

Original Article

A 6 hour therapeutic window, optimal for interventions targeting AMPK synergism and apoptosis antagonism, for cardioprotection against myocardial ischemic injury: an experimental study on rats

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Abstract: The time relation between autophagy and myocardium ischemia (MI) has never been documented. Therefore, the present study was conducted to find out the exact timings and specific roles that AMP-activated protein kinase (AMPK)-mTOR signaling pathway plays on autophagy and apoptosis in rats' ischemic heart. 36 male Sprague Dawley (SD) rats were divided randomly into control and MI groups (each = 6). MI models were created by ligating left anterior descending artery (LAD) of rat hearts and the right myocardium were harvested at 0.5 h, 1 h, 3 h, 6 h, 12 h after ischemia. Expressions of Phosphorylated-AMPK (p-AMPK) and Phosphorylated-mTOR (p-mTOR) were determined by immunohistochemistry (IHC), western blotting (WB) and quantitative real-time PCR (Q-PCR) methods. LC3 expression was determined by WB and Q-PCR. The level of cell apoptosis was measured by the terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) method. p-AMPK was activated significantly in ischemic myocardium and its expression at MI groups showed a time dependent pattern with a fluctuating pattern compared to the control group. p-AMPK levels were seen to rise at 0.5 h followed by a fall at 1 h after MI, which again gradually peaked at 6 h and finally decreased at 12 h. While, p-mTOR levels suggested a constant declining trend with time. Autophagy related protein LC3 had a sustained up-regulation with time. TUNEL method suggested that apoptosis increased at 0.5 h, then decreased at 1 h and 3 h after MI and finally showed a continuous rising trend. Activation of protective autophagy that occurred during the initial phases of ischemic insults was within 6 hours. When the ischemia was prolonged, after 6 hours, although autophagy increased, cardiomyocyte death followed via the activation of apoptosis. Thus, limiting autophagy within 6 hours would give us double benefits. It would prevent the death related autophagy and prevent apoptotic cellular death. This 6 hours time period could serve as a landmark for therapeutic application for achieving cardioprotection from ischemic insults.

Keywords: Myocardium ischemia, autophagy, apoptosis, timings, AMPK, mTOR, LC3

Introduction

Ischemic Heart Disease (IHD) is the leading cause of mortality worldwide [1]. It results from a mismatch between the supply and demand of nutrient and oxygen which finally can lead to catastrophic cell death of a cardiomyocyte. Clinically, the sequel of myocardial damage may manifest as acute coronary syndromes (ACS), cardiac dysfunction, ventricular dilation and heart failure. Currently, alleviating ischemia by means of drugs, reopening of the occluded vessel or bypassing the occlusion are the only available modes of cure. However, all

of these methods are associated with drawbacks and limitations. For the last 2 decades, researches have centered on discovering and manipulating the micro-molecular processes pertaining to myocardial ischemic pathogenesis and limiting reperfusion injury. Till date an active search for therapeutic target has been unsuccessful.

At the onset of cardiac ischemia, a unique process called autophagy, an evolutionarily conserved intracellular process in eukaryotes, has been seen to be activated. This process replenishes ATP in an attempt to maintain energy

homeostasis at the expense of organelles especially by degradation and recycling of long-lived proteins and damaged organelles for cellular survival [2-4]. However, its dysregulated controls have been associated with varieties of cardiac diseases and cellular death. Autophagy with its dual nature of cytoprotection and death induction has been an area of intense research of today. Moreover, activation of the process not only during ischemia but also during reperfusion has lead to a newer form of cellular death distinct from the primary insult, the lethal reperfusion injury [5]. Numerous studies have demonstrated the cytoprotective effects of autophagy during situations of energy depletion and fasting [6], in vivo animal models as well as in cell culture models [7]. Some of them have even elaborated this effect and have demonstrated the cytoprotective autophagy to be active only during mild oxidative stress (Chen et al). When excess oxidative stress occurs or when the exposure time is prolonged, then apoptosis comes into play, that is a programmed death process within the cell [8]. There appears to be a relation between autophagy and apoptosis that controls cellular death or cellular survival. Researches conducted by Yan et al have demonstrated an inverse control between autophagy and apoptosis in a porcine model [9].

At the subcellular level, autophagy activation, during myocardial ischemia, occurs when the ratio of ATP/AMP declines [10], sensed by AMP-activated protein kinase (AMPK), a member of serine/threonine protein kinase family. AMPK stands in the middle, activating and inhibiting different intracellular molecules, so as to maintain cellular energy homeostasis [11-14]. Its direct role on survival autophagy has been validated by demonstration of reduced autophagy in transgenic mice overexpressing a dominant negative AMPK [7]. It is also expressed in cardiomyocytes, fibroblasts, endothelial cells and smooth muscle cells. AMPK mediated survival autophagy has been demonstrated to play a vital role in maintaining cell energy homeostasis and myocardial function during MI [15, 16]. Increased AMPK is likely to compensate for the ischemic conditioning by upregulating Glucose transporter type 4 (GLUT4) translocation and enhancing fatty acid oxidation [17, 18]. AMPK has also been seen to regulate the expression of p27 [19], which is a genetic regulator, for manufacturing autophagic machinery. AMPK

has also been demonstrated to negatively regulate mTOR (mammalian Target Of Rapamycin) [20, 21], which is an inhibitor of cellular autophagy. This fact has been demonstrated by Matsui et al. in a glucose deprived cardiomyocyte [7].

mTOR is another powerful sensor of nutrient abundance, cellular energy, amino acid levels, which shut off autophagy. Similarly, another molecule, microtubule-associated protein 1 light chain 3, MAP1-LC3 (LC3) is also seen to rise during autophagy and the ratio of LC3-II to LC3-I specifically correlates with autophagy. In this study we utilize these basic autophagic molecules and try to pinpoint of time of activation of AMPK, mTOR, LC3 and activators of apoptosis, in a rat model. In this study, we hope to expose a suitable, critical time window where therapeutic intervention could be applied for cardioprotection against I/R injury.

Material and methods

Experimental animals

The current study was performed in accordance with the NIH guidelines for the care and use of laboratory animals. All experimental procedures were approved by the Animal Care and Use Committee of Anhui Medical University. 36 SPF grade male Sprague-Dawley (SD, 250-300 g) rats were purchased from Experimental Animal Center of Anhui Medical University (Hefei, China). They were maintained at 25°C with a 12-h light/dark cycle and had access to food and tap water. 30 rats were assigned as MI groups by ligating the left anterior descending artery (LAD) with 6-0 silk sutures, while the rest were assigned as sham groups. Surgical operations were conducted with the assist of a small animal respirator (TaiMeng Corporation, Chengdu, China) and intraperitoneal chloralhydrate anesthesia. Demonstration of ST segment elevation after LAD ligation, in lead II of an electrocardiogram was the guide while creating a MI model. These MI models were randomly divided into five groups (n = 6) and respectively were sacrificed at 0.5 h, 1 h, 3 h, 6 h, 12 h. The ischemic parts of left ventricles, as observed by distinct colour and ST segment change, were removed and the half was stored in 10% neutral formalin liquid while the other half was preserved in liquid nitrogen and then stored at -80°C for further tests. Likewise, the sham con-

trols that only received exposure of LAD were treated with the same method as above. The obtained samples of all groups with different timings were then analysed with different stains and procedures.

Hematoxylin-eosin (HE) staining

Myocardial tissues fixed in 10% neutral buffered formalin solution for at least 24 hours were subsequently dehydrated, embedded in paraffin blocks and sliced at six micron-thick paraffin sections. These sections were then dewaxed in xylene followed by dehydration in alcohol solutions and finally were stained with hematoxylin and eosin. The slides were then studied under a microscope for morphological changes.

Immunohistochemical (IHC) staining

The paraffin embedded slides were dewaxed in xylene and dehydrated in alcohol. The process of antigen retrieval was achieved by microwaving in 10 mM citrate saline (pH 6.0) solution for 7 min at high fire grade with a 4 minutes interval for another 4 minutes. These sections were then washed with phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked by means of 0.3% hydrogen peroxide at 37°C for 15 mins. After rinsing, the sections were further blocked with 5% goat serum for 30 min at 37°C and then treated with the following primary antibodies at 4°C overnight: rabbit anti-phosphorylated mTOR (p-mTOR) (1:100, Ser2448, abcam, USA); rabbit anti-phosphorylated AMPK α 2 (p-AMPK α 2) (1:50, Thr172, Affinity, USA). Next day, after an incubation at 37°C for 30 min, the sections were treated with goat anti-rabbit IgG secondary antibody for 30 min at 37°C, which was followed by the incubation alongwith horseradish enzyme tag chain enzyme avidin working fluid for 30 min at 37°C. Simultaneously, after each incubation, the slides were washed with PBS solution. The antigen target protein stainings were visualized after dipping the slides into 3-3'diaminobenzidine (DAB) (ZSGBBIO, CHINA). Counterstaining was done with hematoxylin. These slides were then dried, cover slipped and observed under a microscope. Application of control serum instead of the primary antibody on alternative sections of the same heart provided the negative controls.

Western blotting (WB) analysis

Total proteins were extracted from the myocardium tissue blocks in RIPA lysis buffer (KeyGEN BioTECH, CHINA) containing protease inhibitor cocktail tablets (Roche,USA), phosphatase inhibitor cocktail (KeyGEN BioTECH, CHINA) and phenylmethanesulfonyl fluoride (PMSF, Sigma, USA). Homogenates were centrifuged at 12000 rpm for 15 mins at 4°C and then stored at -80°C. BCA protein assay kit (Pierce, Rockford, USA) was used to evaluate the protein content in the samples. An electrophoresis gel was created along with 8 lanes using standard protocols. For each lane, 40 μ g protein was loaded and separated on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (12% for GAPDH and LC3, 10% for p-AMPK, 6% for p-mTOR), which was then transferred onto polyvinylidene difluoride membranes (PVDF, Millipore Corporation, USA). After blocking in 5% nonfat milk for 3 hours at room temperature (RT), the membranes were washed and incubated overnight at 4°C with the following primary antibodies: rabbit anti-phospho-mTOR (Ser2448) antibody (1:1000, Abcam, USA); rabbit anti-phospho-AMPK α 2 (Thr172) antibody (1:1000, Affinity, USA); rabbit anti-GAPDH antibody (1:1000, KeyGEN, TECH, CHINA); rabbit anti-LC3 antibody (1:1000, Sigma, USA). Next day, the membranes were washed followed by further incubation with a secondary antibody (1:10000, Affinity, USA) for 1 hour at RT. The immunocomplexes were visualized with an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, USA) and quantitative analysis of specific band density was performed by using a ImageJ software. Protein levels were normalized to GAPDH as a loading control. All experiments were repeated at least three times to ensure reproducibility of the results.

Quantitative real-time PCR (Q-PCR) analysis

Total RNA was extracted from rat heart tissues using animal tissue total RNA extraction kit (TIANGEN, CHINA) and RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used to synthesize the first-strand complementary DNA (cDNA) according to the manufacturer's protocol. Q-PCR was performed with the SYBR Green qPCR kit (Toyota, Japan) on 5100 Thermal Sycler RT-PCR System (Thermo Fisher Scientific, USA). The reactions were performed

Table 1. Primers used for Q-PCR

| Genes | Forward | Reverse |
|------------------------------|-------------------------------|------------------------------|
| GAPDH ^a | 5'-CCATCACTGCCACTCAGAAGA C-3' | 5'-TCATACTTGGCAGGTTTCTCCA-3' |
| AMPK α 2 ^b | 5'-TGGAGGTGAATTGTTGACTACAT-3' | 5'-ACAGTAGTCCACGGCAGACAGA-3' |
| mTOR ^c | 5'-GGCCACCGTGTGTGAAGAA-3' | 5'-GACCCTGCACTG AGATCCTG-3' |
| LC3II ^d | 5'-GATGTCCGACTTATTCGAGAG C-3' | 5'-TTGAGCTGTAAGCGCCTTCTA-3' |

^aGlyceraldehyde phosphate dehydrogenase; ^bAMP-activated protein kinase α 2; ^cmammalian Target Of Rapamycin; ^dLight Chain3 II.

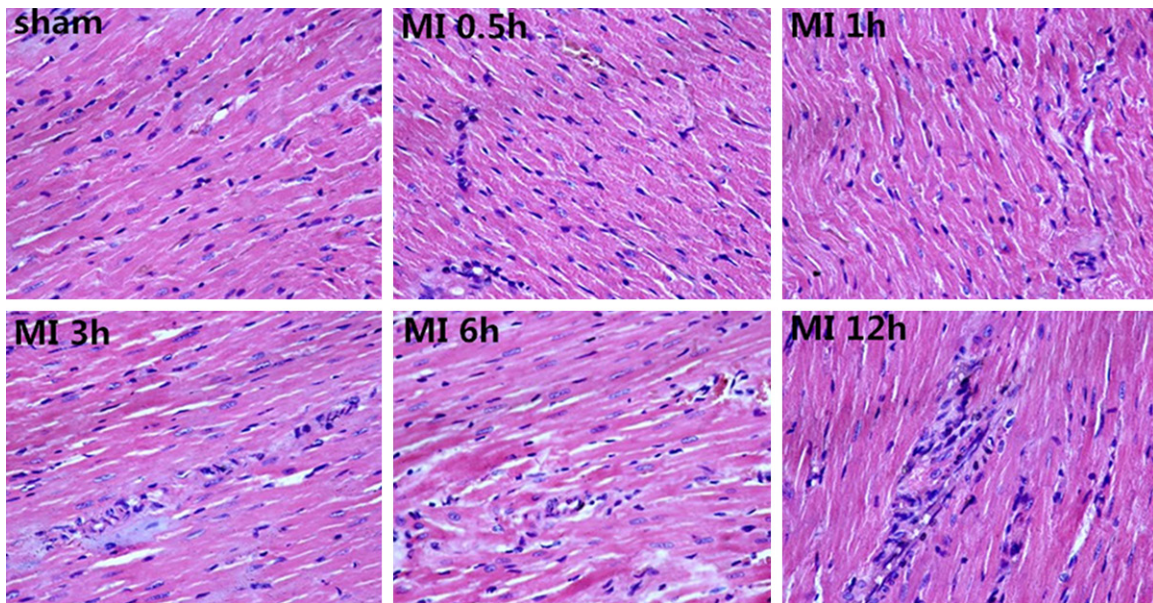


Figure 1. (HE×400). The morphological changes between MI and sham groups. The progression of nuclear clumping increases with time. Onset of cellular dissolution and cellular death can be seen in the images corresponding to 3 hours and 6 hours respectively.

in a 10 μ l volume mixture containing 5 μ l SYBR Green II mixture, 0.6 μ l primers (10 μ M), 2 μ l cDNA and 2.4 μ l sterile water. Cycling conditions were set as follows: 95°C for 30 s, 40 cycles of amplification at 95°C for 5 s, 60°C for 30 s, data acquisition, 60°C for 30 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. The experiment was repeated at least three times from three independent RNA samples, each of which was run in three tubes. After PCR amplification, datas were analyzed with the Piko RealTM Software 2.1 and the fold-change for mRNA relative to GAPDH was determined by the formula: $2^{-\Delta\Delta Ct}$. A primer pair for the detection of GAPDH was used as the internal control. Negative control in PCR reaction was done by replacing cDNA with ultrapure water. PCR primers were as follows in **Table 1**.

Terminal deoxynucleotidyl transferase- mediated DUTP-Nick end labeling (TUNEL) analysis

One Step TUNEL Apoptosis Assay Kit (KeyGEN BioTECH, CHINA) was used to detect the apoptotic cells in the myocardial tissues from both sham and MI groups. Sections were firstly deparaffinized, dehydrated, and then treated with protein K (20 μ g/mL) for 30 min at 37°C. After washing, they were treated with 0.1 M citrate solution at high fire grade in microwave for 8 minutes. The sections were incubated at 37°C for 30 min with DNase I working solution (4000 U/100 μ l) and then washed with PBS solution. After an incubation in a lucifuge box at 37°C with marked pre-mixed solution for 1 h, the sections were washed and then incubated with DAPI working solution for 10 min, which was finally covered and observed using a fluorescence microscope under a lucifugal circumstance.

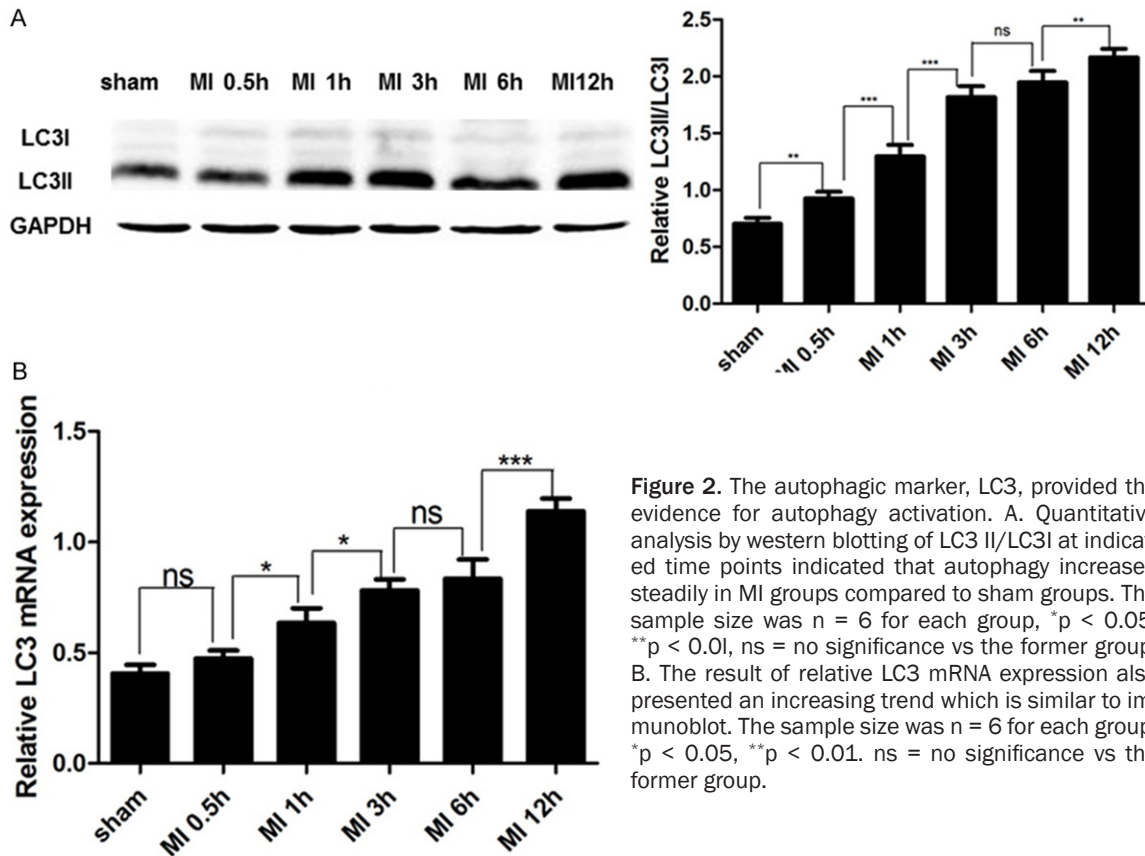


Figure 2. The autophagic marker, LC3, provided the evidence for autophagy activation. A. Quantitative analysis by western blotting of LC3 II/LC3I at indicated time points indicated that autophagy increased steadily in MI groups compared to sham groups. The sample size was $n = 6$ for each group, $*p < 0.05$, $**p < 0.01$, ns = no significance vs the former group; B. The result of relative LC3 mRNA expression also presented an increasing trend which is similar to immunoblot. The sample size was $n = 6$ for each group, $*p < 0.05$, $**p < 0.01$. ns = no significance vs the former group.

Pathological verifications

In order to verify the findings, 3 pathologists from Anhui Medical University were consulted for their opinions on different results. The findings, figures and statistical analysis are expressed in Huang with their opinions.

Statistical analysis

All the datas were expressed as mean \pm standard deviation (SD). Statistical analysis was performed by using SPSS 16.0 software. Statistical comparisons were performed using one-way ANOVA with Student-Newman-Keuls (SNK) post hoc test. Statistical significance was defined as $p < 0.05$.

Results

Morphological changes

The morphology of myocardial cells belonging to sham groups showed normal physiological architecture with regular distribution of clear nucleus and cytoplasm. On the contrary, the cells that were exposed to ischemia showed

nuclear aggregation that started at 0.5 h after MI, which became more apparent as time prolonged. As time progressed after 3 hours, the dissolution of myocardial cells began to appear. Evidence of initiation of cell death was witnessed at 6 hours depending on the severity of exposure to ischemia. These changes are depicted in **Figure 1**.

Evidence of autophagy activated in response to prolonged MI

LC3, a mammalian homolog of yeast Atg8 (autophagy-related gene 8) is an excellent marker for the detection of autophagosomes [22]. LC3 has two types, namely LC3-I (cystolic form) and LC3-II (processed form located on the autophagosomal membrane). The ratio of LC3-II/LC3-I is correlated with the extent of autophagosome formation [23]. Immunoblot provided a quantitative figures indicating that the expression of LC3 was significantly increased in MI groups and climbed steadily with ischemia prolongation compared to controls, further supporting the presence of autophagy during ischemia and showing a time-dependent manner in MI

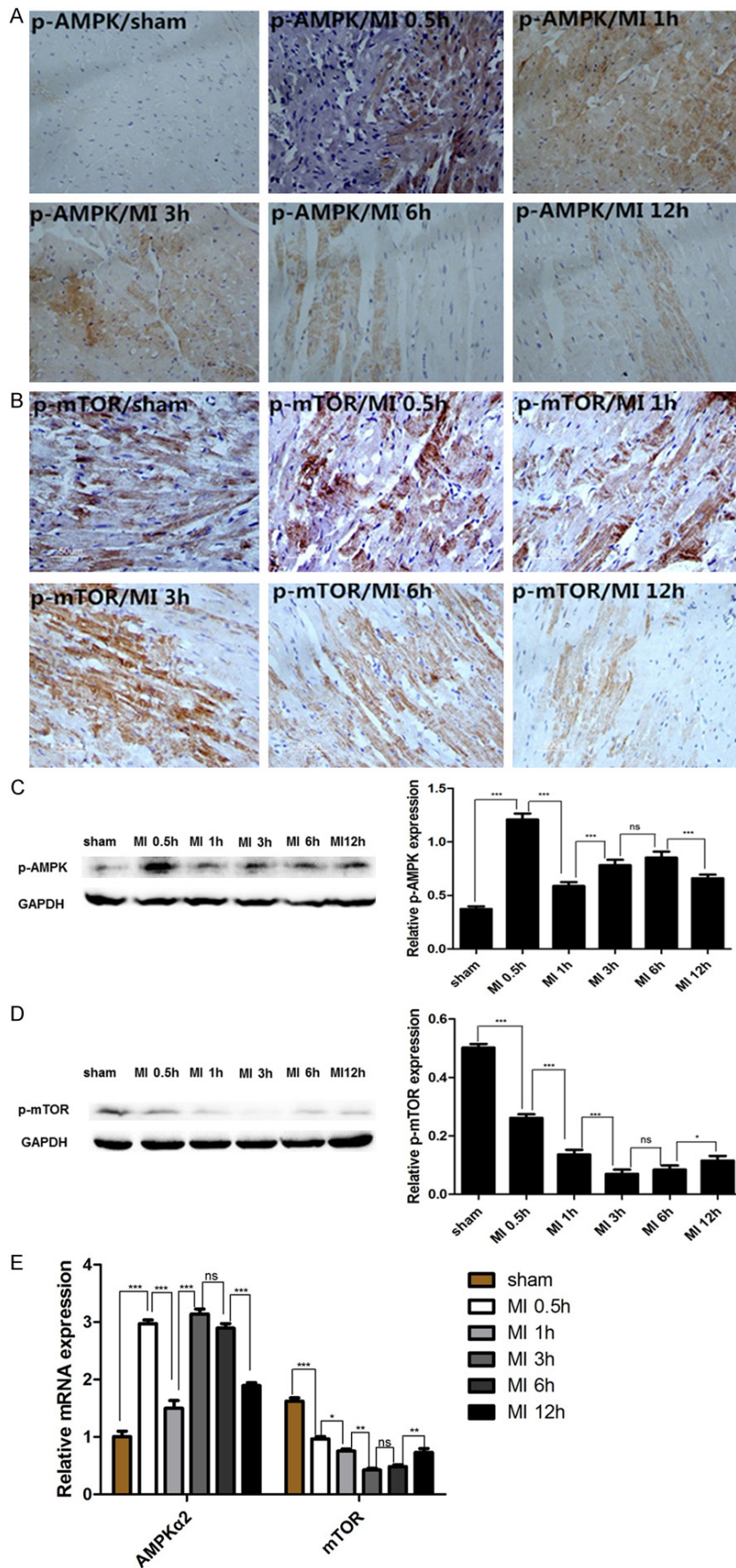


Figure 3. The AMPK-mTOR signaling pathway was activated in response to MI. A and B (SP×400) provided the representative immunohistochemical images of p-AMPK and p-mTOR expressions, p-AMPK expression showed an increasing trend while the p-mTOR expression was reverse. C and D showed the immunoblotting results that confirmed the results above as well as E, on the level of mRNA translation. The sample size was $n = 6$ for each group, $*p < 0.05$, $**p < 0.01$, vs the former group.

(Figure 2A). Likewise, the relative LC3 mRNA expression was also proved to support WB results (Figure 2B).

The role of AMPK-MTOR signaling pathway in regulating autophagy during MI

The expressions of p-AMPK and p-mTOR were detected by the IHC and WB. The IHC results showed that there was barely expression of p-AMPK at sham groups throughout the allotted timeframe while its expression started to increase at 0.5 h after MI and peaked at 1 h, with a slight fall at 6 h and 12 h as shown in Figure 3A. Similarly, the expression of p-mTOR, a negative regulator of autophagy, was reverse. It was expressed in control groups but was significantly down-regulated in MI groups as shown in Figure 3B. The opposite direction of these two molecules proved the opposite roles during autophagy.

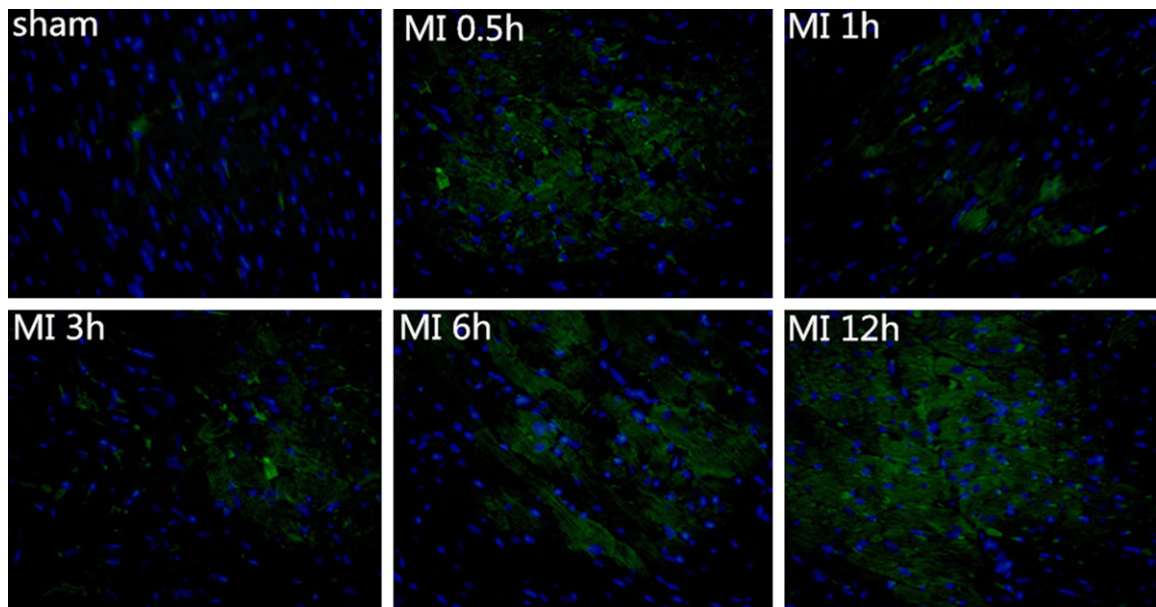


Figure 4. (TUNEL×400). Effect of autophagic regulation on cell apoptosis during MI. TUNEL staining supplied a visualized result of cell apoptosis. It was apparent from the merged immunofluorescence images that apoptosis were induced during MI. It was found that, full blown apoptosis process with highest activity occurred at MI 12 h groups. This fact made us realize that autophagy preceded apoptosis or we can say that autophagy activation was for cytoprotection, the protraction of which lead to cellular death due to apoptosis.

agy and reinforced the fact that AMPK negatively regulates mTOR. p-AMPK and p-mTOR were localized in the cytoplasm, as seen on staining. Furthermore, the evidence of time dependent rise of p-AMPK was also seen on immunoblotting of heart tissue homogenate, which was seen in **Figure 3C**. The p-mTOR expression during tissue ischemia also exhibited the same pattern with IHC that was seen previously in our experiment, which were visualised in **Figure 3D**. These observations of time dependent rise and fall of both p-AMPK and p-mTOR respectively were also seen with Q-PCR and WB, demonstrating the time dependent influence of ischemia at the genetic levels and mRNA expressions of p-AMPK and p-mTOR which has been demonstrated in **Figure 3E**.

The effect of autophagy enhancement on cell apoptosis during MI

To investigate the effect of autophagy activation on MI-induced cell apoptosis, we used TUNEL labeling to detect cell apoptosis in rat hearts. Positive cells were hardly detected in the sham groups before up-regulating significantly from 0.5 h to the end after MI. The number of positive cells peaked at 12 h whereas it decreased at 1 h compared to other MI groups,

it indicated that apoptosis was inhibited by autophagy at the early stage of MI, however, with extension of ischemic time, autophagy seemed to induce apoptosis and be detrimental to ischemic myocardium (**Figure 4**).

Discussion

Autophagy is a stress response mechanism that initially tries to maintain nutritional/energy homeostasis by fueling in organelles [2-4, 8, 24]. Although this process does generate ATP and helps in normal cellular functioning, protracted and prolonged autophagy, as seen with myocardial ischemia, can also lead to mitophagy, and degradation of critical organelles like ER, nucleus, cell membranes, leading to the generation of reactive oxygen species, uncontrolled CA^{2+} production and shifting to acidotic environment. All these events cause cellular death, ultimately. Thus, the effects of protracted autophagy can be seen as a synergistic action to death autophagy which is undesired and unwanted. Moreover, researches have demonstrated that ischemic cells receive further stress with the onset of reperfusion which starts a complex cascade of intracellular reactions, that further gives rise to another form of injury called as reperfusion injury. So, we can

see a checkpoint where protective autophagy stops and degradative autophagy starts in a cardiac cell exposed to ischemia. It has been a known fact that autophagy emerges as a powerful intracellular catalytic process mechanism in cardiomyocytes exposure to ischemia. Although previous studies have demonstrated the protective effects of autophagy during early stages of myocardium ischemia [25], the timings of this exact switch between the two effects and the standpoint of apoptosis in ischemia have not been explored in a rat model.

Our study has been able to demonstrate that AMPK-mTOR signaling pathway is involved in the regulation of autophagy which has a positive correlation with apoptosis in the initial stages and an inverse relation during the later stages of myocardial ischemia. All of the observations were compared with the sham groups. It has further established that during the initial 30 mins of ischemia exposure, p-AMPK and apoptosis increase while within 1 hour the autophagy as well as apoptosis decreases. That is during the first hour both autophagy and apoptosis follow the same path of activation and inactivation respectively. Activation of autophagy in these hours with reference to previous findings is for cellular survival and supercedes the actions of apoptosis. Similarly, by the time ischemia reaches 6 hours, we witnessed an inverse relationship between these two processes. Once again AMPK was activated while the level of apoptosis declined. The activation of autophagy during these hours were not for cellular survival but for cellular death as evidenced by the rising trend of Beclin 1. Similarly with further advancement of ischemia, apoptosis was seen to take over and cause cellular death that logically correlated with a decline of autophagy activity in almost dead cells. Simultaneously, it was also observed that with the increase of p-AMPK levels, mTOR levels were absent in MI groups which was in accordance with the findings of professor Matsui [7].

The severity of pathological changes such as nuclear aggregation and cytoplasmic dissolution were positive related to ischemic time. However, the excellent autophagic marker, LC3 was up-regulated at MI groups and showed an increasing trend with time. As ischemic time prolonging, the level of apoptosis increased again and showed a positive relation to autophagy,

this result may suggest that apoptosis is down-regulated by autophagy activation at the early stage of MI, however, the inverse reaction happened while the ischemic time prolonging. Autophagy plays a protective role in maintaining myocardial function and preventing cell death during MI.

Conclusion

As evident from above results, the intracellular machinery tries to maintain the integrity of the cell up to 6 hours mediated by AMPK, after which autophagy cannot be protective and cell death is evident by apoptosis. Thus this 6 hours time period can be taken as a window period and our molecular target would be promoting AMPK activation. It also can be said that the sooner the better care of the cells. Similarly, as apoptosis gets its full throttle after the 6 hours period, cells that are exposed to ischemia could be protecting by deploying anti-apoptotic molecules within this time frame.

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Disclosure of conflict of interest

None.

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