed by 120 min of reperfusion with treating the rats by retroviral vector expressing livin 24h before left coronary artery occlusion. Both caspase-3 and livin mRNA expression were detected by real time PCR and the caspase-3 protein was detected by immunohistochemical study; Cardiomyocyte apoptosis was evaluated with TUNEL assay. Myocardial infarction size were detected by TTC dyeing method. Caspase-3 mRNA expression increased during IR and decreased significantly after the transfection of retroviral vector expressing livin. Meanwhile the apoptosis index and MI size were increased in IR group and decreased significantly in livin group. Livin overexpression could down-regulate the expression of caspase-3, attenuate myocardial apoptosis, and decrease myocardial infarction size.

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**Key words:** myocardial infarction; acute; livin; apoptosis

### 1. Introduction

Myocardial reperfusion after acute myocardial infarction(AMI) could reduce the mortality, improve myocardial function and attenuated cardiac arrhythmia. However, the occurrence of myocardial ischaemia reperfusion injury(IRI) following the reperfusion treatment will result in myocardial dysfunction, arrhythmia, myocardial stunning, re-occlusion of coronary arteries, even when revascularization was successful. Some reperfusion injury may occur that transiently impairs myocardial function or results in sudden cardiac death. Therefore, treatment should not only be directed towards the restoration of myocardial blood flow but should also to prevent or alleviate the consequences of myocardial reperfusion injury.

The mechanisms of myocardial ischemia reperfusion injury remain unclear. Several factors such as impairment of cardiomyocyte construction, disorder in cardiac energy metabolism, thrombosis and vasospasm were considered as the causes of IRI. Various strategies to deal with IRI have been developed, but no significant effect was seen in clinical settings. Recent studies reported that apoptotic cardiomyocyte increased in myocardial infarction. This phenomenon suggested that cardiomyocyte apoptosis may play an important role in myocardial ischemia reperfusion injury, and IRI might be attenuated if the cardiomyocytes apoptosis was suppressed.

Apoptosis depends on the activation of caspase-3. Livin is one of the inhibitors of apoptosis protein which could combine with caspase-3 and suppress apoptosis. But no data indicated that whether livin could protect heart from ischemia reperfusion injury or not. The present study, we constructed retroviral vector

expressing livin and transferred it into rat myocardium, is to investigate the changes of Caspase-3 and livin mRNA expression during cardiac ischemia reperfusion and the effects of livin over-expression on the expression of caspase-3 and cardiomyocytes apoptosis in myocardial ischemia reperfusion process.

### 2. Materials and methods

#### (1) Materials

Retroviral vector pLNCX<sub>2</sub> and RetroPack™ PT67 cell line were from Clontech Company. Colibacillus strain JM109 was from Takara Co. Caspase-3 antibody was bought from Santa Cruz Company in USA.

#### (2) Livin gene clone and subclone

Livin RNA from mice MA782 cell was extracted according to QIAGEN RNA extraction procedure, the cDNA was obtained by reversible transcription polymerase chain reaction (rt-PCR) technique, livin gene was amplified (primer, 5' ACTCGAGATGGGGCCTGAGAGTAGGGCCAG 3' XhoI, and L2 5' AAAGCTTTTAGGACAGGAATGTGCGTACAC 3' HindIII) and first cloned into pGEM-T easy plasmid then digested by XhoI/HindIII and identified by PCR. The pGEM-T-livin was subcloned into the retroviral vector pLNCX2. The pLNCX2-livin was transferred into packaging cell PT67 after XhoI/HindIII digestion and identified by PCR. A pure packaging cell line pLNCX2-Livin cells expressing livin was cultured and frozen at -80 °C.

**(3) Transfection rat myocardium**

All the animal experiment and care were approved by the guidelines on the Use of Laboratory Animals in Zhengzhou University Animal Care Committee (China, Zhengzhou). Eighty healthy SD rats were divided into Control group, IR group, empty vector group and Livin group. Rats were subjected to 30 min of left coronary artery occlusion followed by 120 min of reperfusion with treating the rats with 50 $\mu$ l(3.9 $\times$ 10<sup>7</sup>IU/ml) pLNCX2-Livin (livin group) or with 50 $\mu$ l(3.7 $\times$ 10<sup>7</sup>IU/ml) pLNCX2 (empty vector group) by intramyocardium injection 24h before left coronary artery occlusion. Rats in IR group underwent the IR process but no treating with vectors. The samples were isolated and freezeed in -196 liquid nitrogen.

**(4) Livin and caspase-3 mRNA detection by real time PCR**

Rat myocardium RNA was extracted in trizol method and the cDNA was obtained by reversible transcription with catalysis in AMV and the primer was OLIGO hexamer: livin, up, 5' atggggcctgagagtagggccag 3' , 169 bp ; down, 5' GACAGAGGCCGAAGCTGGCC 3'. Caspase-3, up 5' gtcgatgcagctaacctcag 3' ,156 bp, down 5' GACTTAGAATCACACACAC 3' . -actin, up, 5' atggatgacgatatcgtgctg 3' , 247 bp, down 5' TCCaTatCGTCCCagttggtg 3'. According to real time PCR kit procedure, reaction system was 50 $\mu$ l. Livin gene amplification was compared with -actin amplification. The pGEM-T- -actin and pGEM-T-livin standard curve in different dilution were set up. The relative level of livin expression was calculated by the ratio of livin expression and -actin expression.

**(5) Myocardial apoptosis detected by TUNEL assay:**

The infarcted and ischemical myocardium was fixed in formaldehyde and embedded in paraffin and then cut into 4  $\mu$  m slices along the long axis of left ventricle. The apoptotic myocardial cells were detected in TUNEL method. The samples in control group experienced the same procedure but without Tdtase. Three slices from one sample were observed for 10 fields under microscope of 10 $\times$ 40 to count the positive apoptotic myocardial cells and total myocardial cells. Apoptotic index(AI) was the ratio of positive apoptotic myocardial cells and the mean of myocardial cells in 10 fields.

(6) MI size was evaluated by 2, 3, 5 triphenyltetrazolium chloride (TTC) dyeing method: Evan's blue 3ml (2 g in 100 ml) was injected into left ventricle after the IR, then 5 ml KCL (10 g in 100 ml)

was injected into ventricle. The heart was isolated and cut into 5 slices , then put the ischaemic and infarcted myocardium into TTC (1 g in 100 ml) at 37 ~ 40 for 10 min. The infarcted regions was negative but the risk and normal regions were stained brick red by TTC.

**(7) Statistics:**

The *P* values for the experiments were calculated using the unpaired Students *t* test. The statistical software was SPSS Version 13.0. Data were indicated as SD $\pm$ s,  $\alpha$  = 0.05.

**3. Results****(1) Livin amplification results**

Electrophoresis result: bright strap at 1000bp is accordance with theoretical value 816bp, seen in figure 1A.

(2) Electrophoresis results of recombinant plasmid pGEM-T-livin amplification production. Positive clone at 992bp (Fig 1B).

(3) Electrophoresis results of recombinant plasmid pGEM-T-livin digested by XhoI and HindIII : 2 straps were seen at 816bp and 3027bp ( Fig 1C ) .

(4) Recombinant clone pLNCX2-Livin identified by enzyme digest(Fig 1D).

(5) Positive recombinant clone pLNCX2-Livin identified by PCR:positive clone pLNCX2-Livin, Fig 1E.

(6) Real time PCR amplification results: Livin mRNA expression increased significantly in livin group compared with those in IR group, control group and empty vector group. Caspase-3 mRNA expression increased during IR, but decreased significantly in livin group (Table 1).

(7) Results of Caspase-3 protein expression: The levels of caspase-3 protein expression was 91.39  $\pm$  4.82 in control group, 103.39  $\pm$  8.24 in IR group, 97.43  $\pm$  11.15 in livin group and 102.28  $\pm$  7.83 in empty vector group, respectively. The expression levels of caspase-3 protein were increased significantly in IR group and in empty vector group compared with that in control group. And the caspase-3 protein decreased after transfecting retroviral vector expressing livin (Fig 2, Fig 3).

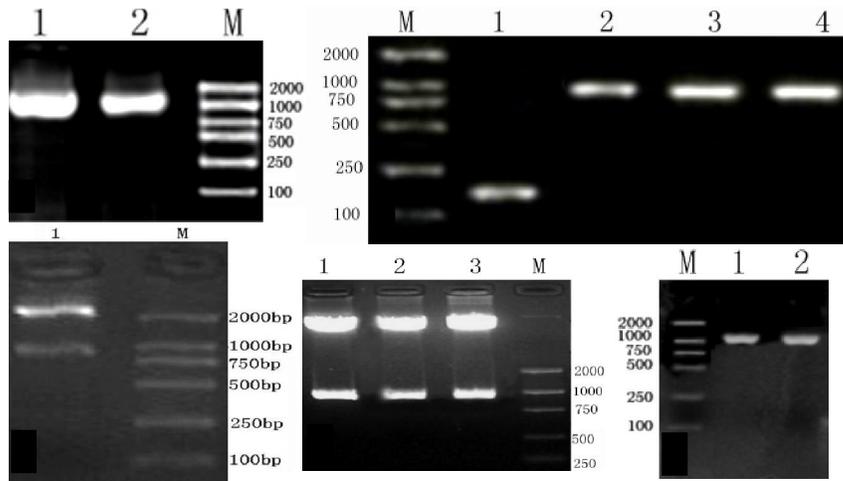


Fig 1A. Livin amplification results 1,2:livin amplification production ; M:Marker

Fig1B. Electrophoresis results of recombinant plasmid pGEM-T-livin amplification production.

M : DNA marker ; 1: pGEM-T ; 2-4: positive clone pGEM-T-livin.

Fig 1C : Electrophoresis results of recombinant plasmid pGEM-T-livin digested by XhoI and HindIII:1 : XhoI and HindIII digested pGEM-T-livin ; M : DNA Marker.

Fig 1D: Recombinant clone pLNCX2-Livin identified by enzyme digest:

M : DNA Marker; 1-3 : Electrophoresis results of recombinant clone pLNCX2-Livin digested by XhoI and HindIII enzyme.

Fig 1E: Positive recombinant clone pLNCX2-Livin identified by PCR: M : DNA Marker;

1-2 : positive recombinant clone pLNCX2-Livin identified by PCR (primer: Pln1/Pln2 ).

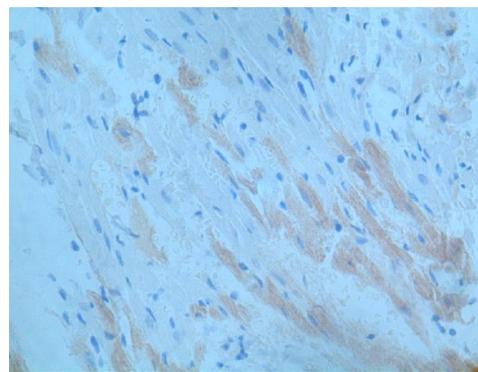
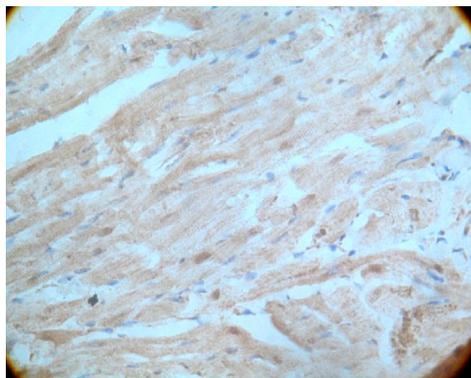


Fig 2. expression of caspase-3 in IR group.

Fig 3. expression of caspase-3 in livin group. (10)

Myocardial apoptosis: The apoptosis index (AI) was ( 1.1±0.42 )% in control group, ( 10.35±3.34 )% in IR group, ( 1.7±1.57 )% in livin group, and (9.62±3.68)% in empty vector group respectively. AI increased significantly in IR group but decreased after transfecting the retraviral vector expressing livin.

(11) MI size: The MI size was 27.82%± 9.77% in IR group and 19.55% ±2.82% in livin group. The MI size decreased significantly in livin group.

Table 1. Livin and Caspase-3 mRNA expression

group	n	Livin mRNA ( $\times 10^{-3}$ )	Caspase3 mRNA ( $\times 10^{-3}$ )
Control group	20	8.41 $\pm$ 1.39	5.12 $\pm$ 2.11
IR Group	20	7.82 $\pm$ 3.22	92.1 $\pm$ 34.6 *
Livin Group	20	145 $\pm$ 89 * #	56.2 $\pm$ 21.1 * #
Empty vector Group	20	7.45 $\pm$ 2.51	94.1 $\pm$ 18.1*

\* compared with control group  $P < 0.01$ , # compared with IR group  $P < 0.01$ .

#### 4. Discussion

Acute myocardial infarction was frequently followed by myocardial ischemia reperfusion injury (IRI) because of the opening of occluded coronary arteries<sup>[1-3]</sup>. The mechanisms of IRI have not been elucidated. Recent studies reported that cardiomyocyte apoptosis increased in ischemia reperfusion (IR)<sup>[4]</sup>. We detected the caspase-3 mRNA expression in normal rat myocardium and in ischemic reperfusion model. There was a significantly increase of caspase-3 expression in rat heart after 30 minutes ischemia and followed by 2 hours reperfusion compared with that in the control conditions. This supported the report that cardiomyocyte apoptosis play an important role in the myocardial ischemic reperfusion injury<sup>[5-7]</sup>.

Apoptosis is a process whereby cells undergo programmed death. The mechanism that apoptosis taken place is unclear yet. Apoptosis relies on activation of distinct signalling pathways. The activation of caspase-3 has been found in many models of apoptosis and it may play a pivotal role in a downstream event of caspases cascade and the occurrence of apoptosis<sup>[8-10]</sup>. Inhibitor of apoptosis protein (IAP) can combine to caspase and suppress caspase-3. Livin was one of IAP family discovered recently. Livin contain a baculoviral inhibitor of apoptosis repeats (BIR domain) and a ring zinc finger domain (RING). BIR domain is the functional area by which livin could combine with caspase and suppress its activity and suppress apoptosis<sup>[11,12]</sup>. Livin was seen in tumour tissue and was very few in myocardium. It is unknown that whether livin could suppress myocardium apoptosis in rat or not so far.

We measured the expression of livin mRNA in normal myocardium and in ischemic reperfusion myocardium in rat. The results indicated that there was little livin expression in normal rat heart and the livin expression did not increase during the IR process.

Retroviral vector is a stable and effective gene expression vector. In the present study we constructed and produced a retroviral vector

expressing livin and transferred it into rat myocardium. By real-time PCR detection we found that the transfection livin vector into rat myocardium was successful. And caspase-3 expression decreased significantly after the transfection of retroviral vector expressing livin. These indicated that livin overexpression could suppress the expression of caspase-3.

Using TUNEL assay we evaluated the changes of myocardial apoptosis during IR. The results indicated that apoptosis index increased during the IR process but decreased significantly in livin group. This suggested that livin overexpression could suppress myocardial apoptosis during IR process and this might be actualized through the suppression of caspase-3.

Myocardial ischemia reperfusion injury is complex and hard to cure. Methods used in present clinical such as vasodilation, thrombolysis and so on were ineffective. Anti-apoptosis is a new idea to prevent the IRI and its effect is worth to expect.

In conclusion, the present study provided a clear evidence that cardiomyocytes apoptosis during myocardial ischemia reperfusion is related to the myocardial ischemia reperfusion injury. Livin overexpression could suppress the activation of caspase-3 and then downregulate cardiomyocyte apoptosis. These indicate that transfecting retroviral vector expressing livin might be useful for the treatment of myocardial ischemia reperfusion injury.

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