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Effects of miR-21 on hypertensive rats through PTEN/PI3K/Akt/mTOR signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effects of micro-ribonucleic acid (miR)-21 on hypertensive rats through the phosphatase and tensin homolog deleted on chromosome ten (PTEN)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway.

MATERIALS AND METHODS: A total of 10 spontaneously hypertensive rats (SHRs) were selected as the model group. Meanwhile, 10 rats with the same age were enrolled in the normal control group. Real Time-fluorescence quantitative Polymerase Chain Reaction (qRT-PCR) was performed to detect the mRNA level of miR-21 in rats of the SHR model group and control group. The tail arterial diastolic pressure of rats in the awake and resting state was measured in both groups, respectively. Pathological sections were prepared to evaluate pathological changes in myocardial tissues. Subsequently, myocardial cells were isolated, cultured and transfected with miR-21 mimics and miR-21 inhibitor, respectively. Transfection efficiency was verified using fluorescence quantitative PCR. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was utilized to determine the apoptosis level of myocardial cells. Furthermore, the expression levels of the signaling pathway-related proteins were detected via Western blotting assay.

RESULTS: Fluorescence quantitative PCR results revealed that the expression level of miR-21 was significantly higher in the SHR model group ($p<0.05$). The diastolic pressure increased markedly in the SHR model group when compared with that in the control group ($p<0.05$). Subsequent hematoxylin and eosin (HE) staining indicated apparent myocardial tissue injury in the SHR model group ($p<0.05$). After transfection, the results showed that miR-21 inhibitor could effectively down-regulate the expression level of miR-21 in myocardial cells ($p<0.05$). Meanwhile, TUNEL staining revealed that the number of apoptotic cells in the miR-21 inhibitor group was remarkably higher than that of the other two groups ($p<0.05$). In addition, Western blotting results manifested that the protein expression levels of PTEN, PI3K, Akt and mTOR were significantly lower in the miR-21 mimics group ($p<0.05$), whereas was remarkably higher in the miR-21 inhibitor group ($p<0.05$).

CONCLUSIONS: MiR-21 is involved in regulating the pathological symptoms and myocardial cell apoptosis in hypertensive rats through the PTEN/PI3K/Akt/mTOR signaling pathway.

Key Words:
MiR-21, Hypertension, PTEN/PI3K/Akt/mTOR signaling pathway.

Introduction

Hypertension, with fairly high incidence and mortality rates, has always been a major public health burden[1]. Patients with hypertension are often accompanied by cardiac structural and functional abnormalities, including left ventricular hypertrophy and systolic/diastolic dysfunction. Meanwhile, heart failure occurs in many cases[2]. In spite of certain progression in the development of anti-hypertensive drugs, the number of hypertension patients is constantly rising. The pathogenesis of hypertension has not been fully clarified yet. Multiple factors can lead to an increase of blood flow and resistance in cardio-cerebral vessels, eventually inducing hypertension. Therefore, most treatment methods cannot efficiently ameliorate the pathological state of patients at present[3,4]. Understanding the pathogenesis of hypertension is of great significance for formulating new prevention and treatment strategies.

Micro-ribonucleic acids (miRNAs) are members of non-coding RNAs, whose roles in the pathogenesis of different diseases have been widely explored[5]. Previous studies[6] have indicated that they participate in the pathological processes of a variety of diseases. Early studies[7] have discovered that miRNA expression is

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associated with hypertension, arrhythmia and fibrosis, among which miR-21 in cardiovascular diseases has been extensively researched. MiR-21 can participate in vascular tension and extension as well as vascular remodeling, and reduce vascular cell apoptosis through several signaling pathways[8]. However, apoptosis eliminates harmful substances in cells, responds relevantly to the invasion of the cell body, supplies energy for the production of subcellular structures and metabolism, and even maintains the stability of cells. Parikh et al[9] have demonstrated that the expression level of miR-21 in myocardial cells of rats with myocardial ischemia is remarkably elevated. This can significantly decrease the apoptosis of myocardial cells, exerting protective effects on ischemia/reperfusion[10]. In addition, it has been revealed[11] that silencing of miR-21 with specific antagonists *in vivo* can reduce fibrosis and dysfunction of heart with pressure overload.

The mechanism of the phosphatase and tensin homolog deleted on chromosome ten (PTEN)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway in the onset of hypertension has been investigated recently[12]. The PTEN/PI3K/Akt/mTOR signaling pathway is involved in various biological functions in the life process. Meanwhile, its functions have been gradually studied and clarified[13]. The loss of PTEN function results in increased activity of Akt and target proteins of the mTOR kinase pathway, thereby promoting cell proliferation[14]. A majority of scholars have focused on exploring the impacts of the signaling pathway on cancers and cancer cells. However, there is little research on its regulatory role in hypertension.

In this research, a spontaneously hypertensive rat (SHR) model was successfully established. Subsequently, miR-21 mimics and miR-21 inhibitor were applied to interfere with the expression of miR-21 in myocardial tissues. Hematoxylin and eosin (HE) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay were adopted to detect the pathological changes in myocardial tissues and apoptosis level of myocardial cells, respectively. In addition, the potential role of miR-21 and the important function of the PTEN/PI3K/Akt/mTOR signaling pathway in the pathogenesis of hypertension were investigated.

Materials and Methods

Animal Grouping and Measurement of Diastolic Pressure

A total of 10 SHRs were selected as the SHR model group. Meanwhile, another 10 normal rats with the same age were enrolled in the control group. Rats in both groups were fed separately in an equivalent environment. A week later, the tail arterial diastolic pressure of rats in the awake and resting state was measured in each group, respectively. This study was approved by the Animal Ethics Committee of Chinese PLA General Hospital Animal Center.

Culture and Transfection of Myocardial Cells

Myocardial tissues were first taken out under sterile conditions. A part of the tissues was used to extract total RNA for detection of miR-21 expression, while others were utilized for extraction of myocardial cells. The cells were cultured in medium containing fetal bovine serum (FBS) and dual antibodies (Gibco, Grand Island, NY, USA), and maintained in an incubator. The medium was changed every 48 hours. Subsequently, the second and third generations of myocardial cells were utilized for experiments. After that, the cells were divided into three groups, two of which were transfected with miR-21 mimics and miR-21 inhibitor according to the instructions of the transfection kit, respectively. Meanwhile, the remaining group of cells was set as the control group. After verification of transfection efficiency, subsequent experiments were conducted.

Pathological Changes in Myocardial Tissues of Rats in Each Group

Hematoxylin and eosin staining (HE; Boster, Wuhan, China) was applied to detect the pathological injury of myocardial tissues of rats in each group. The dissected heart was first soaked in formalin for 7 d. Then, myocardial tissues were washed with running water for 24 h, followed by dehydration with graded alcohol to prepare conventional sections. After deparaffinization, the sections were hydrated in 95%, 90%, 80%, 75% and 50% ethanol, followed by clearing, dipping and embedding in paraffin. Next, paraffin-embedded blocks were prepared into pathological sections. Thin sections were then baked dry for HE staining. Finally, mounting and tissue observation were conducted under a light microscope.

TUNEL Apoptosis Assay

Apoptotic DNA fragments were subjected to fluorescein isothiocyanate (FITC)-end labeling according to the instructions of TUNEL apoptosis assay kit (Roche, Basel, Switzerland). The images of FITC-labeled TUNEL-positive cells were observed under a fluorescence microscope, and the number of cells was counted. 10 fields of vision were selected for each sample.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied to extract total RNA in myocardial tissues of rats in each group. The purity and concentration of extracted RNA were determined. Subsequently, total RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) strands, with attention to the use of isopropyl alcohol. Primer sequences of target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed according to GenBank. GAPDH was used as an internal reference. The expression levels of target genes were determined *via* quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Primer sequences were shown in Table I. Finally, the relative expression levels of miR-21 and PTEN in myocardial tissues of rats in each group were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blotting Assay

Cardiac tissues of rats were cut into pieces, weighed and added with radio-immunoprecipitation assay (RIPA) lysis buffer at a ratio of 100 mg: 1 mL for tissue homogenization (Beyotime, Shanghai, China). The concentration of total protein in myocardial tissues in rats of each group was measured *via* bicinchoninic acid (BCA) protein assay kit (Pierce, Waltham, MA, USA). After that, protein samples and gel were prepared and loaded for electrophoresis, followed by membrane transfer and sealing with skimmed milk. After that, the membranes were incubated with primary antibodies overnight, followed by incubation with corresponding secondary antibody for 1 hour. Freshly prepared enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) mixture was added for image development in a dark room, followed by treatment of bands with software. Protein bands were then scanned and quantified using an Odyssey membrane scanner. The level of proteins to be detected was corrected *via* GAPDH. Image Lab software was employed to quantify the bands of Western blotting. Finally, the expression levels of corresponding proteins in each group were calculated.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Experimental results were presented as mean ± standard deviation. *t*-test was used to compare the difference between the two groups. One-way ANOVA was applied to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Changes in Tail Arterial Diastolic Pressure of SHRs

One week later, the tail arterial diastolic pressure of rats in resting state was measured in each group. As shown in Figure 1, the tail arterial diastolic pressure increased significantly in the SHR group when compared with the control group ($p<0.05$).

Expression Level of MiR-21 in Myocardial Tissues of Rats

QRT-PCR results indicated that the expression level of miR-21 in myocardial tissues of the SHR group was notably higher than that of the control group ($p<0.05$; Figure 2).

Pathological Changes in Myocardial Tissues of Rats in Each Group

The pathological injury of myocardial tissues of hypertensive rats in each group was deter-

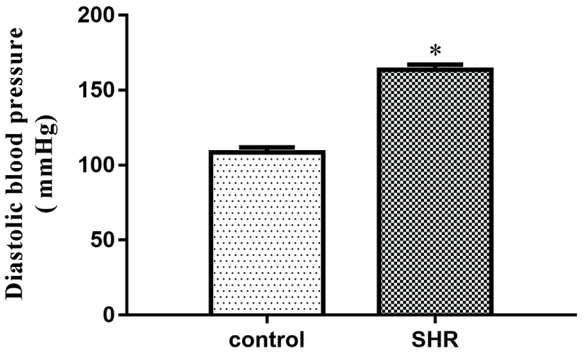

Figure 1. Changes in tail arterial diastolic pressure of SHRs. *$p<0.05$ *vs.* control group.

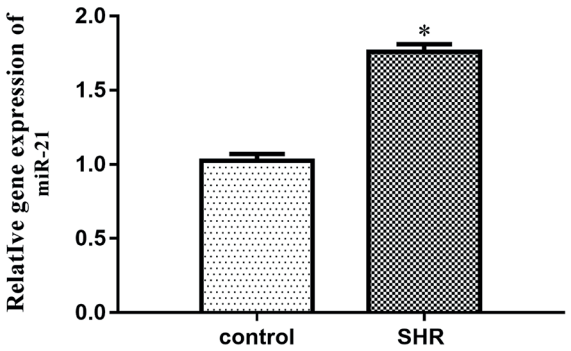

Figure 2. Expression level of miR-21 in myocardial tissues of rats. *p<0.05 *vs.* control group.

mined *via* HE staining. As shown in Figure 3, in comparison with the control group, the SHR group showed a significantly higher degree of myocardial tissue injury, with massive infiltration of inflammatory cells (p<0.05). Combined with Figure 2, it could be found that the expression of miR-21 increased markedly in the SHR group, and myocardial damage was much more severe.

Transfection Efficiency in Myocardial Cells of Each Group

After transfection, the relative expression level of miR-21 was measured *via* qRT-PCR, to verify transfection efficiency. The results demonstrated that the expression of miR-21 in myocardial cells of the miR-21 mimics group was markedly elevated when compared with the control group (p<0.05). However, it decreased significantly in the miR-21 inhibitor group in comparison with the control group (p<0.05; Figure 4).

Apoptosis Level of Myocardial Cells in Rats of Each Group

The apoptosis level of myocardial cells in rats of each group was detected *via* TUNEL staining. The results manifested that there was a small amount of TUNEL-positive cells in the miR-21 mimics group and control group. Moreover, the number of TUNEL-positive myocardial cells in the miR-21 inhibitor group was significantly higher than that of the control group (p<0.05; Figure 5).

Expressions of PTEN/PI3K/Akt/mTOR Signaling Pathway-Related Proteins Regulated by MiR-21

The expression of the PTEN/PI3K/Akt/mTOR signaling pathway-related proteins in myocardial tissues of rats was determined by Western blotting (Figure 6). Compared with the control group, the protein expression of PTEN increased evidently in the miR-21 inhibitor group (p<0. 05), while was markedly reduced in the miR-21 mimics group (p<0.05). Moreover, the protein expressions of PI3K, Akt and mTOR were remarkably up-regulated (p<0.05).

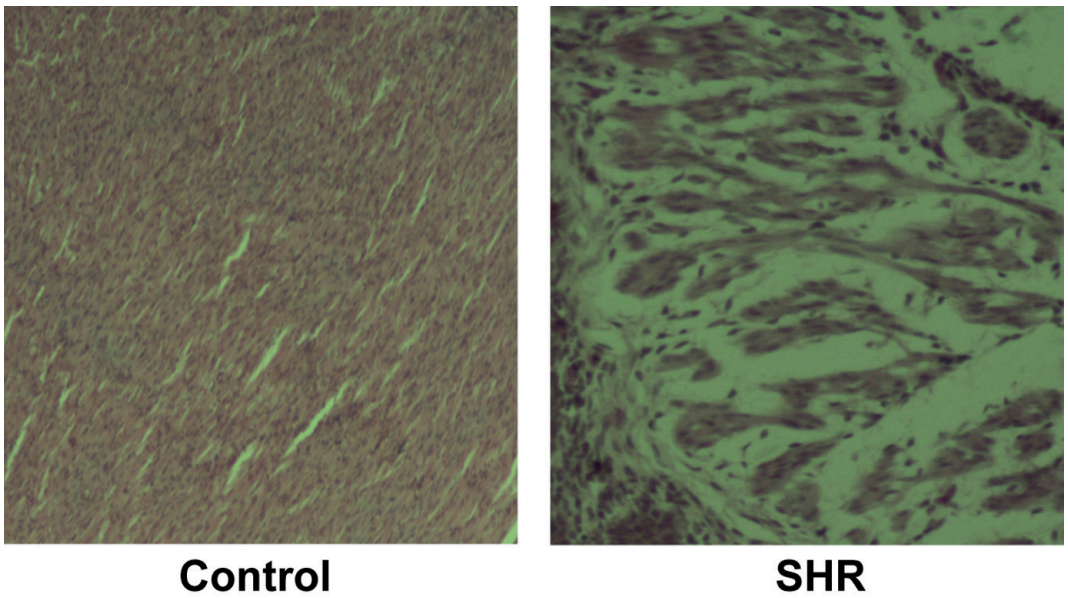

Figure 3. Pathological changes in myocardial tissues of rats in each group detected *via* HE staining (magnification: 100×). SHR group manifested significantly higher degree of myocardial tissue injury than control group, *p<0.05 *vs.* control group.

Discussion

Hypertension is generally accompanied by the occurrence of chronic diseases related to the heart, brain, kidney, etc. As a serious public health problem worldwide, it is estimated that 6% of global deaths are caused by hypertension[15,16]. Currently, the pathogenesis of hypertension remains unclear. A large amount of research evidence has found that miR-21 exerts regulatory effects on various cells, such as myocardial cells, vascular smooth muscle cells and vascular endothelial cells. In recent years, studies have discovered that miRNAs can regulate and participate in diversified cell processes of normal development and disease attack. Meanwhile, they are considered to contribute to the development of multiple diseases. Among them, miR-21 is an important cell regulatory molecule that is widely investigated. Previous studies have indicated that miR-21 can exert its anti-apoptotic effect through PTEN/PI3K/Akt. Akt is activated by many growth factors and cytokines in a PI3K-dependent manner. Tong et al[17] have shown that miR-21 overexpression is closely correlated with myocardial cell proliferation and myocardial remodeling. In this research, an SHR model was successfully established. MiR-21 mimics and miR-21 inhibitor were applied to interfere with the expression of miR-21 in myocardial tissues. To further investigate the impacts of miR-21 on the proliferative capacity of cells, TUNEL assay was utilized to evaluate the apoptosis level in each group. At the same time, HE staining was performed to determine the pathological changes in myocardial tissues. The results showed that the expression level of miR-21 in the SHR model group and miR-21 mimics group was significantly elevated, which was of important significance for the regulatory role of miR-21 in hypertension. Moreover, the tail arterial diastolic pressure increased markedly in the SHR model group. The results of HE staining indicated severe myocardial tissue injury in the SHR model group as well. According to the TUNEL staining results, the number of apoptotic cells increased remarkably. Western blotting demonstrated that the protein expression of PTEN was significantly up-regulated, while the protein expressions of PI3K, Akt and mTOR were markedly down-regulated in the miR-21 inhibitor group, which were consistent with the findings of Roy et al[18] and Shi et al[19]. The above-mentioned results could serve as a basis for the development of therapeutic methods for hypertension. In addition, our findings provided new insights into the regulation of miR-21 on hypertension.

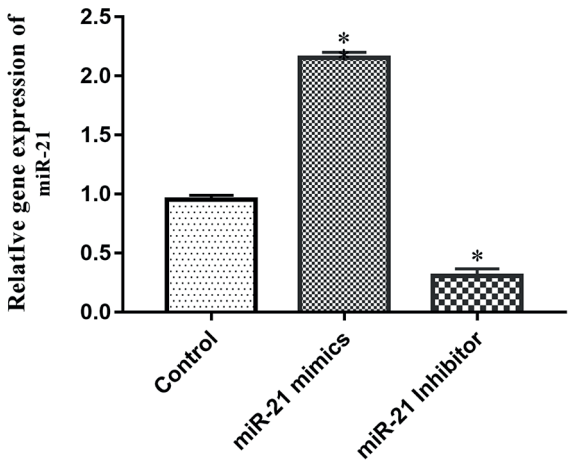

Figure 4. Expression of miR-21 in myocardial cells of each group after transfection. The expression of miR-21 was significantly elevated in miR-21 mimics group, *$p<0.05$ *vs.* control group.

PTEN was first discovered as a suppressor gene involved in the occurrence of a variety of diseases[20,21]. Silencing PTEN expression has been found to accelerate cell proliferation[22]. Researchers have revealed that PTEN inhibits the activity of Akt by repressing PI3K activity. Akt participates in angiogenesis and metastasis, and partially prolongs survival signal[21]. PTEN deficiency may result in continuous activation of the signaling pathways, thereby losing the control on cell growth. Therefore, up-regulation of PTEN enhances the apoptosis of myocardial cells, while its inactivation will reduce cell apoptosis[23]. Previous studies have reported that apoptosis is able to clean harmful substances in cells, respond correspondingly to the invasion of the cell body, supply energy for the production of subcellular structures and metabolism, and even maintain the stability of cells. Activated PI3K/Akt signaling pathway exerts crucial influences on the differentiation, proliferation and apoptosis of smooth muscle cells and vascular fibroblasts[24]. However, the potential role of PTEN/PI3K/Akt/mTOR in anti-apoptosis has not been fully elucidated. Meanwhile, the downstream elements of the PTEN/PI3K/Akt signal need to be defined[25]. Previous studies have manifested that miR-21 is capable of controlling the occurrence of diseases by targeting PTEN. However, it should be realized that there are more than

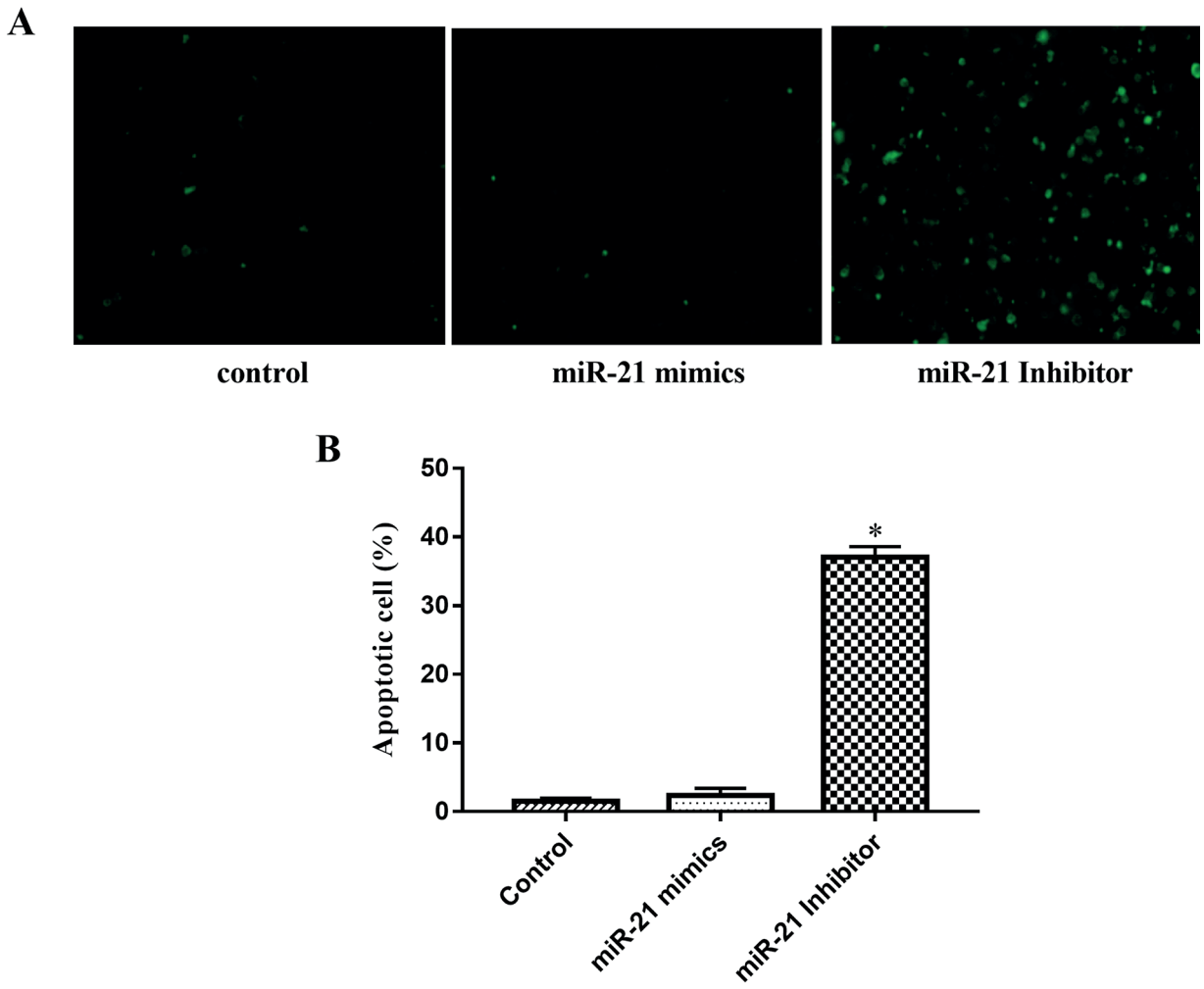

Figure 5. Apoptosis level in myocardial tissues of rats in each group detected *via* TUNEL staining. ***A,*** TUNEL staining (magnification: 40×). ***B,*** Apoptosis rate. The number of TUNEL-positive myocardial cells in the miR-21 inhibitor group was markedly greater than that of the control group.

one target genes of miR-21[26]. A latest study[27] has indicated that miR-21 probably mediate the proliferation, apoptosis, migration, invasion and cell cycle progression of human esophageal cancer cells through targeting key proteins of the PTEN/PI3K/Akt signaling pathway.

Currently, studies[28] have revealed that multiple gene mutations in the PTEN/PI3K/Akt signaling pathway have cumulative impacts, which may further strengthen its predictive ability. The PI3K/Akt/mTOR signaling pathway is a typical pathway regulating various normal cell processes, such as survival, proliferation, growth and movement[29]. In-depth research on the pathway has demonstrated that the PI3K/Akt/mTOR signaling pathway plays an important role in hypertension regulation[30]. In this research, the SHR model was first successfully established. The expression of miR-21 in myocardial tissues interfered with transfection of miR-21 mimics and miR-21 inhibitor, respectively. Furthermore, HE staining and TUNEL assay were employed to examine the pathological changes in myocardial tissues and apoptosis level of myocardial cells in rats, further verifying the regulatory role of the PTEN/PI3K/Akt/mTOR signaling pathway in hypertension. Our findings provided new perspectives for the regulation of miR-21 on the pathogenesis of hypertension. In addition, combined with clinical practice verification, our research might lay a certain theoretical basis for related studies, as well as prevention and treatment of hypertensive disorders as a biomarker.

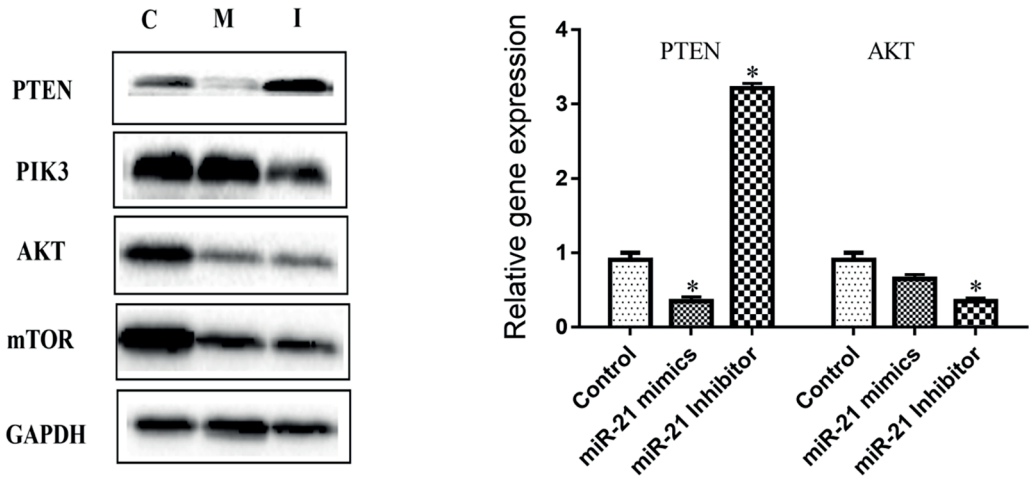

Figure 6. Relevant protein expressions detected by Western blotting. C: control group. M: miR-21 mimics group. I: miR-21 inhibitor group. *$p<0.05$ *vs.* control group.

Conclusions

We found that miR-21 is involved in regulating the pathological symptoms and myocardial cell apoptosis in hypertensive rats through the PTEN/PI3K/Akt/mTOR signaling pathway.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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