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Anti-viral effects of curcumin on influenza A virus-induced myocarditis via inhibiting Wnt/β-catenin signaling

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Abstract

Influenza A virus (IAV) is a widespread human pathogen which plays an important role in the development and exacerbation of myocarditis and causes a significant impairment of the cardiac function and even mortality. Curcumin is an important component of traditional Chinese and Indian medicine with well-documented anti-inflammatory effects to prevent the cardiovascular disease. However, no study to date has addressed the effects and possible mechanism of curcumin on IAV-induced myocarditis in mice. In this study, a mouse model was firstly established for the study of IAV-induced myocarditis. H1N1-infected mice were apparently ill with lethargy, poor coat condition, anorexia, irritability, back arching and even survival. After treatment with curcumin (100 mg/kg/day), the weight loss and survival rate were ameliorated throughout the study. On day 5 after infection, both the heart weight to body weight ratio and the left ventricular weight to body weight ratio were significantly decreased in mice treated with curcumin. The area of myocardial necrosis was significantly smaller in the hearts of mice treated with curcumin compared to IVA-infected mice. Gene expression of NS1, TNF-α, IL-6 and IL-1β, I collagen, and MMP-2 mRNA in the heart tissue was markedly increased in IAV-infected mice, while curcumin significantly attenuated the expression of these genes. RT-PCR analysis revealed that curcumin inhibited the mRNA expression of Wnt3, Tcf4 and β-catenin. Curcumin also suppressed mRNA levels of Wnt target genes, c-Myc and cyclin D1. Our results provided the first evidence for the effect of curcumin to treat IVA-induced myocardial damage in particular via inhibiting Wnt/β-catenin signaling.

Key words: *curcumin, A/PR8(H1N1), viral myocarditis, Wnt/β-catenin.*

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Introduction

Viral myocarditis (VMC) is an important cause of cardiac diseases including chronic myocarditis, dilated cardiomyopathy, congestive heart failure, and even can result in death due to the impaired cardiac function [1]. Pandemic H1N1 virus leads to reversible cardiac dysfunction with direct damage of myocardial cells [2] and H1N1 viral myocarditis has become one of the common and sometimes fatal complications of influenza virus infection [3]. Many studies reported the development and exacerbation of heart disease induced by influenza virus infection [4, 5]. Because of absence of the influenza virus-induced myocarditis animal model, the molecular mechanisms of influenza virus-induced myocarditis remain unclear.

Previous studies had indicated that one of the major mechanisms of pathogenesis of cardiac and vascular dysfunction caused by influenza virus infection is nuclear factor κB (NF-κB) signaling-mediated virus-cytokine-protease cycle [6]. Significant increases in levels of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and IL-1β after infection with influenza A virus had been shown previously to direct

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the attack on myocardial cells to remodel the cardiac structure and function [7, 8], while the upregulation of TNF-α, IL-6 and IL-1β was significantly suppressed by treating with N-acetyl-L-cysteine against NF-κB activation [9]. These results suggest that proinflammatory cytokines may be an important cause of influenza virus-related heart dysfunction via NF-κB signaling [10, 11]. Recently, a systemic profiling has shown that Wnt/β-catenin signaling pathway may play an important role in influenza replication in primary human bronchial epithelial cells [12]. Wnt/β-catenin signaling pathway is a multifunctional pathway, which is crucial for embryonic heart development and cardiovascular disease [13-15]. However, there is no direct evidence of the molecular relationship between influenza A virus and Wnt/β-catenin signaling on pathogenesis of VMC caused by influenza virus infection.

Curcumin, a natural polyphenol derived from the *Curcuma longa* L plant, is used as a herb in traditional Indian and Chinese medicine. Curcumin can affect the metabolism of cells and organisms in a number of ways, including inhibition of proliferation and DNA topoisomerase II, to treat a variety of disorders, such as inflammatory disorders, infections and digestive diseases [16, 17]. Clinical studies have shown that curcumin was a highly pleiotropic molecule with immunomodulatory effects to prevent cardiovascular disease [18, 19]. Curcumin protected against cardiac inflammation through suppression of GATA-4 and NF-κB in rats [20]. Furthermore, increasing evidence indicates that curcumin has anti-viral effects: curcumin and quercetin target downstream β-catenin activity to effectively repress HBx-mediated regulation of c-MYC and E-cadherin [21]. In addition, curcumin may be potentially useful as a novel anti-HCV reagent via suppressing the Akt-SREBP-1 pathway [22]. Those findings suggest that curcumin may be a promising new strategy for inflammatory cardiovascular disease caused by viral infection through suppressing virus replication.

To our knowledge, no study to date has addressed the effect of curcumin on experimental VMC caused by influenza A virus in mice. In the present study, we established an IVA-induced myocarditis mouse model. Using this model, we found that treatment with curcumin during IVA infection could attenuate viral myocarditis. Further analysis showed that the effects of curcumin on IVA-induced myocarditis were a Wnt signaling pathway-mediated molecular process.

Material and methods

Viruses and mice

Influenza virus strains used in this study is a mouse-adapted A/PR/8/34 (H1N1) virus, which had been through repeated lung-to-lung passages and adapted in mice. They were frozen at –70°C until use. SPF female BALB/c mice, aged 6 weeks, were purchased from the Center for Disease Control and Prevention in Hubei Province, China. They were bred and maintained in SPF conditions all along. All the experiments on mice in this study followed the Chinese regulations on the administration of laboratory animals [23].

IVA-induced myocarditis mouse model

A total of 40 mice were anesthetized and inoculated with 5 LD_{50} A/PR8(H1N1) by intranasal route. The control mice were given 0.1 ml of distilled water (n = 20). The IVA-induced myocarditis mouse model was described as follows: (1) morphological parameters and physiological variables were measured; (2) histopathological observations of heart tissues were characterized by the prominent cardiac inflammation area, as confirmed by histological examination of hematoxylin and eosin-stained sections; (3) the hearts were used for analysis of gene expression, including NS1 gene, proinflammatory cytokines, I collagen, MMP-2 and Wnt/β-catenin signaling.

Curcumin treatment

The curcumin was purchased from Sangon Biotch (Shanghai, China). Curcumin was given orally by gavage at a dose of 100 mg/kg per day (n = 20), beginning from the day of viral inoculation. Five days after the infection, the mice were anaesthetized with chloroform and incised ventrally along the median line from the xiphoid process to the point of the chin.

Histopathology

We examined the histopathologic changes on day 5 after infection. Heart and body weight were measured and the heart weight/body weight was calculated. The hearts were cut into 5 μm sections at various depths and stained with hematoxylin & eosin according to the standard techniques. Histopathological change was observed by using Microanalysis (Nikon, Tokyo, Japan) and the level of inflammation was evaluated according to the conventional point-counting method, as reported earlier [24].

RT-PCR

Total RNA from mouse hearts was extracted using TRIzol reagent (BioFlux, Tokyo, Japan) according to the manufacturer's instructions. Quality control was carried out using BioAnalyzer 2100 (Agilent, Palo Alto, CA). RT-PCR was performed in a 50 μl reaction mixture using one-step RNA PCR kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. The primers were designed by Primer Premier 5.0 (Table 1). The reaction repeated for 35 cycles was carried out. Ten microliters of the amplified DNA products and six microliters of the DNA marker DL2000 (TaKaRa) were electrophoresed through 2% agarose gel (Shanghai Yito Enterprise, Shanghai, China) and visualized by ethidium bromide staining.

Table 1. Sequences of primers for RT-PCR

Gene	Sequence (5'-3')	Length (bp)
GAPDH (forward)	5' GCAGTGGCAAAGTGGAGATT 3'	490
GAPDH (reverse)	5' TCTTCTGGGTGGCAGTGAT 3'	
NS1 (forward)	5' CAGCACTCTTGGTCTGGACAT 3'	408
NS1 (reverse)	5' CCGATGAGGACTCCAACTGCAT 3'	
TNF-α (forward)	5' AGTCCGGGCAGGTCTACTTT 3'	174
TNF-α (reverse)	5' TTGGACCCTGAGCCATAATC 3'	
IL-1β (forward)	5' CAGGATGAGGACATGAGCACC 3'	447
IL-1β (reverse)	5' CTCTGCAGACTCAAACTCCAC 3'	
IL-6 (forward)	5' CACAGAAGGAGTGGCTAAGGACCA 3'	103
IL-6 (reverse)	5' ACGCACTAGGTTTGCCGAGTAGA 3'	
I collagen (forward)	5' AACTTTGCTTCCCAGATGTCCT 3'	330
I collagen (reverse)	5' TCGGTGTCCCTTCATTCCAG 3'	
MMP-2 (forward)	5' ACTTTACTCAAGGAGCAACC 3'	146
MMP-2 (reverse)	5' ACGGCTGTTCTCCTGATTTA 3'	
Wnt3A (forward)	5' GGAGTTTGCCGATGCCAGGGAG 3'	354
Wnt3A (reverse)	5' GGGTTAGGTTCGCAGAAGTTGGGTAG 3'	
β-catenin (forward)	5' GCAACCCTGAGGAAGAAGAT 3'	503
β-catenin (reverse)	5' AGTCCCAGCAGTACAACGAG 3'	
Tcf4 (forward)	5' TGGATTTCAGTGCGATGTTT 3'	824
Tcf4 (reverse)	5' GGAAGAGGTGCTGTAATGGTT 3'	
C-myc (forward)	5' CCAAGGGAAGACGATGACGG 3'	638
C-myc (reverse)	5' AGTGGGCTGTGCGGAGGTTT 3'	
Cyclind1 (forward)	5' GAGGAGCAGAAGTGCGAAGA 3'	398
Cyclind1 (reverse)	5' GAGGGTGGGTTGGAAATGAA 3'	

Statistical analysis

Results are presented as mean ± SD. The data of the experimental groups were analyzed by one-way ANOVA using SPSS 19.0. If the *P*-value was less than 0.05, the difference among the groups was considered statistically significant.

Results

Development of IVA-induced myocarditis mouse model

An IVA-induced myocarditis mouse model was established in the present study. Compared to the control group, the experimental group infected by H1N1 virus were apparently ill with lethargy, poor coat condition, anorexia, irritability and back arching. Statistical differences were observed on body weight loss and survival rate between the H1N1 group and the control group (Fig. 1). Especially five days after infection, the morphological measurements including body weight, body length, BMI, and the rates of lung weight/body weight, liver weight/body weight, kidney weight/body weight, thymus weight/body weight, spleen weight/body weight were significantly lower in the H1N1 group than in the control group (Table 2). HW/BW (P = 2.14E-06) and LV/HW (P = 0.0012) ratios were significantly increased in the H1N1 group as compared to the control group (Table 3). Histopathological observations of heart tissues were characterized by the prominent cardiac inflammation areas, as confirmed by histological examination of hematoxylin and eosin-stained sections. In the IVA-induced myocarditis group, inflammatory cell infiltration, necrosis and fibrosis lesions in hearts with an increased cell size were observed (Fig. 2). The cardiac pathological scores of VMC in IVA-induced myocarditis group were bigger than that in the control group (Fig. 2). The gene expression levels of NS1, TNF-α, IL-1β, IL-6, I collagen and MMP-2 in the IVA-induced myocarditis group were significantly upregulated. The results indicated that an IVA-induced myocarditis mouse model was developed.

Curcumin alleviated the severity of VMC

Data showed that IVA-induced myocarditis mice treated with curcumin reduced weight loss and improved survival ratios (Fig. 1). Morphological and physiological measurements were ameliorated after treatment with curcumin (Table 2). Heart morphological measurements on HW/BW (P = 6.78E-05) and LV/HW (P = 0.0122) on 5 days after infection was significantly decreased in mice treated with curcumin as compared to IVA-induced myocarditis mice (Table 3). The inflammatory cell infiltration, necrosis and fibrosis lesions in hearts with cell size were gradually ameliorated by treatment with curcumin. The cardiac pathological scores of VMC in the IVA-induced myocarditis

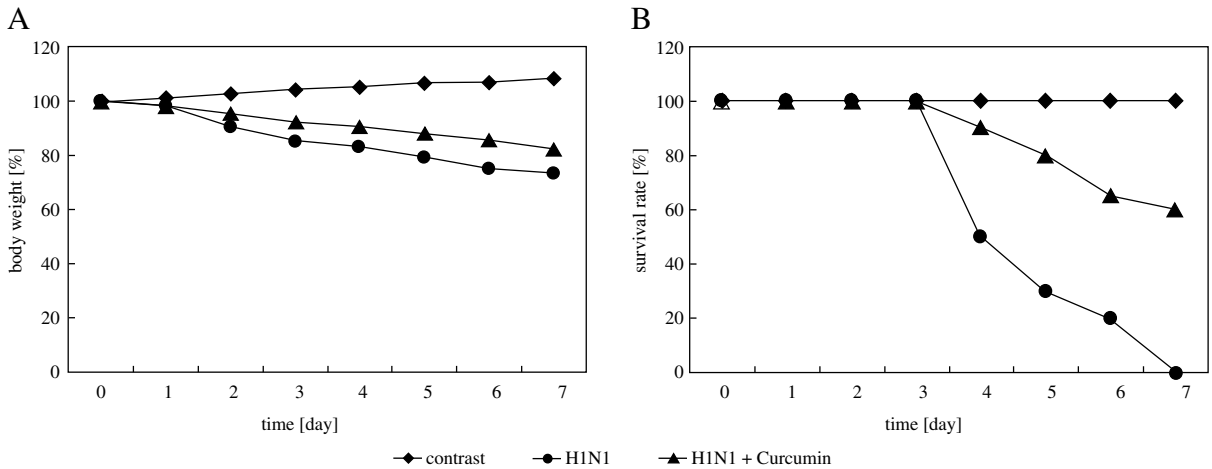

Fig. 1. Body weight and survival rate changes in the IVA-induced myocarditis mouse group, curcumin treatment group and control group (n = 20)

Table 2. Comparison of morphological measurements among the IVA-induced myocarditis mouse group, curcumin treatment group and control group

	Control group (n = 3)	**H1N1 group (n = 3)**	**Curcumin group (n = 3)**	***P* value**	***P* value**	***P* value**
				A and B	**B and C**	**A and C**
Body weight, g	18.00 ±0.301	13.52 ±0.597	15.41 ±0.432	3.70E-14	2.07E-07	6.90E-18
Body length, cm	17.32 ±0.569	12.61 ±0.592	15.77 ±0.643	5.21E-13	1.13E-09	1.19E-15
BMI, g/(cm^2)	0.06 ±0.004	0.09 ±0.008	0.06 ±0.005	5.95E-08	3.54E-07	2.32E-10
Lung weight/body weight, %	0.55 ±0.034	0.71 ±0.068	0.62 ±0.051	2.99E-06	0.0044	1.38E-06
Liver weight/body weight, %	4.50 ±0.376	5.13 ±0.138	4.80 ±0.240	9.13E-05	0.0011	7.22E-05
Kidney weight/body weight, %	1.36 ±0.072	1.52 ±0.124	1.40 ±0.047	0.0032	0.0138	0.0014
Thymus weight/body weight, %	0.18 ±0.028	0.28 ±0.051	0.20 ±0.046	4.15E-05	0.0015	3.78E-05
Spleen weight/body weight, %	0.25 ±0.029	0.35 ±0.049	0.29 ±0.034	1.60E-05	0.0040	7.30E-06
Brain weight/body weight, %	2.23 ±0.063	2.31 ±0.064	2.28 ±0.069	0.0594	0.4296	0.1630

Notes: All data are presented as Mean ± SD. One-way analysis of variance (ANOVA) was used to compare the mean differences in three groups. The least significant difference (L. S. D.) was used for multiple comparison. A: control group, B: H1N1 group and C: Curcumin group

Table 3. Comparison of heart measurements among the IVA-induced myocarditis mouse group, curcumin treatment group and control group

	Control group (n = 3)	**H1N1 group (n = 3)**	**Curcumin group (n = 3)**	***P* value**	***P* value**	***P* value**
				A and B	**B and C**	**A and C**
Heart weight, g	0.10 ±0.004	0.09 ±0.003	0.09 ±0.003	1.24E-06	0.5751	2.19E-08
Heart weight/Body weight, %	0.55 ±0.003	0.66 ±0.004	0.58 ±0.003	2.14E-06	6.78E-05	1.92E-08
Left ventricular weight, g	0.06 ±0.005	0.06 ±0.005	0.06 ±0.004	0.0058	0.6278	0.0055
LV weight/Body weight, %	3.56 ±0.029	4.23 ±0.047	3.76 ±0.027	0.0012	0.0122	0.0006
LV weight/Heart weight	0.64 ±0.492	0.64 ±0.444	0.65 ±0.571	0.9949	0.7410	0.9248
Relative inflammatory area	0.00 ±0.00	3.80 ±0.422	1.50 ±0.707	1.98E-16	5.8E-08	9.05E-16

Notes: All data are presented as Mean ± SD. One-way analysis of variance (ANOVA) was used to compare the mean differences in three groups. The least significant difference (L. S. D.) was used for multiple comparison. A: control group, B: H1N1 group and C: Curcumin group

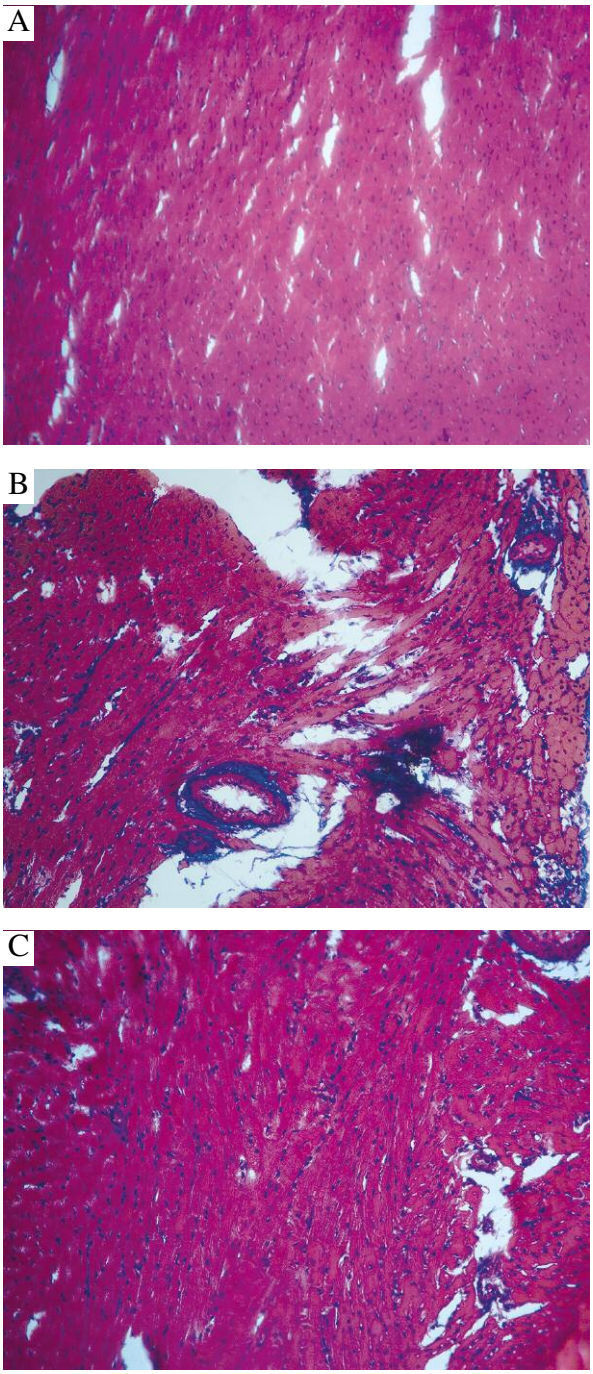

Fig. 2. Histopathologic evaluation of the severity of myocarditis in heart tissues of the control group (A, $n = 12$), IVA-induced myocarditis mouse group (B, $n = 12$) and curcumin treatment group (C, $n = 4$) (original magnification × 200)

group were significantly bigger than these in the hearts of IVA-induced myocarditis mice treated with curcumin ($P = 1.98E-16$) (Fig. 2, Table 3). As shown in Fig. 3, curcumin can inhibit A/PR8(H1N1) replication in heart tissue, in which the NS1 gene mRNA expression level was significantly lower as compared to IVA-induced myocarditis mice (Fig. 3). Our results showed that curcumin alleviated the development of myocarditis.

Expressions of genes proinflammatory cytokines, collagen I and MMP-2 in IVA-induced myocarditis mice treated with curcumin

To determine whether curcumin was involved in the molecular regulation of VMC, we used RT-PCR to test the gene expression levels of proinflammatory cytokines such as TNF-α, IL-1β and IL-6. The expression of these genes was significantly decreased in IVA-induced myocarditis mice hearts after being treated with curcumin (Fig. 3). An increase in circulating levels of I collagen led to increased cardiac inflammation and the development of vascular disease, and excessive accumulation of collagen in tissue fibrosis is an important component of the LV remodeling process [25]. The gene expressions of MMP-2 could assist inflammatory cell migration through extracellular matrix (ECM) in cardiac remodeling [26]. The results also showed that I collagen and MMP-2 mRNA levels were significantly decreased in IVA-induced myocarditis mice hearts after being treated with curcumin (Fig. 3).

Curcumin ameliorated IVA-myocarditis by inhibiting Wnt/β-catenin signaling

Furthermore, to determine whether curcumin inhibition effects on IVA-myocarditis were related to Wnt/β-catenin signaling pathway, Wnt3A gene and its receptors were analyzed by RT-PCR in the present study. Data showed that gene expression levels of Wnt3A, β-catenin and Tcf4 were strongly decreased in IVA-induced myocarditis mice hearts after being treated with curcumin (Fig. 4). Cyclin D1 and c-myc genes, as the Wnt/β-catenin target genes, were further evaluated by RT-PCR analyses. We also found that curcumin reversely regulated expression levels of cyclin D1 and c-myc (Fig. 4). Together, these results demonstrated that curcumin might ameliorate IVA-myocarditis by inhibiting the transcriptional activity of Wnt/β-catenin signaling.

Discussion

Curcumin is a well-known herb in traditional Indian and Chinese medicine which has been demonstrated to target several molecules including growth factors, transcription factors, cytokines, and enzymes involved in the etiology of diverse diseases [27, 28]. Many studies have shown that curcumin has both anti-inflammatory and anti-viral effects, but its effects on virus-induced myocarditis remain unclear.

In order to study the anti-viral effects of curcumin on virus-induced myocarditis, an IVA-induced myocarditis mouse model was firstly established in the present study. The effects and possible mechanisms of curcumin in IVA-induced myocarditis were further investigated. Using the IVA-induced

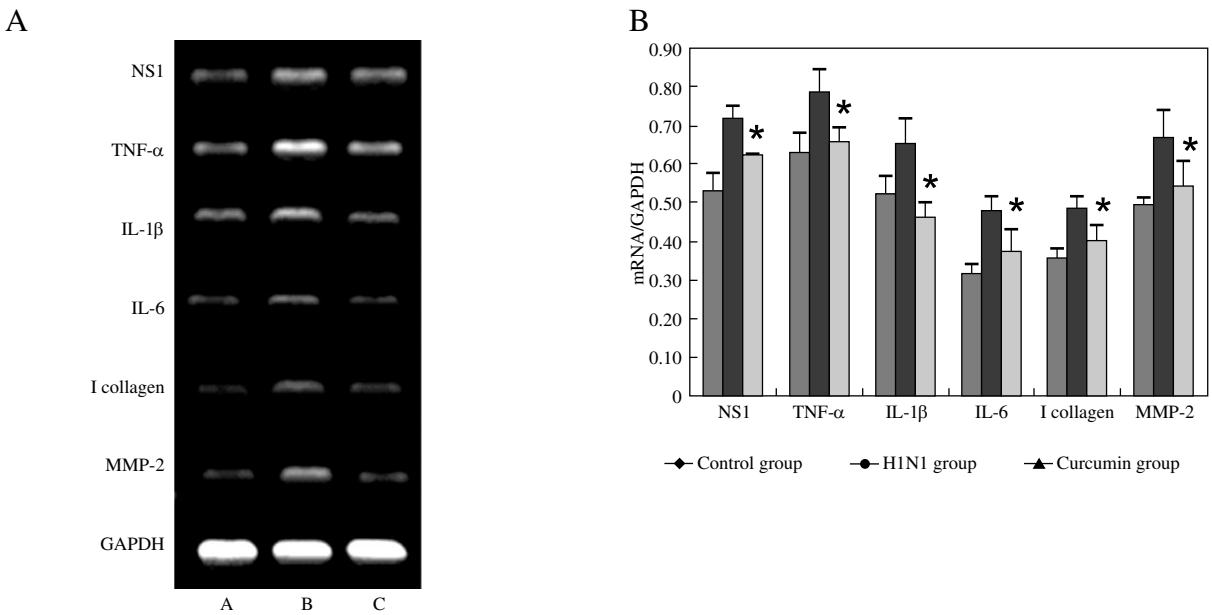

Fig. 3. Gene expressions of NS1, TNF-α, IL-1β, IL-6, I collagen and MMP-2 in heart tissues of the control group (A, $n = 12$), IVA-induced myocarditis mouse group (B, $n = 12$) and curcumin treatment group (C, $n = 4$)

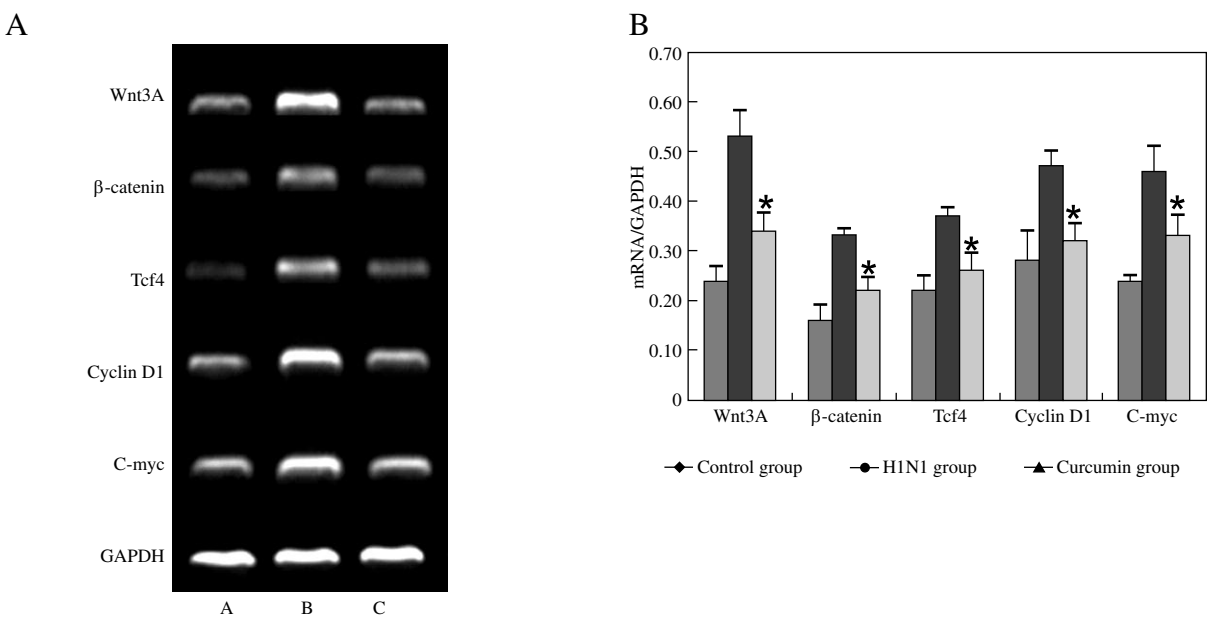

Fig. 4. Gene expressions of Wnt/β-catenin genes in heart tissues of the control group (A, $n = 12$), IVA-induced myocarditis mouse group (B, $n = 12$) and curcumin treatment group (C, $n = 4$)

myocarditis mouse model, we found that curcumin could significantly suppress influenza virus infection and improve mouse survival rate with obvious morphological changes. To our knowledge, although H1N1 virus can lead to many heart diseases in clinical studies, the molecular mechanisms of IVA-induced myocarditis remain unclear because of absence of an IVA-induced myocarditis animal model. The influenza myocarditis mouse model in this study can be a good tool to clarify the pathological mechanism of IVA-induced myocarditis, and this is the first report showing that curcumin can suppress influenza A virus infection to improve progressive myocarditis by cardiac remodeling in a mouse model.

Many experiments had indicated that one of the major mechanisms of the VMC was relevant to proinflammatory

cytokines. Influenza A virus can direct attack on myocardial cells accompanied by inducing the expression of proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β which have an important role in the treatment and prevention of cardiovascular disease [29]. Inflammatory cytokines can increase the production of MMPs to assist inflammatory cell migration through extracellular matrix (ECM) in cardiovascular disease [30, 31]. In this study, curcumin can decrease proinflammatory cytokines such as TNF-α, IL-6 and IL-1β in IVA-infected heart tissue, and inflammatory cell infiltration was reduced in the myocardial heart. There was also a trend toward reduced heart fibrosis by inhibiting I collagen production and improved cardiac remodeling via inhibiting MMP-2 to assist inflammatory cell migration. Our results demonstrated that curcumin plays a potential role in the treatment of IVA-myocarditis.

It is well-known that the Wnt/β-catenin signaling pathway plays an important role in metabolic homeostasis. Wnt/β-catenin signaling pathway is a multifunctional pathway, and many molecules in the Wnt signaling pathways play an important role in many diseases, especially in the cardiovascular disease. Our results showed that curcumin could inhibit the expression of Wnt3A, β-catenin and Tcf4 and the Wnt targets: c-Myc and cyclin D1 to maintain an adequate cardiac function. Firstly, Wnt3A, which promotes Wnt/β-catenin signaling, increased IFN production following influenza infection or vRNA transfection in primary human bronchial epithelial cells [12]. Secondly, previous studies indicated that curcumin could not only activate but also inhibit Wnt/β-catenin signaling. Curcumin was shown to suppress differentiation of adipocytes via activation of the Wnt/β-catenin signaling [32]. However, in the heart, curcumin attenuated response of β-catenin through down-regulation of p300, a positive regulator of Wnt/β-catenin signaling to prevent and reverse heart failure in animal models [33, 34]. Thirdly, another potential mechanism is that curcumin induced caspase-3-mediated degradation of β-catenin, leading to reduced DNA binding activity of TCF/LEF and the Wnt targets: c-Myc and cyclin D1 [35]. In this study, our results showed that curcumin reversely regulated expression levels of cyclin D1 and c-myc, which play an important role in cell proliferation, to inhibit cardiac fibroblast proliferation and enhance collagen degradation by decreasing I collagen and MMP-2 mRNA levels, thereby inhibiting the formation of myocardial fibrosis in mice with heart failure after IAV-induced myocarditis. Therefore, the aberrant activation of Wnt/β-catenin signaling is one of signaling abnormalities known in IVA-induced myocarditis, and curcumin showed its inhibitory effects on Wnt signaling to attenuate IVA-induced myocarditis. Based on these results, we propose that curcumin in the treatment of IVA-induced myocarditis should be a novel inhibitor of H1N1 replication by inhibiting Wnt/β-catenin signaling *in vivo*.

In conclusion, we are currently investigating therapeutic effects of curcumin and whether it may be a suitable medicine to suppress influenza A virus infection and improve IVA-induced myocarditis via inhibiting Wnt/β-catenin signaling. This is the first evidence that treatment with curcumin during IVA infection can attenuate viral myocarditis by inhibiting Wnt/β-catenin signaling in the mouse model. Although further study is needed, curcumin may be a therapeutic modality for myocarditis to progressive cardiac dysfunction associated with cardiac repair. These findings may contribute to new therapeutic approaches for IVA-induced myocarditis in humans.

The authors declare no conflict of interests.

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