European Review for Medical and Pharmacological Sciences 2019; 23: 4406-4413

The expression of overexpressed PTEN enhanced IR-induced apoptosis of myocardial cells

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Abstract. – OBJECTIVE: Myocardial cell apoptosis is an important pathologic basis of ischemia-reperfusion injury (I/R). PI3K/Akt signaling pathway involves in cell growth, survival, and apoptosis regulation, thus playing an important role in the protection of I/R injury. PTEN is a negative regulatory factor of PI3K/Akt signaling pathway. This study established rat I/R injury model after AMI and myocardial cell I/R injury model to explore the regulatory role of PTEN-PI3K/Akt signaling pathway in myocardial I/R injury *in vivo* and *in vitro*.

MATERIALS AND METHODS: Rat myocardial I/R injury model was established. PTEN and p-Akt expressions in myocardial tissue were compared. H9C2 cells were incubated in I/R condition for 12 h, followed by reoxygenation for 12 h. H9C2 cells were divided into three groups, including I/R+pSicoR-Blank, I/R+pSicoR-PTEN, and I/R+pSicoR-PTEN+VO-Ohpic. PTEN, Bcl-2, and Bax expressions were detected. Cell apoptosis was measured by flow cytometry.

RESULTS: PTEN expression significantly increased, while p-Akt level markedly declined in myocardial tissue in I/R group compared with Sham group. Temporary PTEN down-regulation and p-Akt elevation appeared in early I/R. I/R treatment markedly enhanced PTEN and Bax expressions, increased cell apoptosis and reduced p-Akt and Bcl-2 levels. PTEN overexpression significantly enhanced PTEN expression and cell apoptosis, while declined p-Akt and Bcl-2 in H9C2 after I/R. PTEN inhibited by VO-Ohpic markedly downregulated p-Akt and Bcl-2 expressions, whereas reduced Bax level and cell apoptosis.

CONCLUSIONS: PTEN overexpression activated myocardial cell apoptosis after I/R. The overexpressed PTEN enhanced PI3K/Akt signaling pathway and attenuated cell apoptosis induced by I/R.

Key Words:
PTEN, PI3K/Akt, Myocardial cell, Apoptosis, I/R.

Introduction

Acute myocardial infarction (AMI) refers to myocardial necrosis caused by coronary artery acute and persistent ischemia and hypoxia. Coronary artery reperfusion therapy is the most effective way to salvage ischemic myocardium, protect heart function, and prolong patient life[1]. However, re-supply of ischemic myocardium inevitably brings ischemia-reperfusion injury (I/R), which becomes the main restrictive factor affecting the AMI treatment effect. It was showed that myocardial apoptosis is the major pathophysiological process of I/R injury that almost runs throughout the whole process of I/R injury. It is also the main factor of myocardial cell injury[2]. Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB) is an important signaling pathway that participates in cell growth, proliferation, survival, and apoptosis. It can regulate glycogen synthesis and glucose conversion, and is closely related to cell proliferation and apoptosis reduction, thus playing a critical role in the protection of myocardial injury caused by I/R[3]. PI3K can be activated by multiple extracellular factors through the effect of receptor kinase. Activated PI3K can promote PIP2 translating into PIP3, which directly activates the downstream Akt protein. Akt can transmit the signal into the nucleus to promote multiple genes transcription and expression, including factors related to cell proliferation, cycle, and apoptosis. For instance, B-cell lymphoma 2 (Bcl-2) is an important target molecule of the Akt pathway[4].

Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is the only tumor suppressor gene found so far that has the dual activity of protein esterase and phosphatase. It can make the PIP3 dephosphorylate into PIP2, thus nega-

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tively feedback regulating PI3K/Akt signaling pathway. Therefore, it plays a regulatory role on cell survival, proliferation, and apoptosis[5]. Researches[6-8] revealed that PTEN expression and dysfunction was associated with multiple pathological processes of cardiovascular disease, including myocardial remodeling, cardiac hypertrophy, and myocardial fibrosis. Other studies demonstrated that PTEN plays a critical role in I/R induced brain[9], liver[10], and kidney[11] injury. Through the establishment of I/R injury after AMI in rats model and *in vitro* myocardial cell I/R injury model, we discussed PTEN PI3K/Akt signaling pathways regulating role in myocardial I/R injury. We established rat I/R injury model after AMI and myocardial cell I/R injury model to explore the regulatory role of PTEN-PI3K/Akt signaling pathway in myocardial I/R injury *in vivo* and *in vitro*.

Materials and Methods

Main Reagents and Materials

Healthy male Wistar rats (6-8 week and 22 g) were purchased from Tongji Medical C (Hubei, China). Rat myocardial cell line H was got from Beinuobio (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) medium was bought from Gibco (Th Fisher Scientific, Waltham, MA, USA). F e serum (FBS) was obtained from G i B ucts (West Sacramento, CA, US TriPu Isolation Reagent was provide Basel, Switzerland). QuantiTect S BR C PCR Kit was purchased from C n (Hilde ny). PCR primers were hetized by Ge y (Shanghai, China). Ra TEN and p-Akt primary antibodies w got cam (Cambridge, MA, USA). Mouse anti- x, and β-actin primary bodies were ob ed from R&D systems nnea lis, MN, USA). Annexin V-FITC ell a osis detection kit was bought from jing, a). Horseradish peroxidase (eled ndary antibody was provided by S otechnology Co., Ltd (Na hina). T cell apoptosis detectio ot from Beyotime (Jiangsu, China). EN in hpic was bought from M mouth Junction, NJ, USA). H5α competent cell was obtained from Gen Shanghai, China). pSicoR-GFP vector was pu d from Addgene (Edinburgh, UK). Hpa I and Xho I endonuclease were got from New England Biolabs (Ips SA). Lipofectamine 2000 was bou rom I n (Carlsbad, CA, USA).

Ethics Statement

Rats were used fo ll expe all procedures were ap ed by the Eth- ics Committee of jing ildren's ospital, Capital Medical ersit ationa Center for Children's Heal China d the experimental pr edu per ed in strict accordance Legisla ding the Use and Care o oratory Anim f China. Before the exper animals were allowed to suit the new vir for 7 days, and housed in a room under 12 h k cycle, a controlled tem ure at 22± 3 d a relative humidity at 10%.

I/R Modelin

rats wer esthetized by pentobarbital so neal injection. The limbs were conne e electrocardiogram and the endotracheal intubation was connected to the animal breathing machine. The chest was opened 4th intercostal space, and the left anterior descending coronary artery was identified ween pulmonary arterial cone and aorta. The ood supply was restored after blocking for 60 n. Sham group was selected as control.

TUNEL Assay

The rats were killed at postoperative 6 h, 12 h, and 24 h. The myocardium tissue was collected to prepare frozen section. The section was incubated using TUNEL apoptosis detection kit and observed under a fluorescence microscope.

PTEN Overexpression Plasmid Construction

The cDNA of H9C2 myocardial cells was used as the template to amplify the CDS region of PTEN gene. After dual-enzyme digested by Hpa I and Xho I, it was connected to pSicoR plasmid vector at 4:1. After screened by Amp medium, the single colony was picked. The plasmid DNA was extracted and identified by sequencing. The plasmid with correct sequence was amplified and collected.

H9C2 Cell Transfection

Rat myocardial cell line H9C2 was routinely cultivated in DMEM containing 10% FBS and maintained at 37°C and 5% CO_2. The cells in log-

arithmic phase were changed to DMEM without FBS. PTEN overexpression vector pSicoR-PTEN or empty vector pSicoR-Blank was transfected to H9C2 cells using Lipofectamine 2000. After 6 h of incubation, the cells were further changed to DMEM medium containing 10% FBS and collected after 72 h.

I/R Treatment

To stimulate I/R condition *in vivo*, H9C2 cells were treated by I/R. The cells were cultured in low glucose serum-free DMEM to simulate ischemic condition. Next, the cells were maintained in incubator with 5% CO_2 and 95% N_2 to simulate hypoxic condition. The cells were changed to routine medium after 12 h and further cultured in normal condition for 12 h.

H9C2 Cell Grouping

H9C2 cells were divided into three groups, including I/R+pSicoR-Blank, I/R+pSicoR-PTEN, and I/R+pSicoR-PTEN +VO-Ohpic. VO-Ohpic was used to treat cells after transfection at 60 nmol/L.

Flow Cytometry

The cells were digested by enzyme and w[illegible] by precooled PBS. Then, the cells were suspe[illegible]ed by 500 µL binding buffer and incubated in [illegible] µL Annexin V-FITC avoid of light [illegible]om temperature for 15 min. Next, the c[illegible]tained by 5 µL PI and tested on flow c[illegible]metry.

qRT-PCR

QuantiTect SYBR Gree[illegible]RT-P[illegible]as used for qRT-PCR detection. [illegible] primers u[illegible]as follows. The total rea[illegible] system conta[illegible] 10 µL 2×QuantiTect SY[illegible]RT-PCR Master Mix, 1.0 µL positive and rev[illegible]1 µL template RNA, and ddH_2O. [illegible]reactio[illegible]per-formed at 95°C for 15 mi[illegible]lowed by 4[illegible]les of 94°C for 15 s, 60°C f[illegible]and 72°C [illegible]0 s on Applied Biosystems 7[illegible].

Western Blot

Total protein w[illegible]extra[illegible]l and sep[illegible]rated by Sodium Dodec[illegible]ulpha[illegible]PolyA[illegible]ylamide Gel Electrophoresi[illegible]E). T[illegible], the protein was tra[illegible]ferre[illegible]vin[illegible]ene difluoride (PVDF) m[illegible]rane and [illegible]y 5% skim milk at room t[illegible]erature for 60[illegible]. Next, the membrane w[illegible]ted in primary antibody (PTEN, p-Akt[illegible]cl-2,[illegible]d β-actin at 1:200, 1:200, 1:300, 1:300, and [illegible]spectively) at 4°C overni[illegible]nd secondary [illegible]body at 1:5000 at room [illegible]perature for 60 min. At last, the membrane [illegible]s developed by ECL and scanned.

Statistical Analysis

[illegible]yses were performed on SPSS 18.0 [illegible](SPSS Inc., Chicago, IL, USA). The measurement data were presented as mean ± standard deviation and compared by *t*-test. $p < 0.05$ [illegible]dered as statistical significance.

Results

PTEN Level Upregulated and Myocardial Cell Apoptosis Enhanced After I/R Treatment

TUNEL assay showed that cell apoptotic rate significantly increased treated by I/R for 1 h, 2 h, 6 h, and 12 h compared with Sham group (Figure 1A), suggesting that I/R markedly induced myocardial cell apoptosis. qRT-PCR demonstrat-

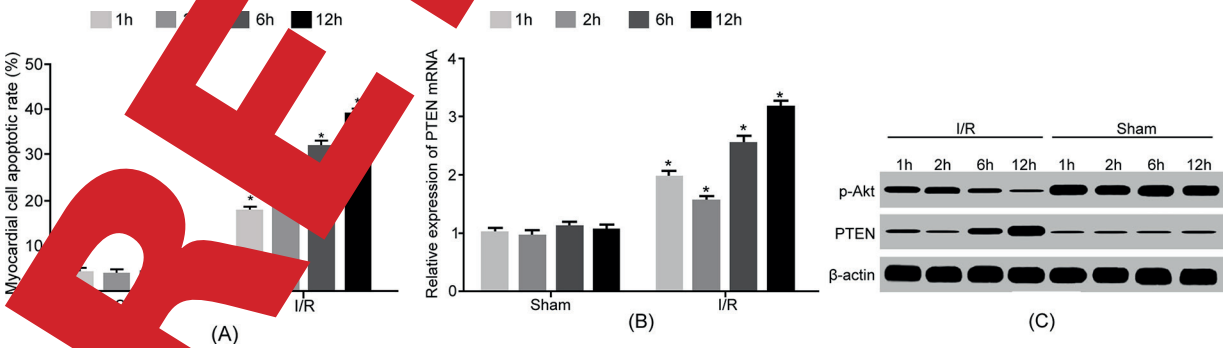

Figure 1. [illegible]upregulated and myocardial cell apoptosis enhanced after I/R treatment. ***A,*** TUNEL detection of cell apoptosis. ***B,*** q[illegible]R detection of PTEN mRNA expression. ***C,*** Western blot detection of PTEN and p-Akt protein levels. * $p < 0.05$ *vs.* Sham g[illegible]oup.

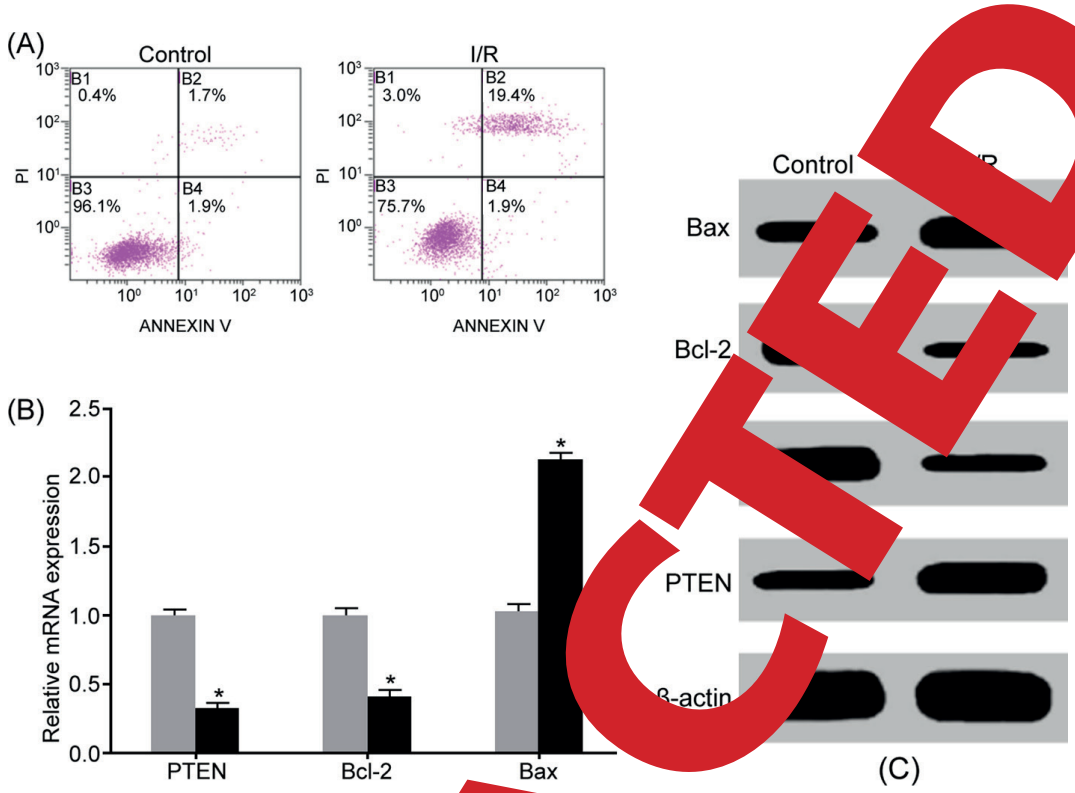

Figure 2. I/R treatment enhanced PTEN, weakened ... cell apoptosis. ***A,*** Flow cytometry detection of cell apoptosis. ***B,*** qRT-PCR detection of mRNA exp... ection of protein level. * $p < 0.05$ *vs.* control.

ed that compared with 1 h after su... PTEN mRNA level in myocardium at po... e 2 h markedly declined. It then gradu... upre... and reached peak at 12 h (Fig... 1B). blot revealed that, compared ... erative 1 h, PTEN protein expres...on i... dium at postoperative 2 h signi...tly redu... it gradually elevated ar...ched peak at... h (Figure 1C). Western b... that Akt phosphorylation did not c...ge i... dium tissue from Sham group, but was high... that in I/R group. P-Akt ...ression slightly i...eased in myocardium tis... from I/R group. It markedly declined at 6 h ... reac... minimum at 12 h.

I/R Treatmen... ced ...N, Weakened Akt ... d Induced Cell Apopt...

Ou... su... showed that PTEN expression and ... apop... at myocardium tissue re... p... ctivity declined at 12 h after ... ent. Thus, H9C2 cells were treated by I/R ... *in vitro* to detect the related indicators. Flow ... metry revealed that compared with control, H9C2 cell apoptosis remarkably ...anced after I/R treatment (Figure 2A). qRT-... R demonstrated that I/R treatment significantly upregulated PTEN and Bax mRNA expression, while decreased Bcl-2 mRNA level in H9C2 cells (Figure 2B). Western blot showed that PTEN protein apparently elevated, Akt phosphorylation activity declined, Bcl-2 reduced, and Bax upregulated in H9C2 cells treated by I/R (Figure 2C).

PTEN Overexpression Promoted Cell Apoptosis Induced by I/R

Flow cytometry revealed that GFP was highly expressed in H9C2 cells transfected by pSicoR-Blank or pSicoR-PTEN plasmids, while no green fluorescence was detected in untransfected cells, indicating high transfection efficacy (Figure 3A). Compared with H9C2 cells transfected with pSicoR-Blank, PTEN protein expression significantly enhanced in H9C2 cells transfected by pSicoR-PTEN (Figure 3B). Compared with empty plasmid group, p-Akt and Bcl-2 markedly downregulated, Bax elevated (Figure 3C), and cell apoptosis (Figure 3D) increased in H9C2 cells transfected with PTEN overexpression. VO-Ohpic inhibition of PTEN activity significantly

increased p-Akt and Bcl-2 expression, reduced Bax level (Figure 3C), and declined cell apoptosis (Figure 3D).

Discussion

Blood reperfusion of ischemic myocardium after AMI inevitably brings I/R injury. The myocardial cell apoptosis degree can reflect the severity of I/R injury at a certain extent. Therefore, alleviation of myocardial cell apoptosis in the process of I/R injury at the same time of blood reperfusion is of great significance to improve the treatment efficacy and prognosis.

PI3K/Akt is a signaling pat exists in multiple tissues and cells con of PI3K could be changed a ctivated u the stimulus of growth facto gen, and ot ac-tors, thus facilitating PI ing in P3. The later can phosph ylate A er473 and Thr308 assist by 3-phosp ide-de-pendent protein ase-1 DK1) a PDK2. Phosphorylated can ulate arious target genes transcri anslat coordinated by multiple ignal ion ors, leading to the regulat f cell cy l, proliferation, and apop A variety o es demonstrated that act PI3K/Akt signaling pathway has the pr tiv n multiple tissues and cells

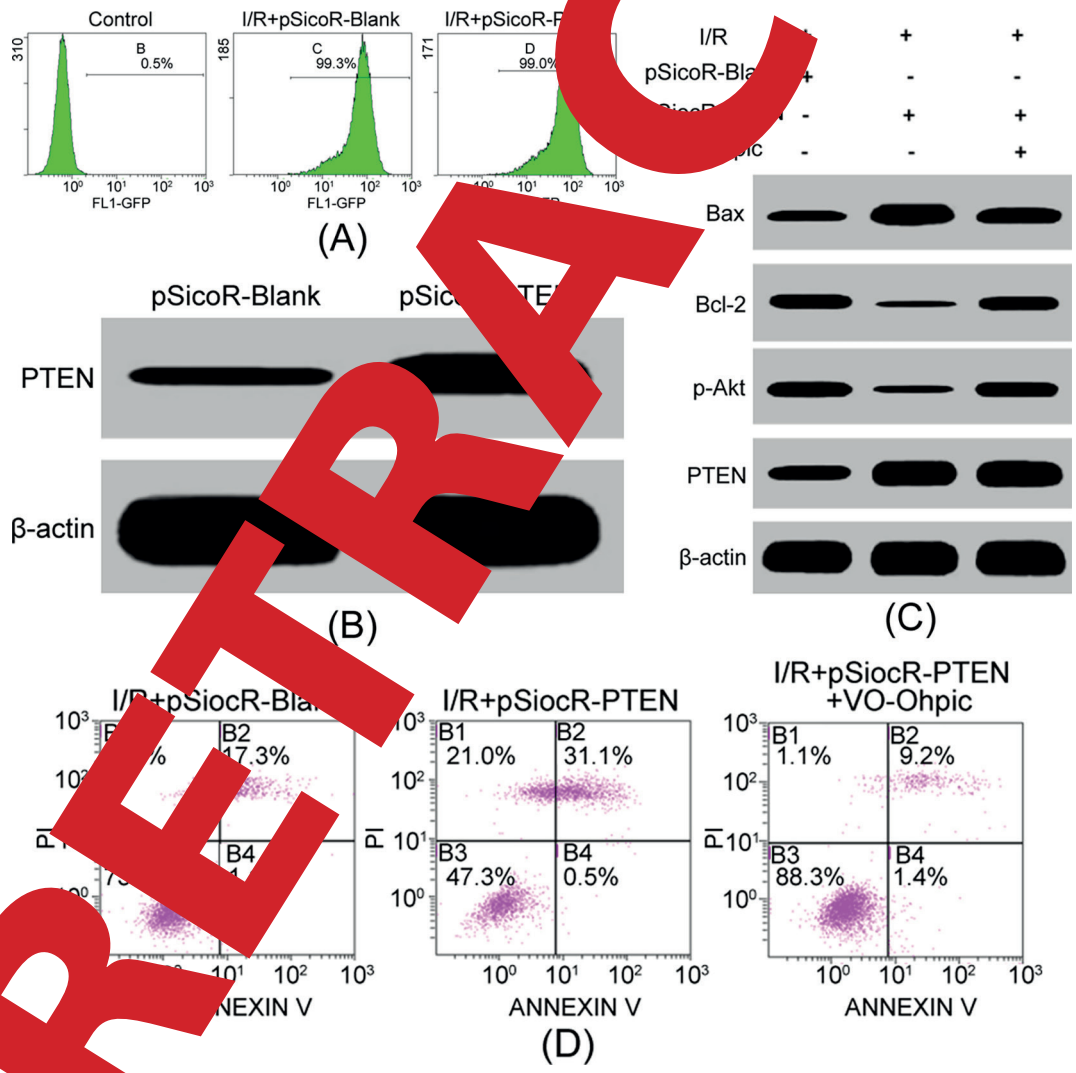

Figure 3. expression promoted cell apoptosis induced by I/R. ***A,*** Flow cytometry detection of GFP expression. ***B,*** Western blo tion of PTEN protein expression. ***C,*** Western blot detection of protein expression. ***D,*** Flow cytometry detection of cell ap ptosis.

I/R injury, such as heart[12], brain[13], lung[14], and kidney[15].

Bcl-2 is an important anti-apoptosis factor by regulating mitochondrial pathway dependent apoptosis signaling pathway[16]. Bcl-2 locates in the nuclear membrane, endoplasmic reticulum membrane, and mitochondrial membrane. It plays an anti-apoptosis function through multiple mechanisms, such as inhibiting cytochrome C release to cytoplasm, blocking the destruction of oxygen radical on cell component, affecting the transmembrane transport of calcium ion, protecting from the damage of DNA clastogen, forming heterodimer with proapoptotic protein in Bcl-2 family to maintain the intracellular location and distribution of proapoptotic protein, and blocking the space displacement and activation of apoptotic protease activating factor-1 (Apaf-1)[17]. Bax is a pro-apoptotic protein belonging to Bcl-2 family that receives most investigation. It locates in the cytoplasm and shifts to mitochondrial membrane when stimulated by cell apoptosis signal, thus forming Bax/Bax homologous dimer to play a promoting role in apoptosis[18]. Bcl-2 elevation may lead to the formation of Bcl-2/Bcl-2 homolo dimers or Bcl-2/Bax heterologous dimer, b which play an inhibitory role to cell apopt Reduction of Bcl-2 and upregulation of Bax le the Bax/Bax homologous dimer at dominance. It further plays proapoptotic function chang- ing the intracellular redox state the mitochondrial permeability, pr ing release, forming channels on t urfac tochondria, damaging the inte chon- drial membrane, and promo g C ase[19]. Several studies[20,21] have nd that signaling pathway can a te cell apopt n I/R injury through im ratio of Bcl-2/ Bax. PTEN gene lo in hromosome 10q23.3 with the transcription pr 515 kb. PTEN can make 3 dephosphory on, thus antagonising th hosp rylation of PIP2 by PI3K and prev ng P formation and activation of Akt a m sig ng pathways. This study esta t I/R ury model after AMI and myocardi jury model to explore th tory rol EN-PI3K/Akt signaling n myocardial I/R injury *in vivo* and *tro*.

sh EN expression and cell apopt n rat myocardium tissue from I/R group we cantly higher than that from Sham group, Akt phosphorylation was significantly lower. It suggested that Akt phosphorylation is of great significance h of survival of myocardial cells. eleva y reduce the activity of PI3K t signaling way, thus inducing myoca ll apoptosis r I/R. A previous study[22] foun ocardi ell apoptosis increased, while PT gu- lated in myocardium ue treated b which was in accordance n our ta. I/R t atment has been reporte o m dly d wnregulate p-Akt expressio tissu addition, Akt phospho latio nifi tly reduced in rabbit m rdium ted by I/R[23]. In this stu -Akt expres was suppressed in rat m tissue treated by I/R, which was sim w us studies[14,23]. In the early sta after surge PTEN level exhibited orarily reduct while p-Akt showed te rarily elevation in myocardium tissue in I roup. PTEN nd p-Akt further upregulated educed in th te stage of I/R (6 h, 12 h), r tively. A ous research[22] reported that P declined in the early stage after treatment. It can protect myocardium through activating PI3K/Akt to inhibit oxi e stress caused by I/R. Moreover, the brain ke PTEN temporarily in dephosphor- y g state (1 h after I/R) as the irritable inher- protection mechanism against I/R injury[24]. e observed that PTEN level exhibited tempo- ily reduction, while p-Akt showed temporari- levation in myocardium tissue in I/R group, which agree with previous studies[22,24]. Myocyte ischemia has been demonstrated to reduce PTEN level and increase p-Akt expression temporarily[25]. As time extended, however, PTEN enhanced to suppress p-Akt expression and promote myocardial cell apoptosis, which was in accordance with our results. *In vitro* I/R cell model showed that PTEN expression was upregulated in H9C2 cells, leading to PI3K/Akt activity reduction, the ratio of Bax/Bcl-2 elevation, and cell apoptosis. Consistently, PTEN level was markedly declined, while cell apoptosis markedly enhanced in H9C2 cells treated by I/R[26]. Further detection demonstrated that PTEN overexpression significantly reduced the activity of PI3K/Akt, declined the ratio of Bcl-2/Bax, and enhanced apoptosis sensitivity to I/R induction. VO-Ohpic antagonized the effect of PTEN overexpression, enhanced the activity of PI3K/Akt signaling pathway, and upregulated the ratio of Bcl-2/Bax, leading to the alleviation of myocardial cell apoptosis. Consistent with this, Salidroside injection to I/R animal model can significantly upregulate the activity

of PI3K/Akt signaling pathway, increase Bcl-2 expression, reduce Bax level, and attenuate cell apoptosis[23]. Consistently, in this study, upregulation of Akt enhanced Bcl-2 level, downregulated Bax expression, and alleviated cell apoptosis. Enhancement of PI3K/Akt signaling pathway has been reported to elevate the ratio of Bcl-2/Bax and reduced neural cell apoptosis induced by I/R[27] and PI3K/Akt inhibitor LY294002 strengthened I/R induced myocardial cell apoptosis[28]. Ke et al[2] showed that miR-93 mimics transfection apparently downregulated PTEN expression and activated PI3K/Akt signaling pathway, thus reduced ROS production and cell apoptosis in H9C2 cells under I/R. In addition, miR-214 mimic declined PTEN expression, enhanced PI3K/Akt signaling pathway, and reduced apoptosis sensitivity of H9C2 induced by I/R[26]. Of note, compared with normal control, PTEN inactivated mice exhibited Bcl-2 upregulation and myocardial cell apoptosis reduction after I/R treatment[29]. We adopted a cell model to reveal the myocardial cell apoptosis induction effect damaged by I/R was enhanced by PTEN overexpression, thus provide theoretical basis to alleviate I/R injury after AMI in cl

Conclusions

We found that overexpression of PTEN aggr vated myocardial cell apoptosis aft /R. Block- age of PTEN enhanced PI3K/Ak g path- way and attenuated cell apopto duc I/R

Conflict of interest

The authors declare no confli interest.

Acknowledgment

This work was supported by with th und of The Pediatric Medical C inated Develop nter of Beijing Municipal A nistration (No. XTYB201819); Research on the a ation c nical characteristics of the Beijing Muni Scien d Tec nology Commission (Z171100001 Mun Education Commission Scien nolog an General Project (KM201910025010) dren's Hospital Affiliated to Medica , National Natural Science F f China (G 1814).

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