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Down-regulation of miR-192 protects against rat ischemia-reperfusion injury after myocardial infarction

J. DONG, Y. ZHAO, X.-K. HE

Department of Three Cardiovascular Ward, The Second Affiliated Hospi[illegible] Shaa[illegible] University of Traditional Chinese Medicine, Xianyang, Shaanxi, China

Abstract. – OBJECTIVE: DJ-1-phosphate and tension homology deleted on chromosome ten/ phosphatidylinositol-3-kinase/protein kinase B (PTEN/PI3K/AKT) signaling pathway plays a role in the regulation of ischemic reperfusion (I-R) injury. Bioinformatics analysis demonstrated that there is a complementary binding site between microRNA-192 (miR-192) and the 3'-UTR of DJ-1 mRNA. This study investigated the role of miR-192 in regulating DJ-1-PTEN/PI3K/AKT signaling pathway and myocardial I-R injury.

MATERIALS AND METHODS: miR-122 and DJ-1 mRNA expressions in myocardial tissue were detected by Real-time PCR (RT-PC[illegible] 1, PTEN, and phosphorylated AKT (p-A[illegible] tein expressions were tested by Weste[illegible]t. Reactive oxygen species (ROS) content[illegible] measured by flow cytometry. Malondialdeh[illegible] (MDA) content and superoxide dismutase (SO[illegible] enzyme activity were detecte[illegible] kits. I-[illegible] treatment was performed at [illegible]ransfec- tion. Cell apoptosis was ev[illegible]ted w[illegible]low cytometry.

RESULTS: Compared[illegible] sham[illegible] 192, PTEN expressio[illegible] a[illegible]ntent were significantly increa[illegible] ($p<$[illegible] while DJ-1, p-AKT levels and[illegible]D activitie[illegible]e signifi- cantly reduced ([illegible]5) in myocar[illegible]sue of I-R group. Co[illegible] control, I-R [illegible]eatment significantly [illegible]egula[illegible]iR-192 level, signifi- cantly dec[illegible]ased DJ-1 a[illegible]KT proteins, sig- nificantly[illegible]ated PTEN exp[illegible]n, and signifi- cantly i[illegible]ced apoptosis and [illegible]OS production in H9[illegible]ells ([illegible]05). Transfection of miR-192 inhi[illegible] signi[illegible]tly enhanced DJ-1 level, de- clin[illegible]EN[illegible]pressi[illegible]levated p-AKT lev- el, an[illegible]ned a[illegible]osis, ROS production [illegible] MDA[illegible]t, a[illegible]romoted SOD activity in [illegible]ells u[illegible]ondition.

C[illegible]CLUSIO[illegible] he expression of miR-192 incr[illegible]ed significantly, while the expression of DJ[illegible]viously during I-R injury after m[illegible]ardia[illegible]arction. Down-regulation of miR- markedly enhanced DJ-1 expression and [illegible]/PI3K/AKT pathway activity, inhibited cell a[illegible]is and ROS generation, and reduced I-R inju[illegible] cardiomyocytes.

Key Words:
MiR-192[illegible]/PI3K/AKT, Ca[illegible]omyocyte, I-R, Apoptosi[illegible]

Introduction

Coronary arte[illegible]eperfusion therapy after acu- yocardial in[illegible]tion (AMI) is the most ef- fe[illegible]method[illegible]ve the ischemic myocardium, prot[illegible]function, and save the lives[1,2]. However, blood recirculation inevitably induces ischemia-reperfusion (I-R) of infarcted myocardium causes more severe damage to the in- farcted myocardium[3,4].

Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT/PKB) signaling pathway is widely expressed in a variety of tissue cells and is involved in cell growth, survival, and apoptosis[5]. 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[6]. DJ-1/Parkinson gene 7 (PARK7) gene is a negative regulator of PTEN, which can suppress PTEN's inhibition on PI3K/AKT signaling pathway activity, thereby indirectly activating the PI3K/AKT signaling pathway, to play a role in anti-oxidation, apoptosis reduction, and cell survival promotion[7]. MicroRNA (miR) is a newly discovered non-coding single-stranded small molecule RNA with a length of 19 to 25 nucleotides in recent years. It is an extremely important gene regulatory substance that regulates about 30% human gene transcription and expression[8,9]. MicroRNA mainly participates in cell growth, differentiation, migration, and apoptosis mainly through anti-sense complementation to the 3'-untranslated region (3'-UTR) of target gene mRNA, leading to target

Corresponding Author: Xuekui He, MD; e-mail: bayanconglinlu@163.com

mRNA degradation or inhibition of post-transcriptional translation[10,11]. MicroRNA.org online analysis demonstrated that there is a complementary binding site between miR-192 and the 3'-UTR of DJ-1 mRNA, suggesting a targeted regulatory relationship between the two. Several studies have shown that miR-192[12-14] and DJ-1[15-17] expressions changed in I-R injury tissues. This study investigated the role of miR-192 in regulating DJ-1-PTEN/PI3K/AKT signaling pathway and myocardial I-R injury.

Materials and Methods

Main Reagents and Instruments

Healthy adult male Sprague-Dawley (SD) rats (6 weeks, body weight 250±20 g) were purchased from Shaanxi University of traditional Chinese Medicine Experimental Animal Center (Xianyang, China). Dulbecco's modified eagle medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). H9C2 cells were purchased from Changsha Winrunner (Cha... China). Rabbit anti-rat DJ-1, PTEN, AKT, and β-actin polyclonal antibodies were pur...ed from Abcam Biotechnology (Cambridge, ... USA). Horseradish peroxidase (HRP)-conjuga... goat anti-rabbit IgG (H+L) secondary antibo... was purchased from Sangon ...logy Co. Ltd. (Shanghai, China). Typ... colla...ase and glucose were purchased f... Sigma-...ich (St. Louis, MO, USA). Trans... reag... was purchased from In...trog... ...chnologies (Carlsbad, CA, USA... meSci... kit was purchased from TaK... Dalian, Chi... ...ciferase activity assay k... ...rchased fro... romega (Madison, W... SA)... R-Report Luciferase, miR-NC, ...iR-192 mim... ...tagomir-NC, and antagomi... R-192 were pu... ...d from Ribobio (G... zhou, China). Lipid peroxidation products ...londia... yde (MDA) and superoxide dis... e (SO... detecti... kits were purchased from ... Biotech... gy (Shanghai, China). DCFH-D... resce... ye was purchased from ... Aldri... uis, MO, USA). Annexin V/P... Apopto... etection Kit was purchased fro... iindo (Tokyo, Japan).

...ed for all experiments, and all ...edures were approved by the Animal Ethics ...mittee of the Second Affiliated Hospital of S... University of traditional Chinese Medicine.

Rat Myocardial I-R Model Establishment

The rat was anesthetized by using ... drate intraperitoneal injection. Th... ...bs w... connected to the SurgiVet V340... ...pe electrocardiogram and the endotrache... ...ubation was connected SANS SA430 type a... ...reathing machine. The chest was op... ...d on th... ...th in... tercostal space and the ... anterior de... coronary artery was i... ...fied b... ...ween pu... nary arterial cone a... ...rta. Ne... ...ne artery was ligated by using 6-... ...e. ... mode... ...succ... cess was judged ... ST ... ...t arch ... ...0.1 mV on Q lead or T... ...ve high-... ...ud... ...yocardial color wanin... ...d pulse wea... ...The blood supply wa... ...after blocking ...r 60 min and the card... apex ... ...e red was considered as successful reperfusio... ...m group was selected as c... The rats in th... ...group were divided i... ...hree subgroups with six in each subgroup, ...uding postop... ...tive 6 h, 12 h, and 24 h, re... ...ctively.

A... omir M... 92 Treatment

T... ... model rats were randomly divided into 2 groups: one group was given 50 nmol ...tagomir-NC intravenously 24 h before surgery, ... other group was given 50 nmol antagomir ... -192 intravenously 24 h before surgery. The tissues were harvested 24 h after surgery. Protein expression was detected by Western blot, caspase-3 activity was tested by spectrophotometry, reactive oxygen species (ROS) content was determined by flow cytometry, and MDA content and SOD activity were measured.

Caspase-3 Activity Detection

According to the instructions of the kit, the pNA standard product was diluted in concentration gradient to prepare standard products with concentrations of 200 μM, 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, and 0 μM. 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. Then the supernatant was taken to a new 1.5 ml centrifuge tube and quantified by the bicinchoninic acid (BCA) kit. 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 obvious, the plate was measured at 405 nm on a microplate reader (Awareness, 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 using BCA method. MDA and SOD contents were tested in accordance with the instructions to assess oxidative stress conditions and antioxidant capacity.

H9C2 Cell Transfection and I-R Treatment

For I/R treatment, 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 cells in logarithmic phase were divided into two groups, including miR-NC and miR-192 inhibitor groups.

Flow Cytometry Detection of Cell Apoptosis

The cells were washed twice in phosphate-buffered saline (PBS) and digested by trypsin. After centrifuged at 300 ×g for [illegible]in, the cells were re-suspended and added with [illegible] µl binding solution. Then, the cells were added with 5 µl of Annexin V-FITC and 5 µl of propidium iodide (PI) solution [illegible]ce, and incubated avoiding light for [illegible] min. [illegible]er supplementation with 400 µl [illegible]ding solution, the cells were tested on a B[illegible]n Cytocytometer.

Flow Cytometry Detection of

ROS detection in myocardial tissue: the rat tissue was [illegible]ected and cut into pieces. The tissue was digested with [illegible]ollagenase II and hyaluronidase-containing digestive solution for 45 min. After centrifugation at 200 ×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 and resuspended in 500 µl PBS, the tissues were tested on Beckman FC500MCL flow cytometer to detect ROS content.

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.

Luciferase Reporter Gene Assay

1×10^5 H9C2 cells were inoculated in [illegible] plate and incubated for 24 h. pMIR-DJ-1-wt (or pMIR-DJ-1-mut) and miR-192 mimic (or miR-NC) were co-transfected into [illegible]293 cells by Lipo2000 and continued to culture for [illegible]h. Dual-Glo Luciferase Assay System Kit was used to detect dual luciferase activity. The plate was added with 100 µl Passive Lysis Buffer cell lysate and shaken slowly for [illegible] min at room temperature. Next, the 20 µl cell lysate was added with 100 µl luciferase assay reagent II (LARII) to test the firefly luciferase activity on a [illegible]ce luminometer, and then added with [illegible]enilla fluorescein reagent to detect renilla luciferase activity to calculate the relative activity value of firefly luciferase activity to renilla luciferase activity.

Quantitative Real-Time PCR (qRT-PCR)

PrimeScript™ RT reagent Kit was used to reverse transcribe RNA to complementary DNA (cDNA) for qPCR reaction. qPCR reaction system contained 5.0 µl SYBR Green Mixture, 0.5 µl Forward Primer (5 µM), 0.5 µl Reverse Primer (5 µM), 1.0 µl cDNA, and ddH_2O. The reverse transcribe conditions were 50°C for 15 min and 85°C for 5 min. qPCR reaction were pre-denatured at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min on the Bio-Rad (Hercules, CA, USA) CFX96 Real-Time PCR Detection System. The primers for miR-192 and DJ-1 were listed in Table I.

Western Blot

Cells and tissues were lysed by radioimmunoprecipitation assay (RIPA). 40 µg proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and 4% concentrated gel. Then the protein was transferred to polyvinylidene difluoride (PVDF) membrane at 300 mA for 90 min. Next, the membrane was blocked with 5% skim milk at room temperature for 60 min and incubated in primary antibody (DJ-1, PTEN, AKT, p-AKT, and β-actin at 1:1000, 1:2000, 1:2000, 1:1000, 1:10000, respectively) at 4°C overnight. After that, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:15000) at room temperature for 60 min and finally detected by enhanced chemiluminescence (ECL).

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Measu-

rement data were expressed as mean ± standard deviation (SD). The student's *t*-test was used to compare the differences between two groups. The Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data among groups. $p<0.05$ was considered as statistically significant.

Results

The Targeted Regulatory Relationship Between miR-192 and DJ-1

MicroRNA.org online prediction showed that there is a targeted complementary binding site between miR-192 and the 3'-UTR of DJ-1 mRNA (Figure 1A). Dual luciferase gene reporter assay revealed that transfection of miR-192 mimic significantly reduced the relative luciferase activity of pMIR-DJ-1-wt transfected HEK293T cells. However, miR-192 mimic exhibited no marked effect on relative luciferase activity in pMIR-DJ-1-mut transfected HEK293T cells (Figure 1B), suggesting that there is a targeted regulatory relationship between miR-192 and DJ-1 mRNA.

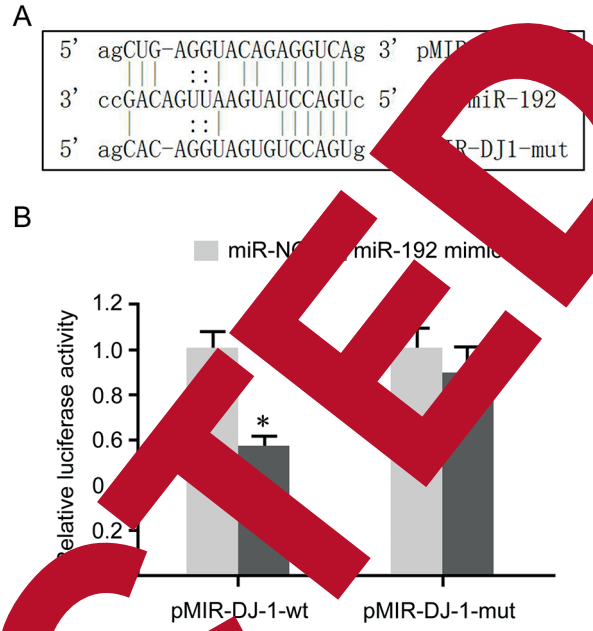


Figure 1. The targeted regulatory relationship between miR-192 and DJ-1. The complementary binding site between miR-192 and the 3'-UTR of DJ-1 mRNA. ***B,*** Dual luciferase reporter assay. $^{*}p<0.05$, compared with miR-NC.

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

Flow cytometry showed that ROS content in myocardial tissue of I-R rats was significantly increased compared with Sham group (Figure 2A). Spectrophotometry revealed that the caspase-3 activity in the I-R rats was significantly enhanced compared with the Sham group (Figure 2B). Lipid peroxidation and antioxidase demonstrated that the MDA content in myocardial tissue of I-R model rats was markedly higher (Figure 2C), while SOD enzyme activity (Figure 2D) was apparently decreased than that in Sham group.

MiR-192 Increased, While DJ-1 Declined in the Ischemic Myocardium

qRT-PCR showed that the expression of miR-192 was significantly higher, whereas the expression of DJ-1 mRNA was significantly decreased in ischemic myocardial tissue of I-R group than that of Sham group. MiR-192 expression gradually elevated, while DJ-1 level gradually declined following I-R time extension (Figure 3A, B). Western blot revealed that DJ-1 and PTEN protein expressions were markedly lower in I-R group than that in Sham group both at 12 h and 24 h (Figure 3C).

Down-Regulation of miR-192 Attenuated I-R Induced Myocardial Cell Oxidative Injury and Apoptosis

qRT-PCR demonstrated that compared with control group, I-R treatment significantly enhanced the expression of miR-192 and weakened the expression of DJ-1 mRNA in H9C2 myocardial cells (Figure 4A). Western blot analysis exhibited that compared with the control group, I-R treat-

Table I. Primers for the qRT-PCR assay.

Genes		Sequences
miR-192	Forwards	GTGGACCTGACCTGCCTGCCGTCT
	Reverse	GGAGGAGTGGGTGTCGCTGT
DJ-1	Forwards	GGGCCTTGTCCCATTCACGGC
	Reverse	TGCCTGCTTCTATATCCCGGCTTGG
GAPDH	Forwards	TGCTGAGTATGTCGTGGAGTCTA
	Reverse	AGTGGGAGTTGCTGTTGAAATC

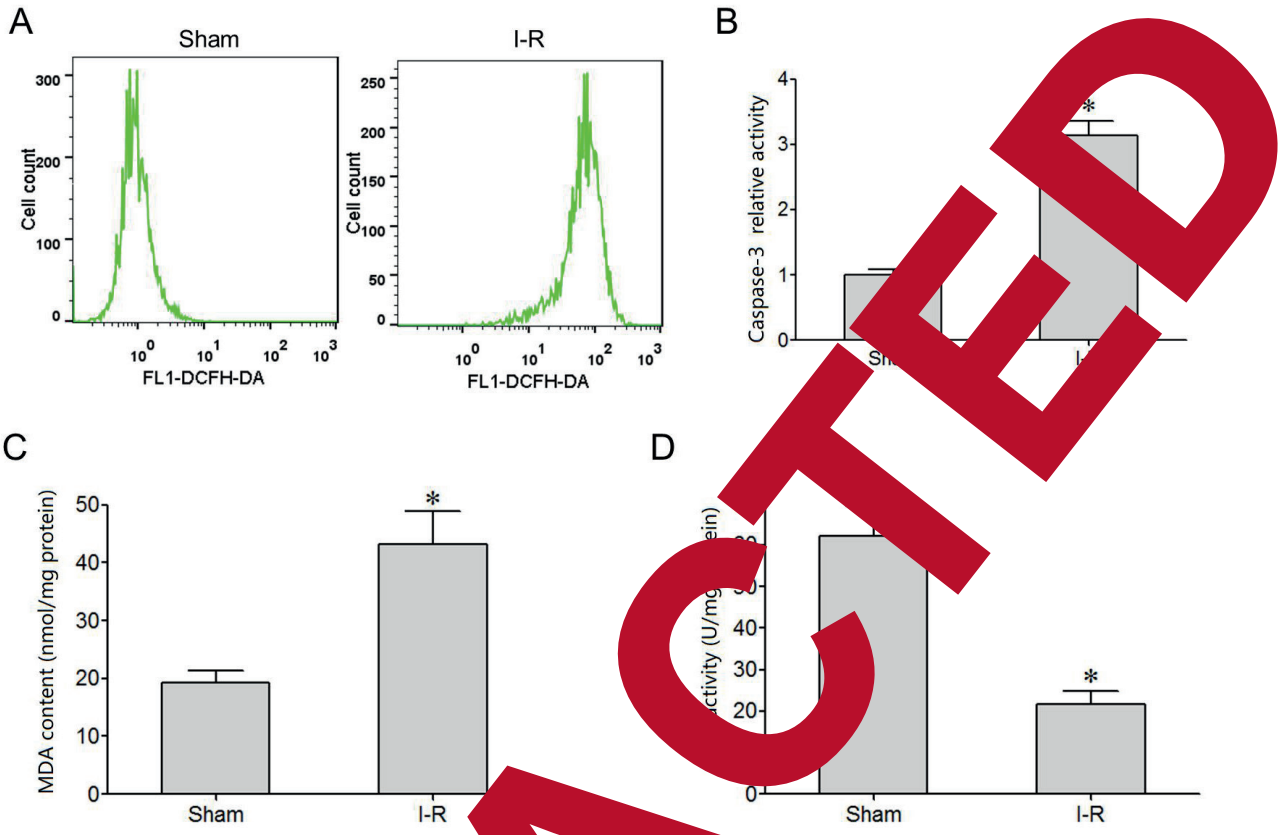


Figure 2. Evident oxidative stress injury of myoca[r]dial tissu[e] I-R[ ]. ***A,*** Flow cytometry detection of ROS content in the myocardium. ***B,*** Spectrophotometry detection of ca[spase] activity. ***C,*** MDA content detection. ***D,*** SOD enzyme activity detection. $^{*}p<0.05$, compared with Sham.

ment markedly declined [the] expressi[on] of DJ-1 and p-AKT proteins, w[hile] elevate[d] sion of PTEN protein [in] H9[c2] (Figure 4B). Transfection of mi[R]-192 mar[kedly] upregulated the expression of [DJ-1] reduced the [expre]ssion of PTEN protein, [and] eased the ex[pre]ssion of p-AKT protei[n] in H9[c2] under I-R condition (Figure 4B). It also relieve[d] ase-3 activity (Figure 4F), [the] apoptosis (Figu[re] reduced MDA content (Figure 4C) and ROS p[ro]duction (Figure 4E), [and] enhanc[ed] OD activity (Figure 4D).

Di[s]sion

[The] PARK7 [gene] is located on human chromos[ome] 1p36.2-36.3, which is about 24 kb in length, [and] a 189-amino acid protein with a [mo]lecular weight of 21 kDa. DJ-1 is a negative [re]gulator of PTEN protein that can enhance the a[ctivity] of PI3K/AKT signaling pathway to suppress [oxi]dation, reduce apoptosis, and promote cell survival[7]. It was showed that the abnormally decreased expression of DJ-1 is closely related to I-R damage in organs such as heart[16] and spinal cord[17], while elevated I-R expression can have a protective effect, indicating that DJ-1 is a protective factor during IR damage. It was found[12,14,18] that elevated miR-192 can be detected during I-R injury in lungs, kidneys, and other organs, suggesting that miR-192 may be a promoting factor for I-R injury. Bioinformatics analysis demonstrated that there is a complementary binding site between miR-192 and the 3'-UTR of DJ-1 mRNA, suggesting a targeted regulatory relationship between the two. This study investigated the role of miR-192 in regulating DJ-1-PTEN/PI3K/AKT signaling pathway and myocardial I-R injury.

Dual luciferase reporter gene assay showed that transfection of miR-192 mimic significantly reduced the relative luciferase activity in pMIR-DJ-1-wt transfected HEK293T cells, but not pMIR-DJ-1-mut transfected, suggesting that there is a targeted regulatory relationship between miR-192 and DJ-1

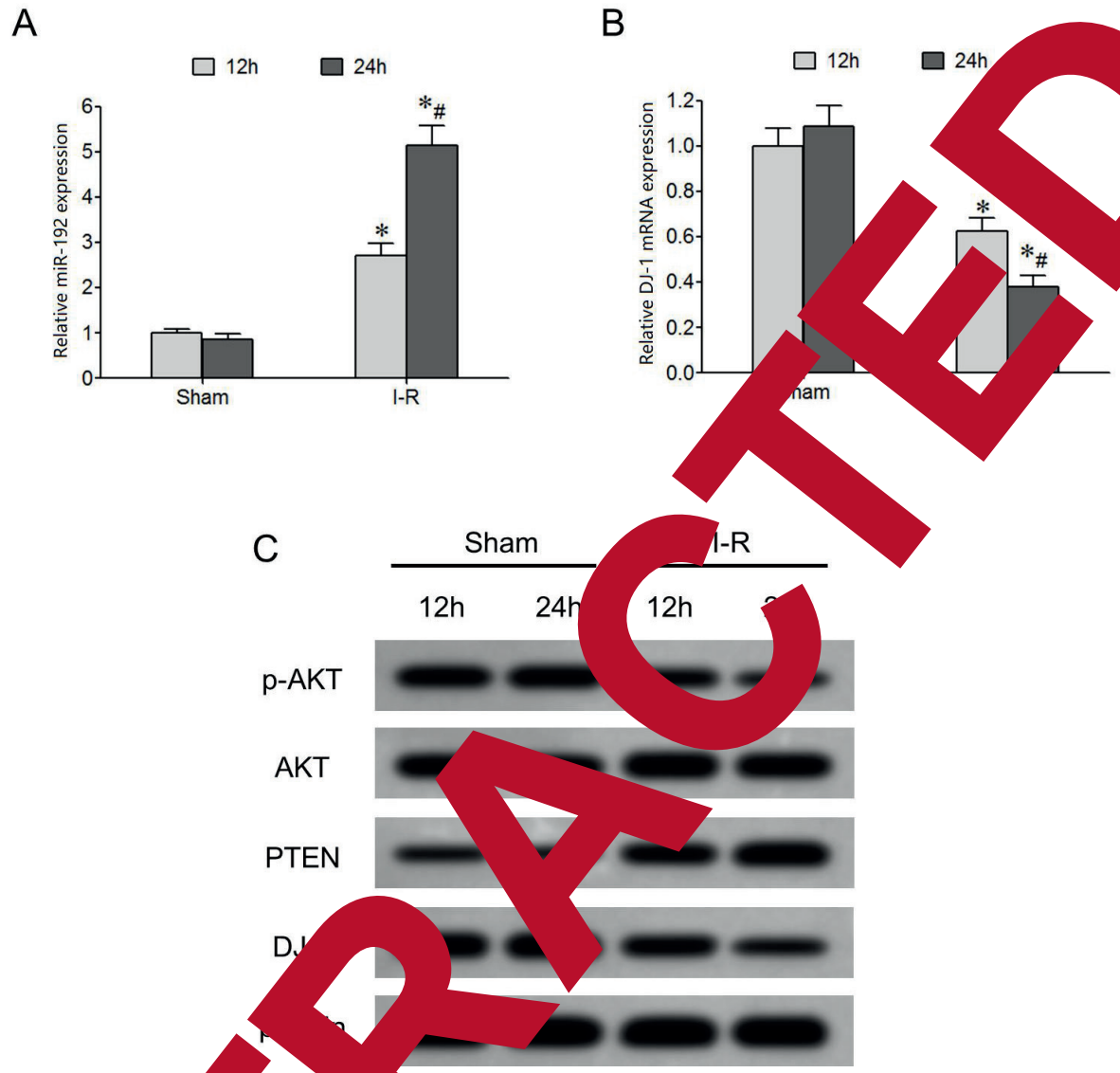
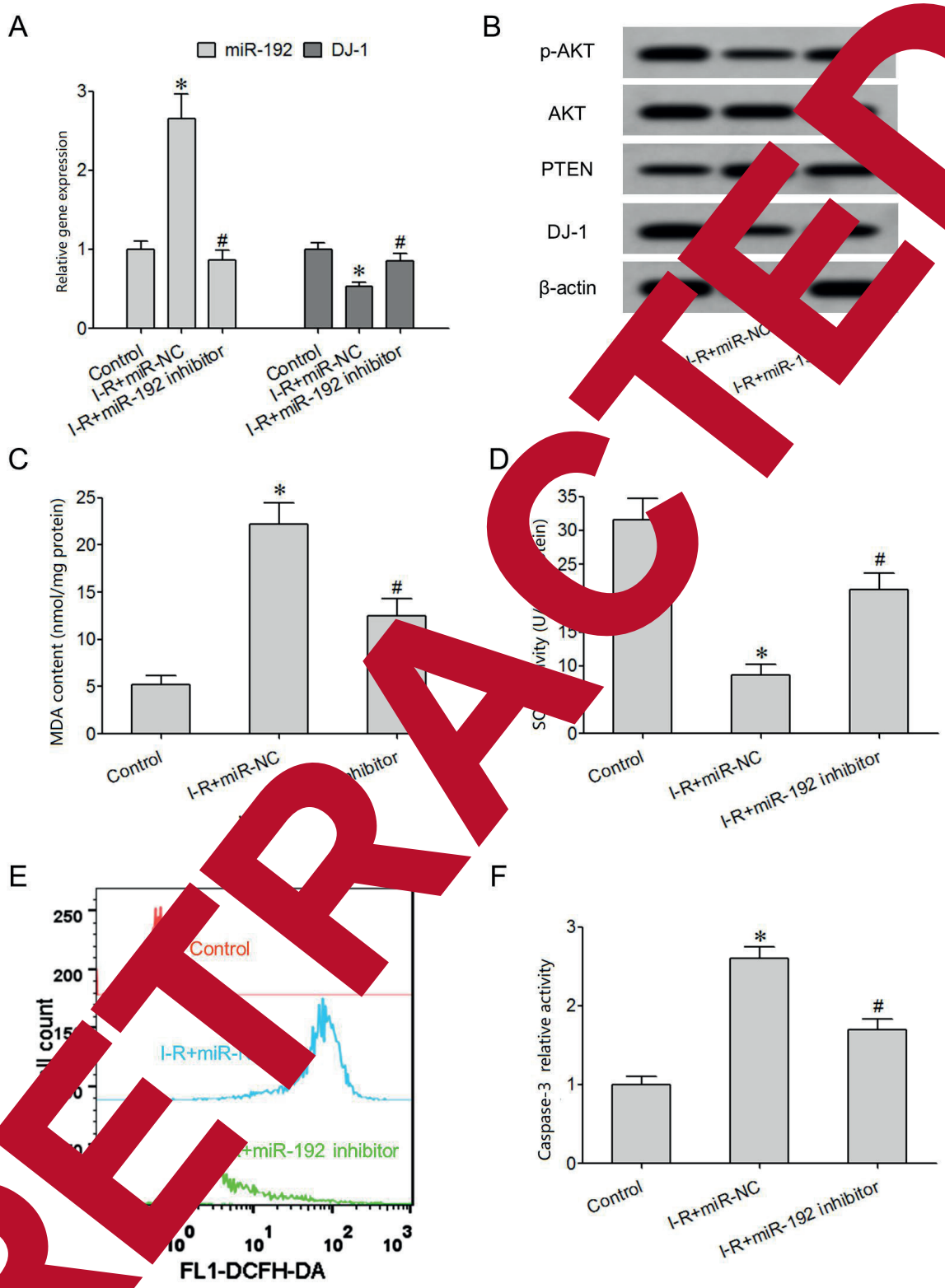


Figure 3. MiR-192 in[illegible]ed, while DJ[illegible]ed in the I-R rat myocardium. ***A,*** qRT-PCR detection of miR-192 expression. ***B,*** qRT-PCR detecti[illegible]J-1 mRNA exp[illegible] ***C,*** Western blot detection of protein expression. $^{*}p<0.05$, compared with Sham, $^{\#}p<0.05$, co[illegible]12 h.

mRN[illegible]After I[illegible]njury, the expression of miR-192[illegible]t my[illegible]dial tis[illegible] gradually increased, while[illegible]ssion [illegible]-1 gradually declined f[illegible]llowing[illegible]exter[illegible], revealing that miR-192 [illegible]press[illegible]lay a role in down-regulatin[illegible]J-1 exp[illegible]on and regulating the myocard[illegible]njury after I-R. The increase of miR-192 is[illegible] factor of myocardial I-R injury. [illegible]e study of the relationship between miR-192 [illegible]I-R injury, Godwin et al[14] reported that com-p[illegible]ith the control group, the expression of miR[illegible] in the renal tissue of mice with I-R injury was significantly increased, and its level gradually upregulated over time. Rancan et al[18] observed that after I-R injury, the expression of miR-192 in lung tissues of pigs elevated significantly. Wang et al[19] demonstrated that compared with the control group, the expression of miR-192 in the peripheral blood of the rats began to increase after 6 h of renal I-R injury and reached a peak after 12 h, and began to decline after 24 h. Zhang et al[12] showed that the expression of miR-192 in peripheral blood of rats with acute renal I-R injury was markedly upregulated with time extension. The expression



re 4. Downregulation of miR-192 attenuated I-R induced myocardial cell oxidative injury and apoptosis. ***A,*** qRT-PCR tion of miR-192 and DJ-1 mRNA expressions. ***B,*** Western blot detection of protein expression. ***C,*** MDA content detection; ctivity detection. ***E,*** Flow cytometry detection of ROS content. ***F,*** Spectrophotometry detection of caspase-3 activity.

Figure continued

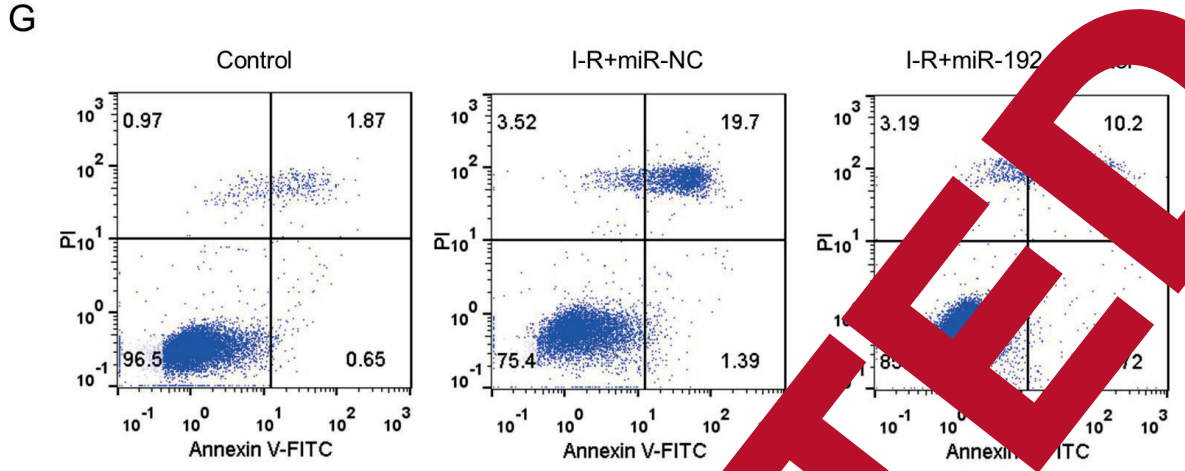


Figure 4. *Continued.* ***G,*** Flow cytometry detection of cell apoptosis. $^{*}p<0.05$ compared with control $^{\#}p<0.05$,compared with I-R+miR-NC.

level of miR-192 was 10 times higher than that of the control group at 12 h after I-R. In addition, in patients with acute renal I-R injury, the expression of miR-192 in peripheral blood was also apparently higher than those without renal I-R injury. [illegible] et al[13] showed *in vitro* that the hypoxia-reoxygenation (H-R) treatment of H9C2 cells markedly induced apoptosis and elevated miR-192 expression. Zou et al[20] revealed that the expression of miR-192 was apparently enhanced in the urine of rats with acute kidney I-R injury, and miR-192 elevation was considered as a biomarker of acute I-R injury. In this study, the expression of miR-192 in the injured myocardium was significantly increased, suggesting that miR-192 elevation may participate in I-R injury. In addition, this study found that the elevated miR-192 expression may play a role in myocardial tissue I-R injury. Shimizu et al[21] showed that compared with wild-type mice, DJ-1 knockout mice exhibited obviously larger myocardial infarct size and apparently decreased ventricular systolic function under I-R injury condition. Dongworth et al[16] reported that DJ-1 knockout mice presented high sensitivity to I-R injury, with a significant increase in infarct size and apoptosis. In this study, the expression of DJ-1 was significantly reduced during I-R injury in myocardial tissue, and it was confirmed that the decreased DJ-1 expression was associated with myocardial I-R injury, which was similar to the study of Shimizu et al[21] and Dongworth et al[16]. Further investigation showed that transfection with miR-192 inhibitor in H9C2 cells significantly elevated the expression of DJ-1, declined the expression of PTEN, enhanced the downstream PI3K/AKT pathway activity, and attenuated intracellular ROS production, oxidative stress injury and apoptosis in cardiomyocytes I-R injury. We observed that miR-192 downregulation can increase the expression of DJ-1, enhance the activity of PTEN-PI3K/AKT pathway, reduce myocardial cell apoptosis, and alleviate I-R injury. Zhang et al[13] showed *in vitro* that over-expression of miR-192 in H9C2 cells significantly aggravated HR injury and cell apoptosis. Downregulation of miR-192 expression significantly attenuated H9C2 cell I-R and apoptosis. Zhang et al[22] revealed that resveratrol can protect I-R injury in cardiomyocytes by up-regulating the expression of DJ-1. Dongworth et al[16] demonstrated that over-expression of DJ-1 significantly increased the apoptosis of mouse cardiomyocyte HL-1 to reduce the sensitivity to I-R injury. Lu et al[23] reported that hypoxic preconditioning prior to ischemia-reperfusion could effectively modulate the endogenous protective mechanisms of the motosome, activate ERK1/2 and up-regulate the expression of DJ-1 in cardiomyocytes, thereby reducing the number of apoptotic myocardial cells in I-R. This study found that elevated expression of miR-192 is a contributing factor to I-R injury after myocardial infarction. DJ-1 can be elevated by decreasing the expression of miR-192, leading to the protective effect of DK-1 in myocardial tissue I-R injury. However, the protective role of miR-192 regulating DJ-1 expression in I-R injury after myocardial infarction still needs to be further studied in animals[24].

Conclusions

We demonstrated that the expression of miR-192 increased significantly, while the expression of DJ-1 reduced significantly during I-R injury after myocardial infarction. Down-regulation of miR-192 markedly enhanced DJ-1 expression and PTEN/PI3K/AKT pathway activity, inhibited cell apoptosis and ROS generation, and reduced I-R damage in cardiomyocytes.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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