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Overexpression of DJ-1 expression protects cardiomyocyte apoptosis induced by ischemia reperfusion

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Abstract. – OBJECTIVE: Phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway plays an important role in regulating cell survival, apoptosis and oxidative stress (OS). Phosphatase and tensin homolog deleted on chromosome ten (PTEN) can negatively regulate PI3K/AKT signaling pathway. DJ-1 is also a key negative regulator of PTEN. DJ-1-PTEN/PI3K/AKT signaling pathway regulates ischemia reperfusion (I-R). This study investigated the role of DJ-1 in affecting myocardial I-R injury.

MATERIALS AND MATERIALS: The rat myocardial I-R injury model was establish[illegible] pression of DJ-1 and PTEN in myocardia[illegible] was detected. The reactive oxidative sp[illegible]s (ROS) content was detected using flow cyto[illegible]try. Caspase-3 activity, malondialdehyde (M[illegible] content, and superoxide dismu[illegible](SOD) a[illegible]tivities were determined by u[illegible]spectro[illegible]photometry. Rat cardiomyo[illegible]H9[illegible]ere cultured *in vitro* and divided i[illegible]contro[illegible]oup, I-R group, I-R+pIRES2-NC [illegible]and [illegible] DJ-1 group. Levels of D[illegible]-1[illegible][illegible]phos[illegible]ylated AKT (p-AKT)[illegible]e det[illegible]Cell apoptosis and ROS cont[illegible]ere eva[illegible]using flow cytometry.

RESULTS: [illegible]with sha[illegible]group, caspase-3 a[illegible]ity, l[illegible]ontent, and PTEN expressio[illegible]ere signifi[illegible]increased, while SOD act[illegible]and DJ-1 lev[illegible]re significantly red[illegible]in myocardial tis[illegible]e of I-R group ($p<0$[illegible] Comp[illegible]d with the control, I-R treatme[illegible]arked[illegible]nduced H9C2 cell apoptosis, decre[illegible]and p[illegible]expression, and enhanced [illegible]produc[illegible]and PTEN expression. [illegible]overe[illegible]si[illegible]pparently down-regulated P[illegible]expre[illegible]levated p-AKT level, and atte[illegible]ted apo[illegible]sis and ROS production in H9[illegible] ($p<0.05$).

CONCLUSIONS: Abnormal expression of DJ-1 plays a regulatory role in the process of myocardial I-R injury. Over-expression of DJ-1 can [illegible]e myocardial cell I-R damage sensitivity by [illegible]biting PTEN expression, enhancing the activity of PI3K/AKT signaling pathway, reducing ROS production, and alleviating apoptosis.

Key Words
DJ-1, PT[illegible]PI[illegible]T, Cardiomyoc[illegible]e, I-R.

Introduction

[illegible]cute myocar[illegible]infarction (AMI) usually oc[illegible]the basi[illegible]coronary atherosclerosis (AS) and [illegible]y various causes, such as overwork, [illegible]stress, overeating, smoking, and heavy drinking[1-3]. The coronary artery reperfusion [illegible]after AMI is the most effective method to [illegible]ischemic myocardium, protect the heart function, and save the patient's life[4,5]. However, blood recirculation inevitably causes ischemia reperfusion (I-R) injury in the infarcted myocardium and causes more severe damage[6,7]. Phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway is widely expressed in a variety of tissue cells and is involved in cell growth, survival, and apoptosis[8-10]. It was showed that the reduction of PI3K/AKT pathway activity is related to the IR damage of various cells, such as cardiomyocytes[11], hepatocytes[12], and brain neurons[13], while enhanced PI3K/AKT pathway plays a role in alleviating I-R injury, indicating that PI3K/AKT has a regulatory role in the process of cellular I-R injury. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) gene is the only tumor suppressor gene with the dual activity of protein esterase and phosphatase that can negatively regulate PI3K/AKT signaling pathway activity[14]. DJ-1 can increase the activity of PI3K/AKT signaling pathway by inhibiting the expression and function of PTEN to antagonize apoptosis, promote cell survival, and accelerate proliferation[15]. This study investigated the role of DJ-1 in affecting myocardial I-R injury via establishing rat myocardial I-R injury model and cardiomyocytes.

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Materials and Methods

Main Reagents and Materials

Healthy adult male Sprague Dawley (SD) rats (6 weeks, weight 250 ± 20 g) were purchased from Shanghai Slake Experimental Animal Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle' medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NJ, USA). Rabbit anti-rat DJ-1 and PTEN polyclonal antibodies were purchased from Abcam Biotech. (Cambridge, MA, USA). Rabbit anti-rat β-actin polyclonal antibody was purchased from GeneTex Inc. (Irvine, CA, USA). Collagenase II was purchased from Gibco (Grand Island, NJ, USA). PrimeScript™ RT reagent kit was purchased from TaKaRa (Dalian, China). Malondialdehyde (MDA), superoxide dismutase (SOD), cell apoptosis detection kit, DCFH-DA, and horseradish peroxidase (HRP) goat anti-rabbit secondary antibody were purchased from Beyotime Biotech. (Shanghai, China). 371 gas Nested CO_2 cell incubator was purchased from Thermo Fisher Scientific (Waltham, MA, USA). FC500MCL flow cytometry was purchased from Beckman Coulter Inc. (Brea, CA, USA). H9C2 cells were purchased from Shanghai Gefan Biological Cell Bank (Shanghai, China).

Rat Myocardial I-R Model Establishment

The rat was anesthetized by 10% chloral hydrate (Beyotime Biotech., Shanghai, China) by intraperitoneal injection. The chest was opened on the left 4th intercostal [illegible] anterior descending coronary artery was identified between pulmonary conus and aorta. Next, the artery was ligated by 6-0 suture. MI modeling success was judged by ST segment arch lift 0.1 mV on ECG lead or T wave highamplitude, myocardial cyanosis, and pulse weakened. The blood supply was restored after blocking for 60 min when the cardiac apex became red was considered successful reperfusion. The sham group was set as control. The rats were killed at 24 h and 48 h after modeling to test the related levels, respectively.

Caspase-3 Activity Detection

According to the instructions of the kit, the pNA standard product was diluted in concentration gradient to prepare standard products. The absorbance was measured at 405 nm to make a standard curve. The tissue was smashed to prepare homogenate. The homogenate was added with caspase lysis buffer on ice for 20 min and centrifuged at 12000 ×g and 4°C for 10-15 min. Next, the supernatant was taken to a new centrifuge tube and quantified by the bicinchoninic acid (BCA, Biyotime Biotech. Shanghai, China) kit. A total of 65 μl assay buffer, 25 μl lysate supernatant, and 10 μl Ac-DEVD-pNA (2 mM) were added to a 96-well plate and incubated for 2 h at 37°C. When the color change was detected, the plate was measured at 405 nm on a microplate reader (Awareness, Hamburg, USA). The relative enzyme activity was calculated based on A405 in the experimental group/A405 in the control group × 100%.

MDA and SOD Detection

Rat myocardial homogenate was prepared and quantified. MDA and SOD contents were tested by using the commercial MDA enzyme linked immunosorbent assay (ELISA) detecting kit and SOD ELISA detection kit (Beyotime Biotech., Shanghai, China).

H9C2 Cell Transfection and I-R Treatment

For I-R treatment, the cells were cultured in 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 cells in logarithmic phase were divided into four groups, including control group, I-R group, I-R+pIRES2-NC group, and I-R+pIRES2-DJ-1 group.

Flow Cytometry Detection of Cell Apoptosis

The cells were washed twice in phosphorylate buffered saline (PBS, Beyotime Biotech. Shanghai, China) and digested by 0.25% trypsin. After centrifuged at 300 ×g for 5 min, the cells were resuspended and added with 100 μl Binding Solution. After that the cells were added with 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) solution in sequence, and incubated avoiding light for 15 min. After supplementation with 400 μ binding solution, the cells were tested on a FC500MCL flow cytometer (Beckman Coulter Inc., Brea, CA, USA).

Flow Cytometry Detection of Reactive Oxidative Species (ROS)

ROS detection in rat myocardial tissue: the rat tissues were collected and cut into pieces. The

tissues were digested with 0.1% collagenase II (Beyotime Biotech. Shanghai, China) and hyaluronidase-containing digestive solution (Beyotime Biotech. Shanghai, China) for 45 min. After centrifugation at 250 ×g for 5 min, the cells were incubated in 0.1% DCFH-DA probe at 37°C avoiding light for 30 min. After washed twice in PBS and resuspended in 500 µl PBS, the tissues were tested on Beckman FC500MCL flow cytometer to measure ROS contents. *In vitro* H9c2 intracellular ROS assay: cells were washed twice in PBS and digested with 0.25% trypsin. After centrifuged at 300 ×g for 5 min, the cells were incubated in 0.1% DCFH-DA probe at 37°C for 30 min. After resuspension in 500 µl PBS, the cells were tested using a Beckman FC500MCL flow cytometer (Brea, CA, USA).

Quantitative Real-Time PCR (qRT-PCR)

PrimeScript™ RT reagent kit (TaKaRa, Dalian, China) was used to reverse transcribe RNA to complementary DNA (cDNA) for qPCR reaction. qPCR reaction system contained 5.0 µl 2×SYBR Green Mixture, 0.5 µl forward primer (5 µM), 0.5 µl reverse primer (5 µM), [illegible] cDNA, and ddH_2O. The reverse transcrib[illegible] tions were 50°C for 15 min and 85°C for [illegible]n. qPCR reaction conditions were pre-denatur[illegible] 95°C for 5 min, followed by 40 cycles of 95°C 15 s and 60°C for 1 min on the [illegible]ad CFX9[illegible] Real-Time PCR Detection Sy[illegible]ad Laboratories, Hercules, CA, US[illegible]he pr[illegible]rs were listed in Table I.

Western Blot

Cells and tiss[illegible]ere lysed [illegible]dioimmunoprecipitation [illegible]RIPA, Beyo[illegible]Biotech. Shanghai, Ch[illegible]40[illegible]oteins were separated by 10% sod[illegible]odecyl [illegible]te-polyAcrylamide gel elect[illegible]oresis (SDS-P[illegible] Beyotime Biotech. S[illegible]hai, China) gel an[illegible]% concentrated gel. [illegible], the [illegible]in was transferred to polyvinyl[illegible] diffl[illegible]ue (PVDF, Amersham Biosciences, Piscataway, NJ, USA) membrane at 300 mA for 90 min. Next, the membrane was blocked with 5% skim milk at room temperatu[illegible] min and incubated in primary an[illegible] 1, PTEN, AKT, p-AKT, and β-a[illegible]at 1:1000, 1:1000, 1:1000, 1:500, 1:5000, re[illegible]vely) at 4°C overnight. After that, the PVDF [illegible]ane was incubated with horseradish [illegible]oxide [illegible] conjugated goat anti-rabbit Ig[illegible]H+L) secon[illegible] tibody (1:10000, Santa C[illegible] Biotec[illegible] Santa [illegible] CA, USA) at room t[illegible]eratur[illegible] 60 min and finally detected by en[illegible]d [illegible]ilumi[illegible]ence (ECL) (Thermo [illegible]enti[illegible]ce, Ro[illegible]d, IL, USA).

Statistical Analysis

All [illegible] analyses [illegible] performed on SPSS 18[illegible]are (SPSS Inc[illegible]Chicago, IL, USA). [illegible]erences among groups were analyzed by using Tukey's pos[illegible] test to validate the one-way [illegible]ysis of vari[illegible] (ANOVA). Student's *t*-test w[illegible]ed to com[illegible]e the differences between two grou[illegible]s considered as statistically significant.

Results

Obvious Oxidative Stress Injury of Myocardial Tissue in I-R Rats

It was demonstrated that the MDA content in myocardial tissue of I-R model rats was markedly higher (Figure 1A), while SOD enzyme activity (Figure 1B) was apparently decreased than that in Sham group. Spectrophotometry revealed that the caspase-3 activity in the I-R rats was significantly enhanced compared with the Sham rats (Figure 1C). Flow cytometry showed that ROS content in myocardial tissue of I-R rats was significantly increased compared with Sham rats (Figure 1D, Table II).

[illegible] Primer[illegible]T-PCR assay.

G[illegible]		Primers
[illegible]1	Forwards	5'-ACCGCGCAGGAAAAACACGC-3'
	Reverse	5'-CTGCCAGACGGCTCTGCAC-3'
[illegible]N	Forwards	5'-GAGCGTGCAGATAATGACAAGGAAT-3'
	Reverse	5'-GGATTTGACGGCTCCTCTACTGTTT-3'
GAPDH	Forwards	5'-GGTATCGTGGAAGGACTCATGAC-3'
	Reverse	5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'

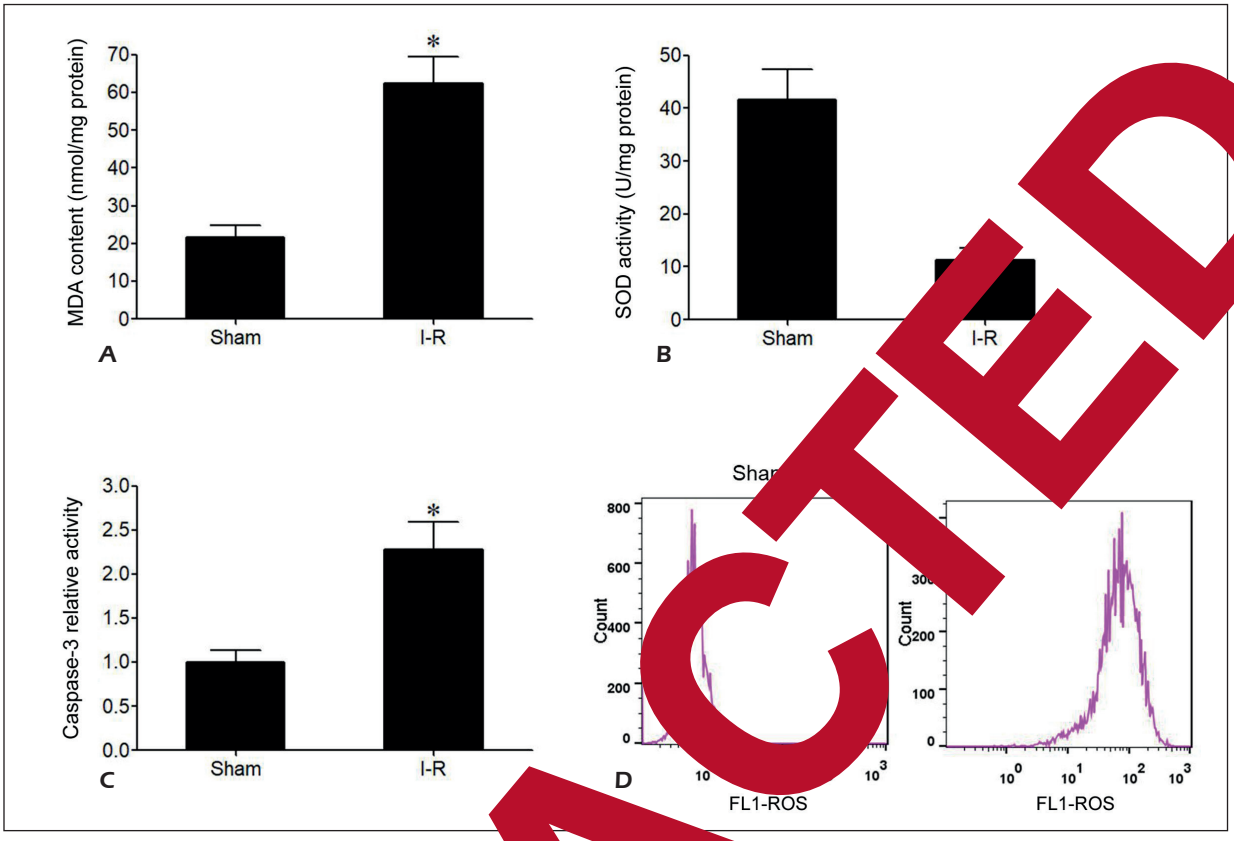

Figure 1. Significant oxidative stress injury of myoc tissu , Spectrophotometry detection of MDA content. **B**, Spectrophotometry detection of SOD activity. **C**, metry detection of caspase-3 activity. **D**, Flow cytometry detection of ROS content. *$p<0.05$, compared with cont

DJ-1 Decreased, While PTEN Elevated in the Rat Myoca m

qRT-PCR sho at the ex on of DJ-1 mRNA was si tly decrea whereas PTEN mRNA s cantly up-re ulated in myocardial tiss e of I-R than that of Sham group w ime depende Figure 2A, B). Weste ot revealed that th J-1 protein expres was m dly lower, while PTEN protei was arently igher in I-R group than that i oup (F 2C).

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qRT-PCR showed that compared with the con- group, the expression of DJ-1 mRNA was si antly declined in H9C2 cells treated by IR (gure 3A). Transfection of pIRES2-DJ-1 on the basis of I-R treatment apparently enhanced the expression of DJ-1 mRNA (Figure 3A). Western blot analysis exhibited that compared with the control group, I-R treatment significantly reduced the expression of DJ-1 protein, increased the expression of PTEN protein, and declined the expression of p-AKT protein in H9C2 cells. pIRES2-DJ-1 transfection significantly enhanced DJ-1 protein expression, reduced PTEN protein level, and up-regulated p-AKT protein expression (Figure 3B). pIRES2-DJ-1 transfection significantly reduced ROS production and cell apoptosis under I-R treatment (Figure 3C, D).

Table II. MFI of ROS in the myocardium.

Group	ROS MFI
Sham	0.86±0.11
I-R	71.53±6.69*

*$p<0.05$, compared with sham.

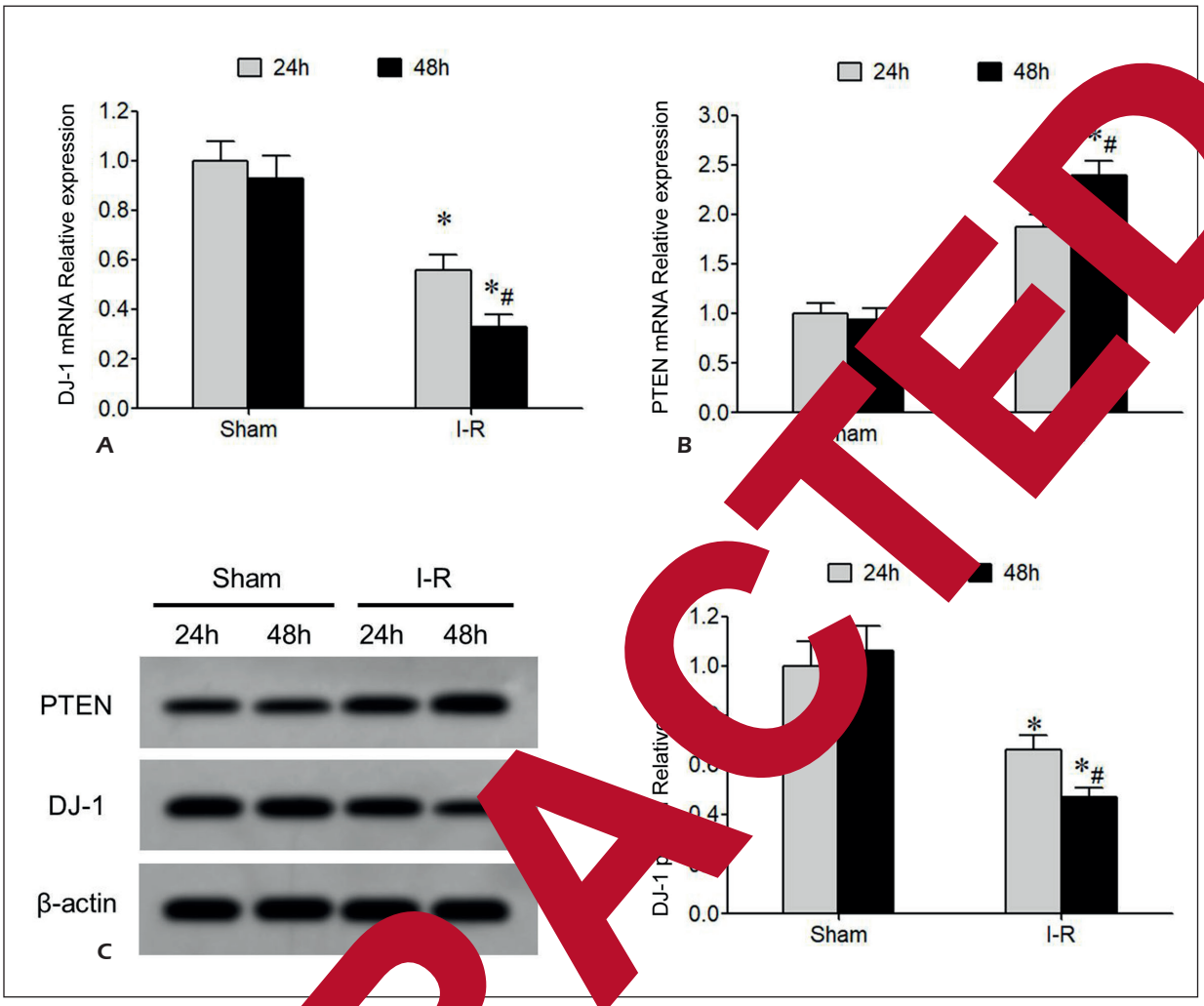
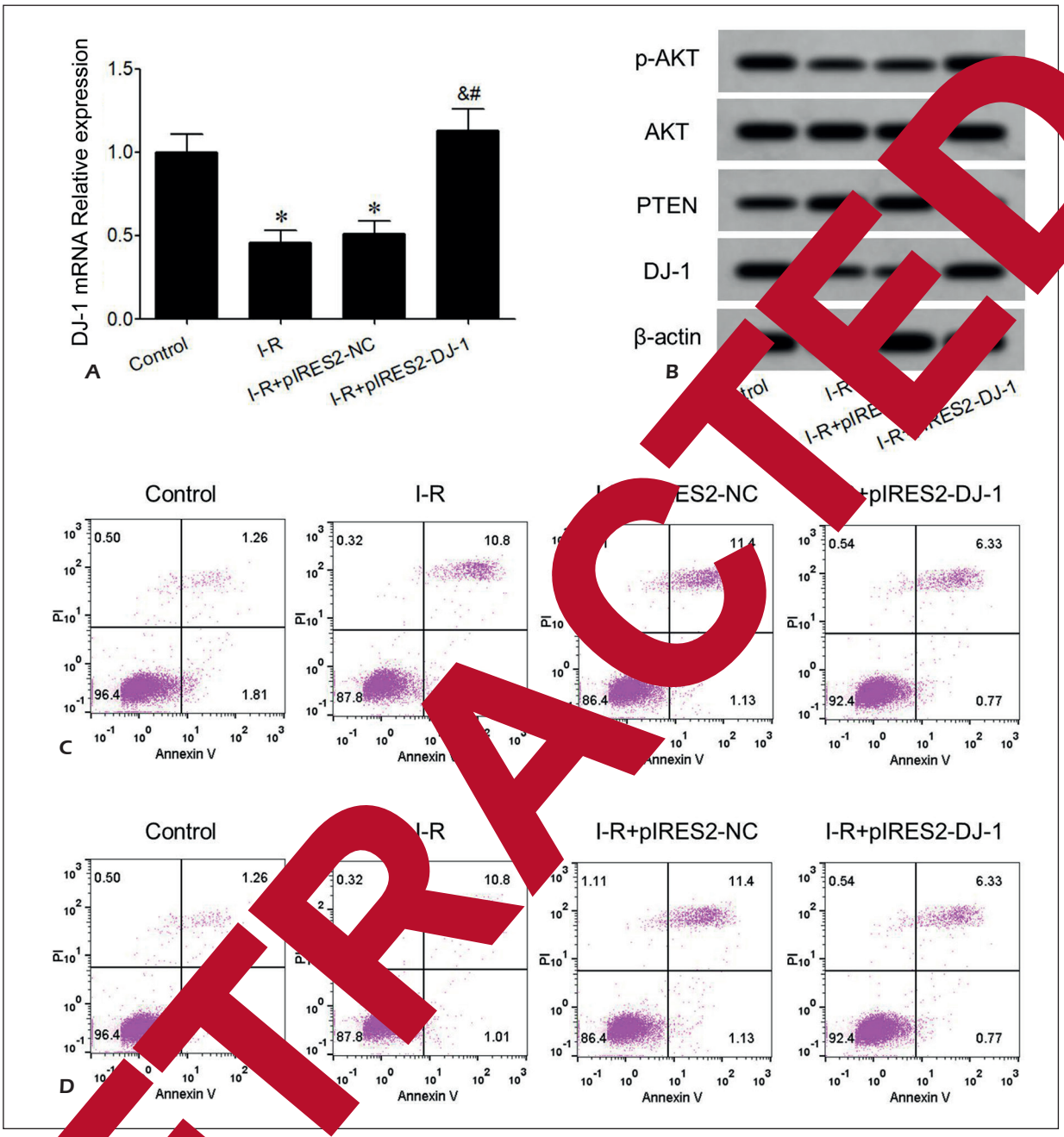


Figure 2. DJ-1 decreased, w[illegible]TEN el[illegible]myocardium. **A**, qRT-PCR detection of DJ-1 mRNA expression. **B**, qRT-PCR detection of P[illegible]EN [illegible]e[illegible]sion. [illegible]stern blot detection of protein expression. *p<0.05, compared with sham. #p<0.05, compared[illegible]24 h.

Di[illegible]ion

PI3K/[illegible] is a signaling [illegible]way that exists in a wi[illegible]nge of tissues and [illegible]s, and involves in th[illegible]gulati[illegible]f various biological processes, [illegible]as c[illegible]growth, proliferation and apoptosis[illegible]phatid[illegible]sitol-3 kinase (PI3K) [illegible]-con[illegible]ion [illegible]ge is activated under [illegible]mulat[illegible]owth factor, mitogen, and oth[illegible]ignaling [illegible]ecules. Activated PI3K can pro[illegible]phosphatidylinositol(4,5)-bisphosphate ([illegible]) [illegible]orylation to phosphatidylinositol-[illegible]3,4,5)-trisphosphate (PIP3) through its p110 [illegible]lytic subunit. PIP3 can act on the PH domain o[illegible]PKB to change conformation. Moreover, it can p[illegible]osphorylate Akt at Ser473 and Thr308 under the effect of phosphoinositide-dependent kinase 1 (PDK1) and phosphoinositide-dependent kinase 2 (PDK2). Phosphorylation-activated Akt regulates the transcription and translation of multiple target genes under the coordinated action of a variety of downstream signaling molecules, thereby achieving the regulation of biological effects, such as cell cycle, survival, proliferation, and apoptosis[18,19]. PTEN antagonizes the phosphorylation of phosphatidylinositol 3-kinase (PI3K) to PIP2 by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP3) to maintain low levels of PIP3 kinase, PI3K), thereby inhibiting PI3K activation of AKT signaling molecules and downstream pathways through PIP3 phosphorylation[20,21]. This study investigated the role of DJ-1 in regulating PTEN-PI3K/AKT signaling pathway and myocardial I-R injury by establishing rat myocardial



Figu Elevat 1 expression attenuated myocardial cell oxidative stress injury and apoptosis induced by IR. **A**, qRT-PC tion mRNA pression in H9C2 cells. **B**, Western blot detection of protein expression in H9C2 cells. **C**, Flow cytom n of RO ent in H9C2 cells. **D**, Flow cytometry detection of H9C2 cell apoptosis.

I-R try mod Our results demonstrated that co with the Sham group, the MDA con- ents, and caspase-3 activities were nificantly increased, while SOD enzyme activ- were significantly reduced in the myocardial t I-R model group, indicating that I-R treatment aggravates the oxidative stress and enhances apoptosis. It was showed that during the course of I-R injury, DJ-1 mRNA and protein expression were significantly reduced, while PTEN mRNA and protein levels were significantly elevated with time dependence, suggesting that expression of DJ-1 was abnormally decreased, resulting in increased expression of PTEN, which may play a role in I-R injury. Shimizu et al[22] revealed that DJ-1 knockout mice exhibited increased sensi-

tivity to I-R injury, increased myocardial infarct size, and reduced cardiac function compared to wild-type mice. Dongworth et al[23] reported that the cardiac I-R injury in DJ-1 knockout mice was significantly higher than that in wild type mice, and the myocardial infarct size was obviously enlarged. Shimizu et al[22] and Dongworth et al[23] showed that the decreased expression of DJ-1 is an unfavorable factor in the process of myocardial I-R injury and can aggravate myocardial I-R injury, which was similar to our results. To further investigate whether the decreased expression of DJ-1 is a promoting factor of myocardial I-R injury, we over-expressed DJ-1 in rat cardiac myocytes H9c2 *in vitro* to observe whether it is possible to alleviate I-R injury. It was observed that after transfection of pIRES2-DJ-1 over-expression plasmid in H9c2 cells to increase the expression of DJ-1, PTEN levels were significantly decreased, p-AKT activity was markedly enhanced, and apoptosis and ROS production were apparently reduced under IR condition. About the relationship between DJ-1 and myocardial injury, Zhang et al[24] found that resveratrol treatment can reduce the effects of apoptosis, ROS produ... and MDA content to alleviate I-R injur... myocardial cells. The injury and its funct... of reducing I-R damage are exerted by up-reg... ing the expression of DJ-1. Wang et al[25] exhibi... that pre-ischemic preconditioni... ignificant... up-regulated the expression ... myocardial tissue, reduced myoca... IR d... ge, and declined MDA, ROS, ap... sis, and ... rdial infarct size, while elevate... c... and glutathione pero... se (G... ivities. Lu et al[26] demonstrated ... hypoxic ... nditioning prior to ischem... rfusion cou... ffectively modulate the ... protective mechanism to activate ER...1/2 and ... gulate the expression of D... cardiomyoc... hereby reducing the nu... of I-R injured myoc... dial cells. Dongwort... al[23] s... sted that over-expression of DJ... nific... reduc... the apoptosis of HL-1 in mo... omyoc... under I-R conditions, ... ich o... ly re... d the sensitivity to I-R ... The ... dies indicated that elevated e... ession o... -1 is a protective factor in the pro... f myocardial I-R injury, and the over-ex... ... observed in this study can alle... e myocardial cell apoptosis and I-R injury. ...izu et al[22] observed that the enhanced sen... of I-R damage to cardiomyocytes caused by t... loss of DJ-1 expression was caused by abnormal small ubiquitin-like modifier (SUMO) modification, which was expressed as abnormal accumulation of SUMO-1 modified protein and decrease of SUMO-2/3 modified protei... et al[24] indicated that DJ-1 protects m... function and reduces ROS producti... decrease... cardiomyocyte apoptosis by bin... ND1 and NDUFS4. Our study revealed that ... egulates I-R injury to cardiomyocyte... y affe... TEN expression and PI3K/AKT ... naling path... tivity. However, wheth... ncreasi... the ex... sion of DJ-1 may pla... ole in ... viating myocardial I-R injury in ... qu... furthe... imal studies and large... de c... validati...

...clusions

We ...ved that ab... l expression of DJ-1 pl... gulatory role in t... process of myocardi... injury. Over-expression of DJ-1 can reduce ...cardial cell... damage sensitivity by inhib... PTEN expr... on, enhancing the activity of P... AKT path..., reducing ROS production, and ... optosis.

...t of Interests

...rs declare that they have no conflict of interests.

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