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Effects of dexmedetomidine on myocardial ischemia-reperfusion injury through PI3K-Akt-mTOR signaling pathway

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Abstract. – **OBJECTIVE:** To explore the role of dexmedetomidine (DEX) in myocardial ischemia-reperfusion (I/R) injury model and investigate its specific molecular mechanism.

MATERIALS AND METHODS: The I/R rat model was established by ligating the anterior descending coronary artery for 30 min and reperfusion for 120 min. In this experiment, all rats were divided into sham operation (SH) group, I/R group, DEX group and I/R + rapamycin (RAP) group. After 120 min of I/R treatment, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximal rates of rise and fall of left ventricular pressure ($\pm dp/dt_{max}$) and ischemic area were detected. Serum samples of rats in each group were collected. The levels of catalase (CAT), glutathione peroxidase (GSH-PX), malondialdehyde (MDA), superoxide dismutase (SOD), creatine kinase (CK), CK-muscle/brain (CK-MB), tumor necrosis factor (TNF) and interleukin-6 (IL-6) were detected using enzyme-linked immunosorbent assay (ELISA). The apoptosis of myocardium in each group was detected according to the instructions of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The expressions of mammalian target of rapamycin (mTOR), phosphorylated-mTOR (p-mTOR), protein kinase B (Akt) and p-Akt in myocardial tissues were detected *via* Western blotting. Moreover, the messenger ribonucleic acid (mRNA) expression level of mTOR in each group was detected using reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: Compared with SH group, LVSP and $\pm dp/dt_{max}$ in I/R group were significantly decreased, whereas LVEDP was remarkably increased in I/R group ($p<0.01$). After DEX administration, LVSP and $\pm dp/dt_{max}$ were remarkably increased, while LVEDP and infarction area were markedly decreased ($p<0.01$). After treatment with mTOR inhibitor rapamycin (RAP), LVSP and $\pm dp/dt_{max}$ were evidently decreased, while LVEDP and infarction area were increased when compared with those of DEX group ($p<0.01$). Compared with SH group, the levels of CK, CK-MB, TNF-α and IL-6 in I/R group were significantly increased. However, the levels of these molecules were significantly decreased after DEX treatment in I/R rats. After the combination of DEX and RAP, the expression levels of these indexes were significantly increased. No significant differences were found between DEX + RAP group and model group, and between I/R + RAP group and model group. MDA level in I/R group was significantly higher than that of SH group, while the levels of SOD, CAT and GSH-PX were notably lower ($p<0.01$). Compared with I/R group, the level of MDA in DEX group was significantly reduced, but the levels of SOD, CAT and GSH-PX were markedly increased ($p<0.05$, $p<0.01$). Meanwhile, compared with DEX group, MDA level in I/R group was significantly increased. However, the levels of SOD, CAT and GSH-PX were remarkably decreased after the application of combined DEX and mTOR inhibitor ($p<0.01$). After the addition of RAP, no significant changes were found in each index compared with I/R group. DEX could alleviate myocardial cell apoptosis caused by I/R treatment ($p<0.01$). The levels of p-mTOR and p-Akt in I/R group were significantly increased when compared with those of SH group. However, the levels of these indexes in DEX group were evidently higher than those of I/R group after DEX administration based on myocardial I/R model ($p<0.01$). After combination of DEX and RAP, the latter canceled the effect of the former on enhancing the expression of p-mTOR and the phosphorylation level of mTOR. Furthermore, there was no significant change in mTOR and its mRNA expression in each group.

CONCLUSIONS: DEX can play a protective role in myocardial I/R rats, improve the cardiac function of I/R rats, eliminate oxygen free radicals, relieve oxidative stress injury, inhibit inflammatory responses and reduce the release of CK and other substances. The myocardial protection effects of DEX are mainly achieved through the phosphatidylin-ositol-3-kinase (PI3K)-Akt-mTOR pathway.

Key Words:
Dexmedetomidine, I/R, PI3K-Akt-mTOR.

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Introduction

With the development of basic research and clinical treatment, drug intravenous thrombolysis, coronary artery bypass grafting and percutaneous coronary angioplasty have been widely used in clinical practice recently. Meanwhile, the prognosis of patients with coronary heart diseases has also been significantly improved. However, subsequent myocardial ischemia-reperfusion (I/R) injury seriously restricts the success rate of these treatments[1]. Zhang et al[2] have shown that myocardial I/R injury may be related to oxygen free radical production, inflammatory responses, calcium overload and other factors. Dexmedetomidine (DEX) is a α_2-adrenoceptor agonist, which is mainly used in anesthesia and sedation[3]. Currently, some researches have revealed that DEX can protect the heart in many ways. It can also alleviate oxidative stress and myocardial I/R injury. However, the specific mechanism of its role is still unclear. In this study, the myocardial I/R injury model was first established. Moreover, we investigated the effects of DEX on myocardial I/R as well as its relationship with phosphatidylin-ositol-3-kinase (PI3K)-protein kinase B (Akt)-mammalian target of rapamycin (mTOR) signaling pathway.

Materials and Methods

Drugs and Reagents

DEX was purchased from Jiangsu Hengrui Pharmaceutical Co., Ltd. (Lianyungang, China); chloral hydrate was bought from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China); creatine kinase (CK), CK-muscle/brain (CK-MB), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), malondialdehyde (MDA), superoxide dismutase (SOD), (GSH-PX) and catalase (CAT) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China); bicinchoninic acid assay (BCA) kits were bought from Beyotime Biotechnology Co., Ltd. (Shanghai, China); and mTOR and phosphorylated-mTOR (p-mTOR) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

I/R Model Preparation

A total of 50 healthy male Sprague-Dawley (SD) rats were anesthetized with chloral hydrate. Needle electrodes were inserted into the limbs of rats. The left common carotid artery of rats was peeled off, and a plastic catheter containing anticoagulant and saline was inserted. Electrocardiograms and hemodynamic changes in rats were recorded. Subsequently, the left anterior descending coronary artery of rats was found. A 4-0-suture thread was then used to pass through the position at 3-4 cm from the initial position of coronary artery. A small catheter was placed under the ligature to block blood flow in the left anterior descending coronary artery. After 30 min, blood flow supply was resumed for 120 min. This study was approved by the Animal Ethics Committee of Harbin Medical University Animal Center (Harbin, China).

Experimental Grouping

50 healthy male SD rats were randomly divided into 5 groups with 10 rats in each group, including: (1) sham operation group (SH group), (2) I/R group, (3) DEX treatment group (DEX group): DEX (2.5 mL/kg) was given before ischemia, and the rest procedures were the same as I/R group, (4) DEX + rapamycin (RAP) group: RAP (1 mL/kg) was given before the administration of DEX, and the rest procedures were the same as DEX group, and (5) I/R + RAP group: RAP (1 mL/kg) was given before ischemia, and the remaining procedures were the same as I/R group.

Hemodynamic Detection

A plastic catheter was first inserted into the left ventricle along the common carotid artery. Subsequently, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and maximal rates of rise and fall of left ventricular pressure (dp/dt_{max}) were recorded using a physiological recorder.

Measurement of Myocardial Infarction Area

The central area of myocardial infarction was first found. Then, the apex of the heart parallel to the atrioventricular groove was cut, followed by incubation with triphenyltetrazolium chloride (TTC) solution (Oxoid, Hampshire, UK) for 15 min. Subsequently, the infarction area appeared white and could be distinguished from normal myocardial tissues. Ischemic and non-ischemic areas were separated, and the volume ratio of infarction area to the left ventricle was calculated as myocardial infarction area.

Biochemical Index Detection

After myocardial I/R injury, whole blood was collected from each rat, followed by centrifugation at 3000 rpm/min for 15 min. Subsequently,

the supernatant was taken and stored in a refrigerator at -20°C. Serum levels of CAT, GSH-PX, MDA, SOD, CK, CK-MB, TNF-α and IL-6 were detected according to the instructions of enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

In Situ Cell Death Detection Kit purchased from Roche (Basel, Switzerland) was applied to measure the apoptosis of paraffin sections. The specific steps were as follows. Briefly, paraffin sections were dewaxed, washed with phosphate-buffered saline (PBS), added with protease K working solution and immersed in sealing solution. Next, the sections were transparentized with Triton X100/sodium citrate (0.1%). Subsequently, Vectashield Hard Set was added to seal the sections. Finally, TUNEL was conducted with fluorescein isothiocyanate (FITC) as fluorescent developer. TUNEL-positive cells were calculated from 10 randomly selected fields.

Expressions of mTOR and p-mTOR via Western Blotting

At the end of myocardial I/R in rats, the tissues below the left ventricular ligature were first treated into fragments in a liquid nitrogen cryogenic environment. Then, the tissues were lysed in lysate containing 1% protease inhibitor. The protein concentration was determined in strict accordance with BCA kit. 50-μg protein sample was boiled in loading buffer and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) after denaturation. Subsequently, PAGE gel containing proteins was placed on NC membranes in a membrane transfer tank. After sealing with 5% skim milk, the membranes were incubated with primary antibodies of mTOR, p-mTOR and internal reference overnight. On the next day, the membranes were incubated with fluorescence secondary antibodies at room temperature for 2 h. After washing with phosphate-buffered saline and Tween-20 (PBST), immuno-reactive bands were detected by Odyssey system.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol was first added to heart tissues and cells (TRIzol kit, Thermo Fisher, Waltham, MA, USA). Then, chloroform was added for centrifugation to obtain the supernatant. Isopropanol was added to obtain total RNA. RNA samples were obtained by treatment with 75% ethanol added with diethyl pyrocarbonate (DEPC) (Beyotime, Shanghai, China). Complementary deoxyribose nucleic acid (cDNA) chain was obtained as templates by reverse transcription PCR. Primers, Taq polymerase, Taq buffer, DNTP mixture and ddH_2O were added to perform PCR amplification by a PCR apparatus. Finally, the product was placed on a quantitative PCR instrument to determine the mRNA expression of target genes. Primer sequences used in this study were as follows: mTOR, F: 5'-CTGTAATTACATCCTCGACTG-3', R: 5'-CGTGTCGTGGTTAGTCG-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Experimental data were represented by ($\bar{x}\pm s$). *t*-test was used to compare the difference between two groups. $p<0.05$ and $p<0.01$ were considered statistically significant.

Results

Effects of DEX on Cardiac Hemodynamic Changes and Infarction Area After I/R Treatment in Rats

As shown in Table I, LVSP and $\pm dp/dt_{max}$ in I/R group were significantly decreased when compared with those of SH group. However, LVEDP in I/R group was remarkably higher than that of SH group ($p<0.01$). After the administration of DEX, LVSP and $\pm dp/dt_{max}$ were markedly increased, whereas LVEDP and infarction area were significantly decreased ($p<0.01$). After the treatment with mTOR inhibitor RAP, LVSP and $\pm dp/dt_{max}$ were evidently decreased, while LVEDP and infarction area were significantly increased when compared with those of DEX group ($p<0.01$). Compared with I/R group, no significant changes were found in the above indexes, indicating that PI3K-Akt-mTOR inhibitor RAP could inhibit the effect of DEX.

Effects of DEX on CK, CK-MB, TNF-α and IL-6 after I/R Treatment in Rats

As shown in Table II, the levels of CK, CK-MB, TNF-α and IL-6 in I/R group were significantly higher than those of SH group ($p<0.01$). However,

Table I. Effects of DEX on cardiac hemodynamic changes and infarction area after I/R treatment in rats ($\bar{x}$±s, n=10).

Group	LVSP/mmHg	LVEDP/ mmHg	$+dp/dt_{max}$/ $mmHg \cdot s^{-1}$	$-dp/dt_{max}$/ $mmHg \cdot s^{-1}$	Infarction area/%
SH group	138.36±9.52	4.69±0.14	4218.11±186.46	-3469.32±195.25	
I/R group	92.56±7.33*	11.82±0.45*	2676.47±227.38*	-2000.18±166.47*	49.46±6.85
DEX group	128.12±8.83#	7.17±0.41#	3418.64±215.89#	-2417.84±170.05#	24.52±3.18#
DEX + RAP group	99.89±7.63Δ	12.37±0.83Δ	2926.17±258.22Δ	-2108.14±186.36Δ	48.23±6.82Δ
I/R + RAP group	97.75±7.49Δ	12.05±0.54Δ	2835.12±256.23Δ	-2009.39±210.51Δ	46.68±4.67Δ

Note: *$p<0.01$ *vs.* SH group, #$p<0.01$ *vs.* I/R group and Δ$p<0.01$ *vs.* DEX group.

Table II. Effects of DEX on CK, CK-MB, TNF-α and IL-6 after I/R treatment

Group	$CK/U \cdot L^{-1}$	$CK\text{-}MB/U \cdot L^{-1}$	$TNF\text{-}\alpha/ng \cdot L^{-1}$	$IL\text{-}6/ng \cdot L^{-1}$
SH group	992.81±98.21	998.82±96.75	34.21±2.12	2.51±0.08
I/R group	3158.52±228.57*	3032.29±212.18*	121.26±11.71*	6.22±0.48*
DEX group	1387.58±152.52#	1517.17±217.24#	69.51±9.76#	3.19±0.26#
DEX + RAP group	2911.46±227.81Δ	3121.25±248.28Δ	124.37±16.40Δ	6.17±0.26Δ
I/R + RAP group	2885.21±283.74Δ	2951.20±214.56Δ	118.27±17.41Δ	6.28±0.52Δ

Note: *$p<0.01$ *vs.* SH group, #$p<0.01$ *vs.* I/R group and Δ$p<0.01$ *vs.* DEX group.

the levels of CK, CK-MB, TNF-α and IL-6 were significantly decreased after DEX administration in I/R rats. After the combination of DEX and RAP, the expression levels of these indexes were obviously increased. However, there were no significant differences between DEX + RAP group and model group as well as between I/R + RAP group and model group. The above results suggested that RAP blocked the effects of DEX on the expression levels of CK, CK-MB, TNF-α and IL-6.

Effects of DEX on Oxidative Stress Indexes of I/R Injury

It was found that compared with SH group, MDA level in I/R group was markedly increased, whereas the levels of SOD, CAT and GSH-PX were significantly decreased ($p<0.01$) (Table III). Compared with I/R group, MDA level was significantly decreased in DEX group, while the levels of SOD, CAT and GSH-PX were increased ($p<0.05$, $p<0.01$). Compared with DEX group, MDA level in I/R group was significantly increased, while the levels of SOD, CAT and GSH-PX were remarkably decreased after the combination of DEX and RAP ($p<0.01$). After the addition of RAP, no significant change was found in each index when compared with I/R group. This suggested that RAP could block the effects of DEX on the above indexes.

Effects of DEX on Myocardial Cell Apoptosis

The results manifested that no TUNEL-positive cells were found in SH group. Moreover, the apoptosis rate of DEX group was significantly lower than that of I/R group ($p<0.01$) (Figure 1).

Table III. Effects of DEX on oxidative stress indexes of I/R injury

Group	$SOD/U \cdot mL^{-1}$	$MDA/\mu mol \cdot L^{-1}$	$CAT/U \cdot mg^{-1}$	$GSH\text{-}PX/U \cdot mg^{-1}$
SH group	115.81±18.84	9.49±1.21	51.72±2.61	18.94±2.58
I/R group	71.54±5.43[1)]	18.31±2.26[1)]	8.45±1.68[1)]	12.11±1.74[1)]
DEX group	114.43±11.74[3)]	11.14±1.21[3)]	46.28±2.85[3)]	15.18±1.55[2)]
DEX + RAP group	79.81±7.32[5)]	16.75±1.64[5)]	9.76±1.46[5)]	11.21±1.18[4)]
I/R + RAP group	78.19±10.26[5)]	17.22±2.25[5)]	9.32±1.32[5)]	12.59±2.75[4)]

Note: [1)] $p<0.01$ *vs.* SH group, [2)] $p<0.05$ *vs.* I/R group, [3)] $p<0.01$ *vs.* DEX group, [4)] $p<0.05$ and [5)] $p<0.01$.

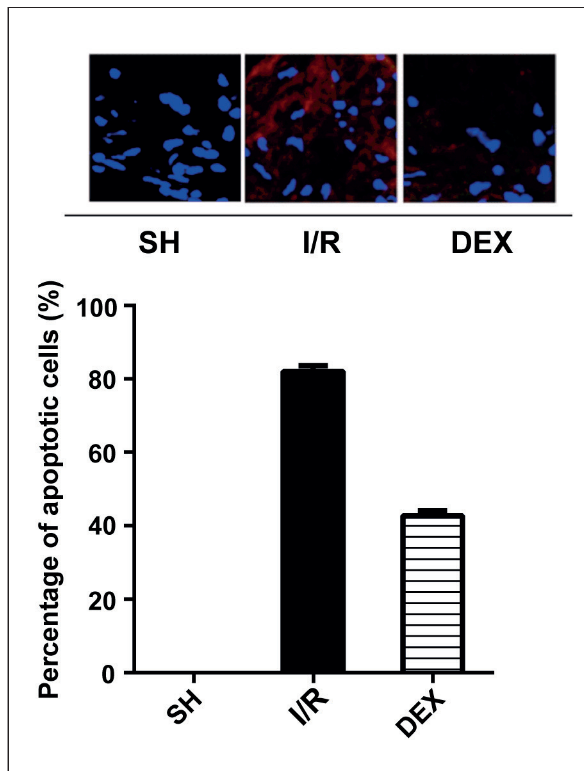

Figure 1. Detection of the apoptosis of rat heart cells via TUNEL assay. Note: Red indicates TUNEL-positive, and blue indicates the nucleus (*p<0.01).

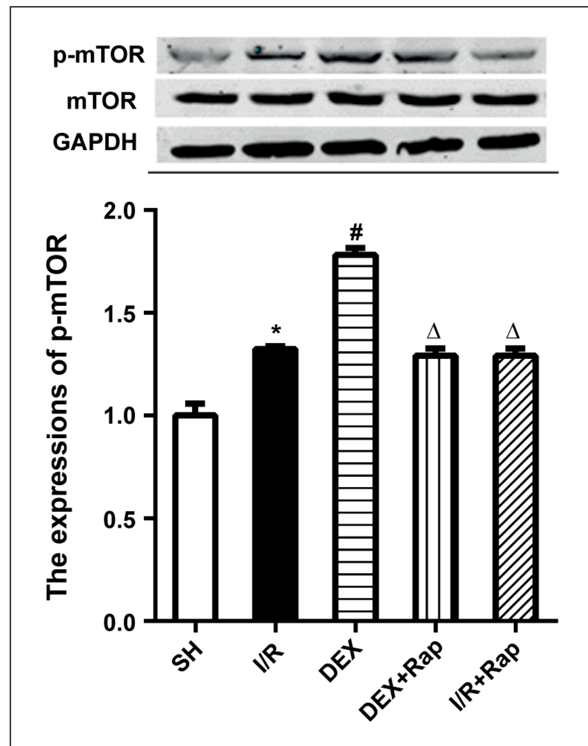

Figure 2. Effects of DEX on the expressions of mTOR and p-mTOR in myocardial I/R rats. Note: *p<0.01 *vs.* SH group, #p<0.01 *vs.* I/R group and Δp<0.01 *vs.* DEX group.

Effects of DEX on Phosphorylation of m-TOR and Akt After Myocardial I/R

As shown in Figure 2-3, the levels of p-mTOR and p-Akt in I/R group were evidently higher than those of SH group (p<0.01). However, the levels of these indexes were significantly higher than those of I/T group after DEX administration based on myocardial I/R model (p<0.01). After the combination of DEX and RAP, the latter canceled the effect of the former on enhancing the expression of p-mTOR and the phosphorylation level of mTOR. However, no significant changes were found in the expressions of mTOR and AKT in each group. The above results indicated that the protective effect of DEX on I/R injury was related to PI3K-Akt-mTOR signaling pathway. In this study, the results revealed that after the establishment of I/R model, DEX treatment and mTOR inhibitor treatment could not significantly change the mRNA level of mTOR (Figure 4). This suggested that the influence of DEX on PI3K-Akt-mTOR signaling pathway could not affect the expression level of mTOR. However, it remarkably promoted the phosphorylation level of mTOR.

Discussion

At present, the treatment of coronary heart disease is mainly to improve coronary artery perfusion through surgery or medicine. However, ischemic myocardial cells usually release a large number of inflammatory mediators during the process of blood flow reperfusion. This may cause metabolic dysfunction of myocardial cells, degeneration and necrosis of myocardial cells and oxidative stress reaction. Eventually, this results in secondary cardiovascular damage, namely, myocardial I/R injury[4]. Therefore, in-depth study on the mechanism and treatment of MIRI is of far-reaching significance for improving the prognosis of cardiovascular diseases[5].

DEX is widely used in clinical anesthesia. It is reported that DEX has a significant improving effect on I/R injury[6]. In this study, the results showed that LVSP and $\pm dp/dt_{max}$ in I/R group were significantly lower than those of SH group, while LVEDP in I/R group was evidently higher (p<0.01). After the administration of DEX, LVSP and $\pm dp/dt_{max}$ were remarkably increased, while

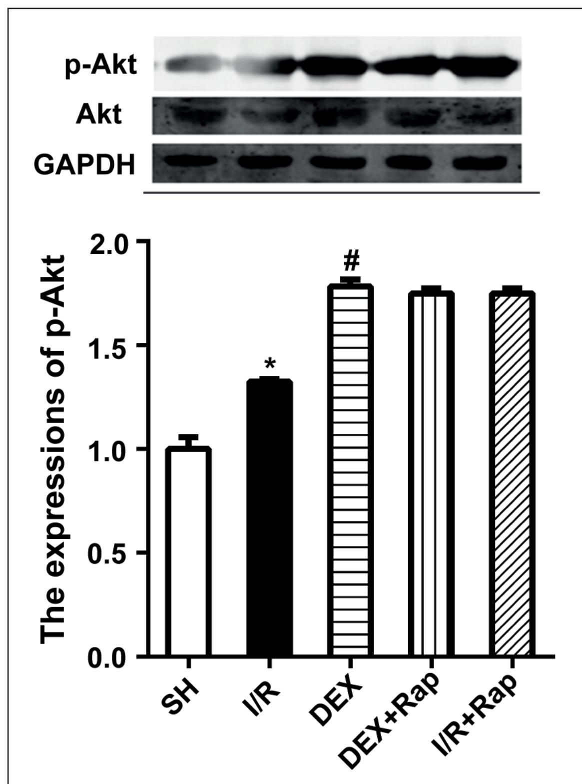

Figure 3. Effects of DEX on the expressions of Akt and p-Akt in myocardial I/R rats. Note: *p<0.01 *vs.* SH group, #p<0.01 *vs.* I/R group and ^Δ^p<0.01 *vs.* DEX group.

LVEDP and infarction area were markedly decreased (p<0.01). The above results indicated that DEX could effectively improve the decline of cardiac function caused by I/R injury, which also exhibited a protective effect on myocardium[7,8]. Meanwhile, it was found that LVSP and $\pm dp/dt_{max}$ were remarkably decreased, while LVEDP and infarction area were increased after treatment with RAP (p<0.01). Compared with those of I/R group, the above indexes did not change significantly. This indicated that PI3K-Akt-mTOR signaling pathway might play an important role in the cardio-protective mechanism of DEX. The PI3K-Akt-mTOR signaling pathway is an important membrane receptor pathway, which plays a key regulatory role in various physiological behaviors of mammalian cells[9]. After activation of PI3K-Akt-mTOR pathway, the anti-apoptosis activity of myocardium can be affected by various ways[10]. Therefore, the cardio-protective effect of DEX may be the role of PI3K-Akt-mTOR signal transduction system. Myocardial I/R injury can cause mitochondrial membrane damage. Meanwhile, it releases CK, CK-MB and other components into the blood. Therefore, the changes of these indexes can reflect the degree of myocardial cell damage[11]. At the same time, it is often accompanied by the release of multiple inflammatory mediators in the process of myocardial I/R[12, 13]. By inducing oxidative stress and cell apoptosis, myocardial injury will continue to be affected[12, 13]. In this study, it was found that the levels of CK, CK-MB, TNF-α and IL-6 in the blood of rats after myocardial I/R were significantly increased (p<0.01). However, the levels of CK, CK-MB, TNF-α and IL-6 were remarkably decreased after DEX administration in I/R rats. After the combination of DEX and RAP, the expression levels of these indexes were significantly increased. However, there were no significant differences found between DEX + RAP group and model group as well as between I/R + RAP group and model group. At the same time, this study revealed that a large number of myocardial cells showed apoptosis after I/R treatment. On this basis, the apoptosis rate of myocardial cells was significantly decreased after the administration of DEX. The above results denoted that DEX could reduce the increased levels of CK, CK-MB, TNF-α and IL-6 caused by I/R. Moreover, PI3K-Akt-mTOR signaling pathway inhibitor blocked the effects of DEX on the expression level of these indexes. Meanwhile, DEX alleviated myocardial cell apoptosis caused by I/R injury. Oxidative stress is one of the key signs of I/R injury. When the myocardium without blood circulation is supplied again, a large number of oxygen free radicals will

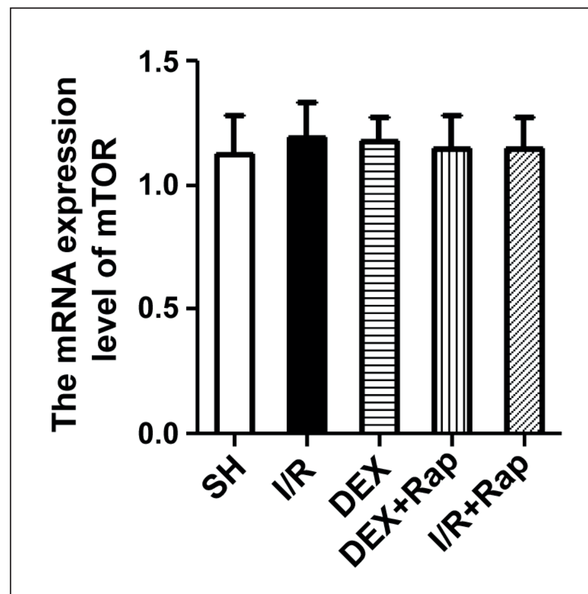

Figure 4. Effects of DEX on the mRNA expression level of mTOR in myocardial I/R rats.

accumulate in the myocardium due to the lack of systematic function of oxygen free radical scavengers. Excessive oxygen free radicals can cause myocardial damage[14,15]. Among them, MDA is an important indicator of lipid peroxidation[16]. Serum levels of SOD, MDA, CAT and GSH-PX in rats can reflect the oxidative stress in myocardium[17,18]. It was found in this study that MDA level in rat serum was notably increased, while the levels of SOD, CAT and GSH-PX were significantly decreased significantly after I/R injury ($p<0.01$). On this basis, after the administration of DEX, MDA level in rat serum was decreased. However, the levels of SOD, CAT and GSH-PX were markedly increased ($p<0.05$, $p<0.01$). After the application of DEX combined with RAP, MDA level was significantly increased, while the levels of SOD, CAT and GSH-PX were remarkably decreased ($p<0.01$). After the addition of RAP, there was no significant change in each index compared with I/R group. This suggested that RAP could block the effects of DEX on the above indexes. To further study the mechanism of DEX against I/R injury, the phosphorylation level of mTOR was detected by Western blotting[19, 20]. According to the results, the level of p-mTOR in I/R group was significantly increased when compared with that of SH group after I/R injury. However, it was evidently increased in DEX group compared with that of I/R group after DEX administration based on myocardial I/R model ($p<0.01$). After the combination of DEX and m-TOR inhibitor, the latter canceled the effect of the former on enhancing the expression of p-mTOR and the phosphorylation level of mTOR. The expression of mTOR did not change significantly in each group. At the same time, RT-qPCR results also showed that the mRNA level of mTOR did not change significantly among groups. This indicated that the effects of DEX on I/R injury was related to PI3K-Akt-mTOR pathway.

Conclusions

In this study we found that DEX, a α_2 receptor agonist, plays a role in myocardial protection against myocardial I/R in rats. It can improve cardiac function in I/R rats, scavenge oxygen free radicals, relieve oxidative stress injury, inhibit inflammatory responses and reduce the release of myocardial enzymes. Furthermore, the effects of DEX on I/R injury are mainly achieved through PI3K-Akt-mTOR pathway.

Conflict of Interests

The authors declared no conflict of interest.

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