Doxycycline Attenuates Atrial Remodeling by Interfering with MicroRNA-21 and Downstream Phosphatase and Tensin Homolog (PTEN)/Phosphoinositide 3-Kinase (PI3K) Signaling Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCDEF Kai Zhang*
BCDEF Linru Zhao*
BC   Zuowang Ma
BC   Weiding Wang
BC   Xiongfeng Li
B   Yue Zhang
B   Meng Yuan
DF   Xue Liang
AG   Guangping Li

Tianjin Key Laboratory of Ionic-Molecular Function of Cardiovascular Disease, Department of Cardiology, Tianjin Institute of Cardiology, The Second Hospital of Tianjin Medical University, Tianjin, P.R. China

Background: Atrial remodeling especially in the form of fibrosis is the most important substrate of atrial fibrillation (AF). The aim of this study was to investigate the effects of doxycycline on chronic intermittent hypoxia (CIH)-induced atrial remodeling and the pathophysiological mechanisms underlying such changes.

Material/Methods: A total of 30 Sprague-Dawley rats were randomized into 3 groups: Control group, CIH group, and CIH with doxycycline treatment group. CIH rats were subjected to CIH 6 h/d for 30 days and treatment rats were administrated doxycycline while they received CIH. After the echocardiography examination, rats were sacrificed at 31 days. The tissues of atria were collected for histological and molecular biological experiments, Masson staining was used to evaluate the extent of atrial fibrosis, microRNA-21, and its downstream target phosphatase and tensin homolog (PTEN), phosphoinositide 3-kinase (PI3K) were assessed.

Results: Compared to the control group, the CIH rats showed higher atrial interstitial collagen fraction, increased microRNA-21, PI3K levels, and decreased PTEN levels. Doxycycline treatment attenuated CIH-induced atrial fibrosis, reduced microRNA-21 and PI3K, and increased PTEN.

Conclusions: CIH induced significant atrial remodeling, which was attenuated by doxycycline in our rat model. These changes may be explained due to alterations in the microRNA-21-related signaling pathways by doxycycline.

MeSH Keywords: Atrial Remodeling • Doxycycline • MicroRNAs • Phosphatidylinositol 3-Kinases • PTEN Phosphohydrolase

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/909800

📄 2083   ▦ 3   📊 5   📖 25

Background

Atrial fibrillation (AF) is one among the most common arrhythmias in clinical practice [1]. Although treatment of AF has substantially advanced in recent years, numerous problems remain, and a fundamental breakthrough has not been made in the treatment of AF. MicroRNA is a class of non-coding ribonucleic acid molecules 22 nucleotides in length, which is usually bound to the 3 'UTR of an mRNA, thereby inhibiting the translation process or promoting the degradation of mRNA [2]. Recently, microRNA-21 and its downstream target PTEN, a potent inhibitor of the PI3K pathway, have been shown to play important roles in various pathophysiological activities [3], and some studies have shown that these factors may be involved in the occurrence and development of AF [4,5].

Doxycycline is a semi-synthetic tetracycline antibiotic. Studies have shown that doxycycline can improve pulmonary fibrosis and prognosis of endoluminal aneurysm repair [6,7], suggesting that doxycycline has an intervention effect on cell injury repair. Matrix metalloproteinases (MMPs) may serve as intervention targets of doxycycline [8]. However, there are few reports on the role of doxycycline in preventing atrial fibrosis. Whether doxycycline can regulate the upstream signaling pathway of MMPs and other signaling pathways is not yet clear. We established a rat model of atrial remodeling by CIH and investigated the intervention effect of doxycycline on atrial remodeling and upstream signaling pathways.

Material and Methods

Experimental protocol

This study was approved by the Experimental Animal Administration Committee of Tianjin Medical University. ARRIVE guidelines were followed in our experiments. Thirty male Sprague-Dawley rats were randomized into 3 groups: a control group, a chronic intermittent hypoxia (CIH) group, and a CIH with doxycycline treatment group. To evaluate the effects of doxycycline on CIH-induced atrial structural remodeling, rats in the treatment group were administrated doxycycline (30 mg/kg/d; intragastrically) for 30 days while receiving CIH. In each group, 5 rats were randomly selected for histological experiments and the other 5 rats were used for molecular biological experiments.

CIH and treatment rats were put into chambers and subjected to CIH. An oxygen concentration the analyzer was installed in the chamber, which is capable of regulating the switch of the charging valve in line with the concentration of oxygen in the chamber. Briefly, this consisted of cycling the oxygen concentration inside chamber at between 21% and 8%. This was achieved by alternately flushing with 100% nitrogen to gradually reduce oxygen concentration to 8%. Chambers were then flushed with oxygen to gradually increase oxygen concentration back to 21%. the time for CIH was 6 h/d for 30 days. The rats were sacrificed after echocardiography was completed at 31 days, and atrial tissues were used for histological and molecular biological experiments.

Echocardiography

After weighing, rats were anesthetized with isoflurane and fixed on the animal platform, and the animal-oriented Doppler ultrasonic machine was used for echocardiography. The VisualSonicsVevo2100 imaging system was used to acquire and anatomize the images. Left atrial diameter (LAD), left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), and pulmonary artery acceleration time (PAT) were assessed. The mean pulmonary artery pressure (mPAP) was acquired in line with the formula proposed by Haham (79-0.45×PAT) and left ventricular ejection fraction (LVEF) was acquired in line with the Teichholtz formula ($V=7.0/(2.4+D)×D^3$), taking the average of 3 cardiac cycles to acquire data.

Sample collection

After the echocardiography was completed, rats were anesthetized with 10% chloral hydrate (1 mg/kg), we cut the skin along the sternal midline, separated the subcutaneous tissues, and exposed the heart. Hearts were quickly removed and rinsed with PBS. Additionally, left atria and right atria comprising atrial appendages were attained and fixed in 10% formaldehyde for 48 h and embedded in paraffin after dehydration. Samples for molecular biological experiments were rapidly frozen in liquid nitrogen.

Histological studies

Samples were embedded in paraffin and stored at 4°C, after which they were sliced into 5-μm sections, heated for 1 h at 60°C, and corresponding staining was performed. Immunohistochemistry staining was used to identify the protein expression of PTEN and PI3K in atrial tissues. Masson staining was used to anatomize the extent of atrial fibrosis; fibers in the tissue appeared blue and myocardial tissue appeared red. We observed the extent of atrial fibrosis by calculating the collagen fraction, defined as [blue area/(red area + blue area)]. Left atria and right atria were cut into least 5 sections per animal. Each section was examined in 5 randomly-selected high-power fields (×400) and middle-power fields (×200) and blood vessels and epicardial tissues were excluded. Stained images were digitized and anatomized using Image-Pro Plus 7.0 software.

Table 1. Primers used for real-time PCR.

Gene	Primers	Temperature (°C)
miRNA-21 stem-loop primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATTTGG	75.04
miRNA-21 forward primer	GCCGAGCTGGTAAAATGGAA	56.00
Universal reverse primer	GTATCCAGTGCAGGGTCCGAGGT	63.77
U6	FP: CTCGCTTCGGCAGCACA	56
	RP: AACGCTTCACGAATTTGCGT	
GAPDH	FP: GGCACAGTCAAGGCTGAGAATG	56
	RP: ATGGTGGTGAAGACGCCAGTA	
PTEN	FP: ACTGCAGAGTTGCACAGTATC	56
	RP: GTCCGTCCTTTCCCAGCTTTA	

F – forward; R – reverse.

Table 2. Weight parameters and echocardiography parameters.

Parameter	Control (n=10)	CIH (n=10)	Treatment (n=10)	P value
Body weight (g)	432.4±12.60	425±5.46	405.6±20.49	0.001
Heart weight (g)	1.24±0.07	1.48±0.07	1.30±0.13	<0.001
LAD (mm)	4.02±0.56	3.89±0.44	3.68±0.46	0.299
LVEDD (mm)	7.03±0.55	6.64±0.70	6.44±0.46	0.085
LVESD (mm)	4.63±0.44	4.55±0.65	4.18±0.54	0.167
PAT (ms)	38.40±2.16	26.86±5.22	26.16±6.17	
LVEF (%)	64±3.76	60.24±8.73	65.28±9.03	0.309
mPAP (mmHg)	52.72±0.97	57.91±2.35	58.23±2.78	<0.001

LAD – left atrial diameter; LVEDD – left ventricular end diastolic dimension; LVESD – left ventricular end systolic dimension;
PAT – pulmonary artery acceleration time; mPAP – mean pulmonary artery pressure; LVEF – left ventricular ejection fraction.

Real-time PCR

Total RNA was extracted with Trizol reagent (Life Technologies, USA), isopropanol, and chloroform, and the RNA was then reverse-transcribed into cDNA using a reverse transcription kit (TIANGEN Biotech, Beijing), RT-qPCR was performed using the 7500 Real-Time PCR System with SYBR green fluorescent dyes (TransGen Biotech, Beijing). All of the reactions were repeated at least 2 times, with at least 3 replicates for every sample. Cycle threshold (Ct) data were collected, and $2^{-\Delta\Delta Ct}$ method was used to compare with mRNA level. U6 and GAPDH were used as internal controls. The primers used are shown in Table 1.

Western blot

Total protein was extracted with RIPA lysate (KeyGEN BioTECH, Nanjing) and PMSF (protease inhibitor, KeyGEN BioTECH,

Nanjing). The lysates were centrifuged at 12 000 rpm for 20 min and the supernatants were collected. Samples (10 µl) were run on a SDS-PAGE gel followed by blotting to a nitrocellulose membrane. Membranes were blocked with 5% skim milk for 2 h at room temperature, then the membranes were incubated overnight at 4°C with the following primary antibodies: β-actin (1: 4000 Proteintech, USA), PTEN (1: 10000 Abcam, USA), and PI3K (1: 2000 Cell Signaling TECHNOLOGY, USA). Before adding secondary antibody (1: 10 000 PROMEGA, USA), we washed the membranes with TBST 3 times followed by incubation for 2 h at room temperature. Rhea ECL (US EVERBRIGHT INC, Suzhou) was used as developer reagent and the band intensity was assessed using Image J lab software.

Statistical analysis

All data are presented as mean ± standard deviation and were compared using one-way ANOVA followed by Bonferroni correction.

Table 3. The atrial collagen fraction in the three groups.

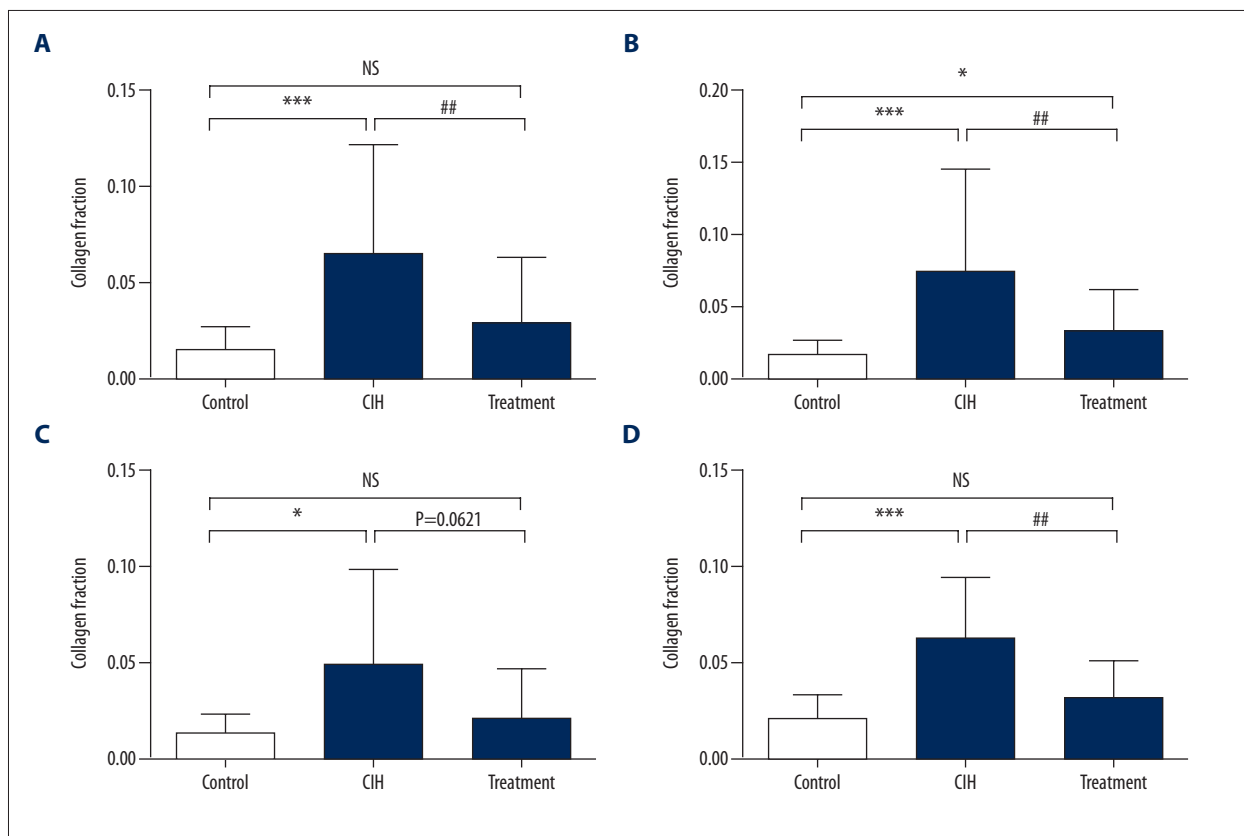| Fields	Control (n=5)	CIH (n=5)	Treatment (n=5)
LA (400×)	0.0150±0.0023	0.0640±0.0084	0.0284±0.0069
RA (400×)	0.0158±0.0022	0.0738±0.0102	0.0324±0.0058
LA (200×)	0.0133±0.0027	0.0485±0.0106	0.0207±0.0068
RA (200×)	0.0202±0.0038	0.0613±0.0065	0.0308±0.0063

LA – left atria; RA – right atria.

Figure 1. Comparison of collagen fraction between 3 groups (n=5). (**A**) High-power fields of left atria (×400); (**B**) High power fields of right atria (×400); (**C**) Middle power fields of left atria (×200); (**D**) Middle power fields of right atria (×200). * Significant difference between the control and CIH groups, ** $P<0.01$, *** $P<0.001$. # Significant difference between CIH and treatment groups, ## $P<0.01$. NS – not significant.
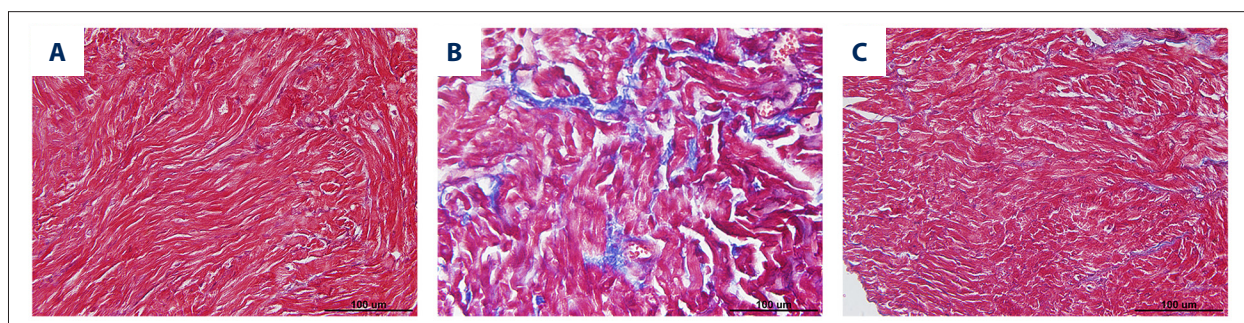

Figure 2. Photomicrographs of Masson staining (n=5). (**A**) Control group; (**B**) CIH group; (**C**) Treatment group.
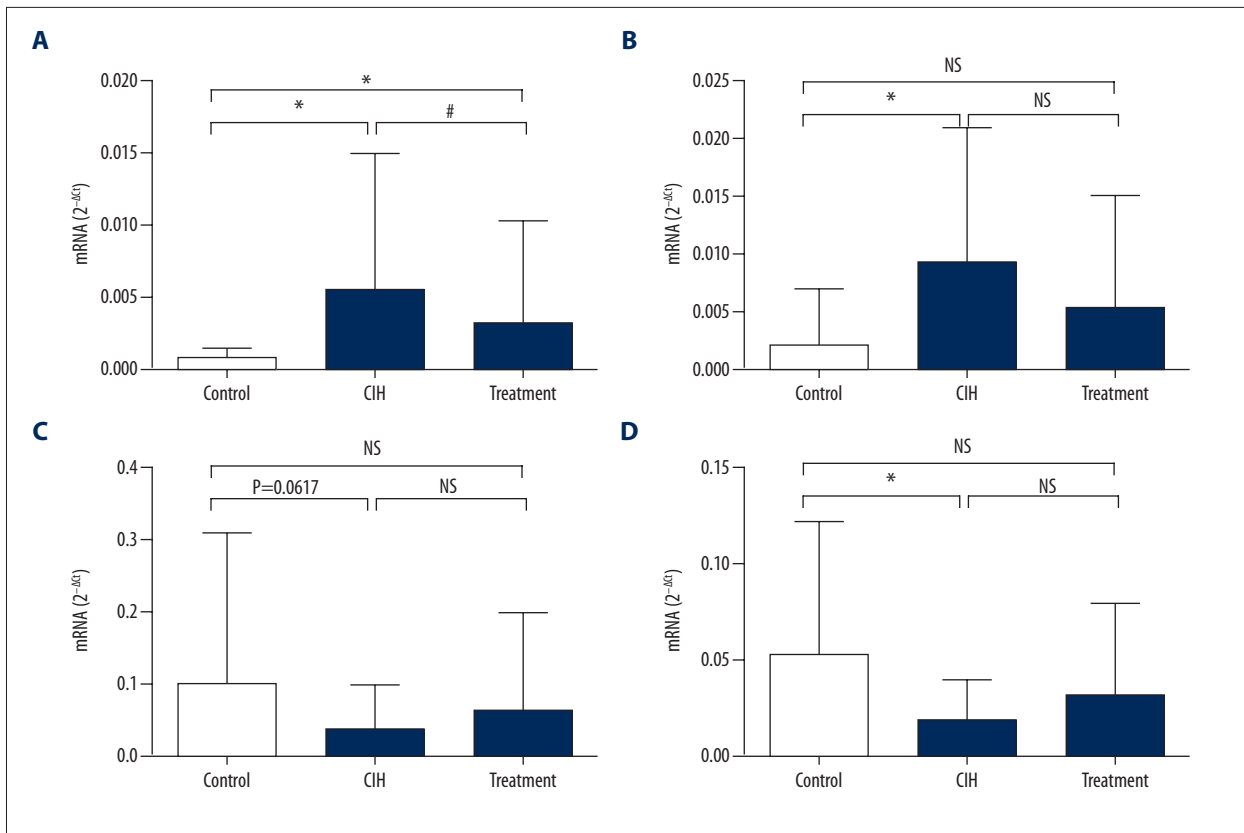
Figure 3. mRNA levels of microRNA-21 and PTEN determined by RT-qPCR (n=5). (**A**) Comparison of microRNA-21 in left atria; (**B**) Comparison of microRNA-21 in right atria; (**C**) Comparison of PTEN in left atria; (**D**) Comparison of PTEN in right atria. * Significant difference between the control and CIH groups. # Significant difference between CIH and treatment groups. NS – not significant.

SPSS 22.0 statistical software was used for data analysis, and P value <0.05 was regarded as statistically significant.

Results

Body and heart weights and parameters assessed by echocardiography

Body weights were higher in the control group compared to the treatment group, and body weights in the CIH group fell between these values. Heart weights in the CIH group were significantly higher than in the control group and treatment group. The findings of echocardiography are detailed in Table 2. The mean pulmonary artery pressures in the CIH and treatment group were markedly higher than in the control group. No significant differences in LAD, LVEDD, LVESD, and LVEF were observed among the control, CIH, and treatment groups.

CIH-induced atrial fibrosis can be attenuated by doxycycline

As exhibited in Table 3 and Figures 1, 2, compared to the control group, CIH induced a significant atrial interstitial collagen deposition, and this change was significantly attenuated by doxycycline.

MicroRNA-21 upregulation and PTEN downregulation were associated with atrial remodeling

The expression levels of microRNA-21 and PTEN are shown in Figure 3. Compared to the control group, microRNA-21 was significantly upregulated in the CIH group and this upregulation was significantly attenuated by doxycycline. PTEN, a downstream target of miR-21, was downregulated in the CIH group compared to the control group and was upregulated in the treatment group.

Consistent with the results of mRNA expression level, PTEN protein levels were significantly lower and PI3K protein levels were significantly higher in the CIH group compared to the
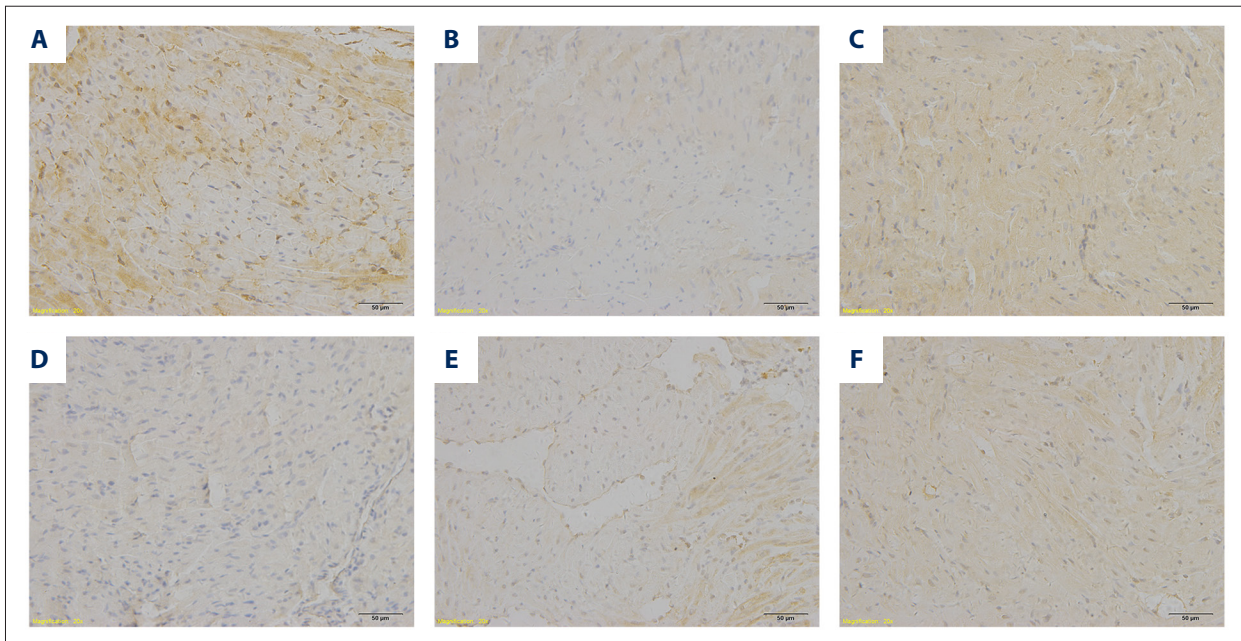
Figure 4. Photomicrographs of Immunohistochemistry staining (n=5). Protein levels of PTEN and PI3K determined by immunohistochemistry staining showed that protein levels of PTEN in the CIH group (**B**) were lower than in the control group (**A**), and protein levels of PTEN in the treatment group (**C**) fell between these values. Protein levels of PI3K in the CIH group (**E**) were higher than in the control group (**D**), whereas protein levels of PI3K in the treatment group (**F**) were lower.

control group. All of these changes were improved in the treatment group (Figures 4, 5).

Discussion

AF is an important problem in cardiovascular diseases in the 21st century. The incidence rate of AF gradually increases with age [9]. Electrical and structural remodeling of the atria are mechanisms and substrates for the occurrence and maintenance of AF [10], and many genes, including microRNAs, are associated with AF [11,12]. Atrial fibrosis is perceived to be the most crucial structural remodeling in AF [13]; therefore, animal models of atrial fibrosis are often used for studying AF. At present, there is still no effective method for the prevention and treatment of AF, but suppression of atrial fibrosis and its signaling pathways may help to prevent and control AF. Related studies have shown that microRNA exerts a crucial role in the pathophysiological mechanism of arrhythmia [14]. Some microRNAs have been confirmed to be related to the occurrence and development of AF [15]. Among them, microRNA-21 is strongly involved in myocardial fibrosis [16]. Cardin et al. [17] reported that microRNA-21 knockout significantly inhibited the process of atrial fibrillation and the induction rate of AF after myocardial infarction. Doxycycline, as an antibiotic, has been applied in clinical practice for many years. Bhattacharyya et al. [18] showed that doxycycline merited an appropriate clinical trial in the management of idiopathic pulmonary fibrosis and related studies suggested that doxycycline

can improve the clinical outcomes of patients with cystic fibrosis exacerbations or lymphangioleiomyomatosis [19,20]. In addition, Hori et al. [21] showed that doxycycline attenuates isoproterenol-induced myocardial fibrosis in rats. Therefore, it is of great clinical significance to exploring the intervention effect of doxycycline on atrial fibrosis.

Our study showed that CIH can promote the atrial structural remodeling in rats. The degree of atrial fibrosis in the model group was significantly increased compared to the control group and treatment group. However, doxycycline improved atrial fibrosis (atrial collagen fractions in the control group, model group, and intervention group were 1.50±0.23%, 6.40±0.84%, and 2.84±0.69%, respectively), perhaps because improving fibrosis is associated with the changes of microRNA-21 and its downstream pathway. Body weights of rats in the model group and treatment group was lower than in the control group, which may be explained by CIH or drug intervention. Additionally, CIH increased of the heart weights of rats in the model group. Moreover, the pulmonary artery pressures of rats in the model group and treatment group were significantly higher than in the control group, which may be the result of CIH.

PTEN/PI3K signaling pathway was involved in a variety of physiological activities, including cell proliferation, differentiation, apoptosis, which participate in the pathophysiology of various diseases [22]. The target genes of microRNA-21, namely, PTEN, are PI3K signaling pathway inhibitor. Overexpression of PTEN inhibit the PI3K signaling pathway. Jiang et al. [23] have
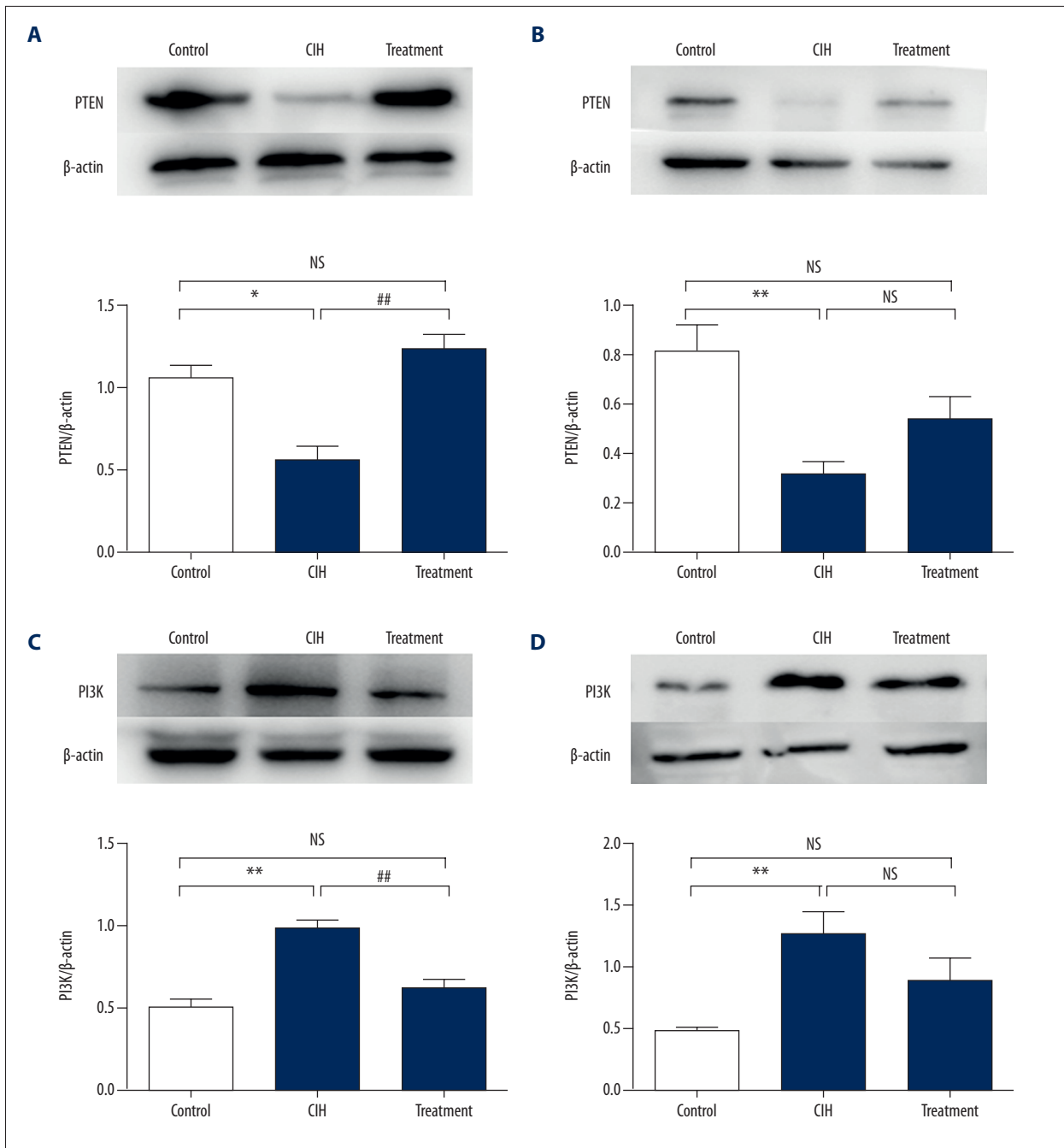
Figure 5. Protein levels of PTEN and PI3K determined by Western blot (n=5). (**A**) Comparison of PTEN in left atria; (**B**) Comparison of PTEN in right atria; (**C**) Comparison of PI3K in left atria; (**D**) Comparison of PI3K in right atria. * Significant difference between the control and CIH groups, ** P<0.01. # Significant difference between CIH and treatment groups, ## P<0.01. NS – not significant.

shown that upregulation of microRNA-21 inhibited PTEN expression and activated the PI3K pathway, thus exerting anti-proliferation effects in rat primary VSMCs. Yang et al. [24] showed that microRNA-21 targeted PTEN increasing the PI3K pathway and finally the activation of this pathway counteracted the apoptotic effect of hypoxia/reperfusion in cardiomyocytes.

Wang et al. [25] also showed that microRNA-21/ERK plays an important role in the pathophysiology of atrial remodeling. The present study showed that microRNA-21 expression in the model group was significantly higher than that in control group. Protein and mRNA expression levels of its target gene PTEN in the model group were decreased, while expression

levels of PI3K were increased. Compared with the model group, expression level of each factor in the treatment group was elevated. Our results suggest that microRNA-21 negatively regulates PTEN. Overexpression of microRNA-21 decreased expressions of PTEN, thereby activating the PI3K signaling pathway, which may play a crucial role in initiation and maintenance of atrial structural remodeling.

Our results show that aberrant expression of microRNA-21 can contribute to cardiovascular diseases by modulating PTEN expression and PTEN-dependent pathways, downregulation of PTEN and overexpression of microRNA-21 may play important roles in atrial remodeling in rats, and doxycycline may improve atrial remodeling by affecting microRNA-21 and its downstream signaling pathway. Further studies are needed to elucidate the mechanism of microRNAs and related signaling pathways in atrial remodeling, which may provide new ideas for clinical treatment of AF.

Limitations

Some limitations should be taken into consideration when interpreting our results. First, we did not perform electrophysiological or AF inducibility studies, so increased inducibility cannot be shown from our results. However, we showed that CIH induced atrial fibrosis, a hallmark of AF and a well-known AF promoter, which can be attenuated by doxycycline. Future studies focusing on AF inducibility and electrical remodeling are warranted. Second, we did not determine whether doxycycline has a direct effect on the expression of PTEN/PI3K.

Conclusions

CIH induced significant atrial remodeling in our rat model, which was attenuated by doxycycline in our rat model. These changes may be explained due to alterations in the microRNA-21-related signaling pathways by doxycycline.

References:

1. Schotten U, Verheule S, Kirchhof P et al: Pathophysiological mechanisms of atrial fibrillation: A translational appraisal. Physiol Rev, 2011; 91: 265–325

2. Santulli G, Accarino G, De Luca N et al: Atrial fibrillation and microRNAs. Front Physiol, 2014; 5: 15–22

3. Yan-nan B, Zhao-yan Y, Li-xi L et al: MicroRNA-21 accelerates hepatocyte proliferation *in vitro* via PI3K/Akt signaling by targeting PTEN. Biochem Biophys Res Commun, 2014; 443: 802–7

4. Shi KH, Tao H, Yang JJ et al: Role of microRNAs in atrial fibrillation: New insights and perspectives. Cell Signal, 2013; 25: 2079–84

5. Zhu HY, Li C, Bai WD et al: MicroRNA-21 regulates hTERT via PTEN in hypertrophic scar fibroblasts. PLoS One, 2014; 9: e97114

6. Hua XF, Li XH, Li MM et al: Doxycycline attenuates paraquat-induced pulmonary fibrosis by downregulating the TGF-β signaling pathway. J Thorac Dis, 2017; 9: 4376–86

7. Hackmann AE, Rubin BG, Sanchez LA et al: A randomized, placebo-controlled trial of doxycycline after endoluminal aneurysm repair. J Vasc Surg, 2008; 48: 519–26

8. Palasuk, J, Windsor LJ, Platt JA et al: Doxycycline-loaded nanotube-modified adhesives inhibit MMP in a dose-dependent fashion. Clin Oral Investig, 2018; 22(3): 1243–52

9. Camm AJ, Kirchhof P, Lip GY et al: Guidelines for the management of atrial fibrillation: The Task Force for the Management of Atrial Fibrillation of the European Society of Cardiology (ESC). Europace, 2010; 12: 1360–420

10. Pellman J, Lyon RC, Sheikh F: Extracellular matrix remodeling in atrial fibrosis: Mechanisms and implications in atrial fibrillation. J Mol Cell Cardiol, 2010; 48: 461–67

11. Pérez-Serra A, Campuzano O, Brugada R: Update about atrial fibrillation genetics. Curr Opin Cardiol, 2017 [Epub ahead of print]

12. de Lucia C, Komici K, Borghetti G et al: microRNA in cardiovascular aging and age-related cardiovascular diseases. Front Med (Lausanne), 2017; 4: 74

13. Everett TH 4th, Olgin JE: Atrial fibrosis and the mechanisms of atrial fibrillation. Heart Rhythm, 2007; 4: 24–27

14. Wojciechowska A, Braniewska A, Kozar-Kamińska K: MicroRNA in cardiovascular biology and disease. Adv Clin Exp Med, 2017; 26: 868–74

15. van den Berg NWE, Kawasaki M, Berger WR et al: MicroRNAs in atrial fibrillation: From expression signatures to functional implications. Cardiovasc Drugs Ther, 2017; 31: 345–65

16. Adam O, Löhfelm B, Thum T et al: Role of miR-21 in the pathogenesis of atrial fibrosis. Basic Res Cardiol, 2012; 107: 278

17. Cardin S, Guasch E, Luo X et al: Role for microRNA-21 in atrial profibrillatory fibrotic remodeling associated with experimental postinfarction heart failure. Circ Arrhythm Electrophysiol, 2012; 5: 1027–35

18. Bhattacharyya P, Nag S, Bardhan S et al: The role of long-term doxycycline in patients of idiopathic pulmonaryfibrosis: The results of an open prospective trial. Lung India, 2009; 26: 81–85

19. Xu X, Abdalla T, Bratcher PE et al: Doxycycline improves clinical outcomes during cystic fibrosis exacerbations. Eur Respir J, 2017; 49: 1601102

20. Pimenta SP, Baldi BG, Kairalla RA et al: Doxycycline use in patients with lymphangioleiomyomatosis: Biomarkers and pulmonary function response. J Bras Pneumol, 2013; 39: 5–15

21. Hori Y, Kunihiro S, Sato S et al: Doxycycline attenuates isoproterenol-induced myocardial fibrosis and matrix metalloproteinase activity in rats. Biol Pharm Bull, 2009; 32: 1678–82

22. Cantley LC: The phosphoinositide 3-kinase pathway. Science, 2002; 296: 1655–57

23. Jiang Q, Han Y, Gao H et al: Ursolic acid induced anti-proliferation effects in rat primary vascular smooth muscle cells is associated with inhibition of microRNA-21 and subsequent PTEN/PI3K. Eur J Pharmacol, 2016; 781: 69–75

24. Yang Q, Yang K, Li AY: Trimetazidine protects against hypoxia-reperfusion-induced cardiomyocyte apoptosis by increasing microRNA-21 expression. Int J Clin Exp Pathol, 2015; 8: 3735–41

25. Wang W, Zhang K, Li X et al: Doxycycline attenuates chronic intermittent hypoxia-induced atrial fibrosis in rats. Cardiovasc Ther, 2018 [Epub ahead of print]

This work is licensed under Creative Common Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0)

5587

Indexed in: [Current Contents/Clinical Medicine]   [SCI Expanded]   [ISI Alerting System]
[ISI Journals Master List]   [Index Medicus/MEDLINE]   [EMBASE/Excerpta Medica]
[Chemical Abstracts/CAS]