

## Original Research Article

# Forsythiaside A inhibits hydrogen peroxide-induced inflammation, oxidative stress, and apoptosis of cardiomyocytes

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### Abstract

**Purpose:** To investigate the effect of forsythiaside A on heart failure.

**Methods:** An *in vitro* cell model of myocardial injury was established by incubating H9c2 primary cardiomyocytes with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Apoptosis was measured by flow cytometry. Expression of inflammatory factors, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6), was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA). Oxidative stress was evaluated by measuring malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) levels by ELISA.

**Results:** Incubation with H<sub>2</sub>O<sub>2</sub> increased H9c2 cell apoptosis ( $p < 0.001$ ). Treatment with forsythiaside A reduced Bax expression and enhanced Bcl-2 expression which suppressed apoptosis of H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells. Forsythiaside A also attenuated the H<sub>2</sub>O<sub>2</sub>-induced increase in TNF- $\alpha$  and IL-6 expressions in H9c2 cells ( $p < 0.001$ ). The H<sub>2</sub>O<sub>2</sub>-induced increase in MDA and decrease in SOD and GSH-Px in H9c2 cells were reversed by treatment with forsythiaside A. I $\kappa$ B $\alpha$  protein expression was downregulated, whereas p65 phosphorylation (p-p65), p-I $\kappa$ B $\alpha$ , nuclear factor erythropoietin-2-related factor 2 (Nrf2), and heme oxygenase 1 (HO-1) were upregulated in H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells. Forsythiaside A increased I $\kappa$ B $\alpha$ , Nrf2, and HO-1 expression and decreased p-p65 and p-I $\kappa$ B $\alpha$  expression in H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells.

**Conclusion:** Forsythiaside A exerts anti-inflammatory, anti-oxidant, and anti-apoptotic effects against H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells through inactivation of NF- $\kappa$ B pathway and activation of Nrf2/HO-1 pathway. These results support the potential clinical application of forsythiaside A for the treatment of heart failure.

**Keywords:** Forsythiaside A, Inflammation, Oxidative stress, Apoptosis, Hydrogen peroxide, Cardiomyocytes

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## INTRODUCTION

Heart failure occurs when the heart is unable to pump blood adequately to maintain the body's needs [1]. Heart failure results in ventricular filling

or impaired ejection and leads to multifactorial disorders [1,2]. Although heart failure associates with high morbidity and mortality, there is currently no ideal treatment regimen for the disease [1].

Oxidative stress and inflammation have attracted extensive attention as important pathophysiological contributors to heart failure syndrome [3]. Excessive and unregulated production of reactive oxygen and reactive nitrogen species in the heart promotes oxidative stress and myocardial cell damage [4]. Moreover, oxidative stress induces inflammation, which contributes to heart failure [4]. Therefore, antioxidants and anti-inflammatory drugs are regarded as potential therapies for heart failure.

Forsythiaside A is the main bioactive component extracted from *Forsythia suspensa* (Thunb.) Vahl and it has hepatoprotective, neuroprotective, antiviral, anti-inflammatory, and antioxidant properties [5]. Forsythiaside A protected mice against ovalbumin-induced asthma through its anti-inflammatory properties [6]. Forsythiaside A also effectively inhibited hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced oxidative stress and apoptosis in PC12 cells [7]. However, there are limited studies on the effect of forsythiaside A on heart failure.

In this study, H<sub>2</sub>O<sub>2</sub>-induced myocardial injury in H9c2 primary cardiomyocytes was established as an *in vitro* cell model of heart failure. The effects of forsythiaside A on inflammation, oxidative stress, and apoptosis of H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells were investigated.

## EXPERIMENTAL

### Cell culture and treatment

The primary cardiomyocyte cell line H9c2 was purchased from the Chinese Academy of Sciences (Shanghai, China) and was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA) in a 37 °C incubator. The cells were treated with 2.5, 5, or 10 µg/mL forsythiaside A (Sigma-Aldrich, St. Louis, MO, USA) for 6 h and then incubated with 150 µmol/L H<sub>2</sub>O<sub>2</sub> for another 24 h.

### Flow cytometry

H9c2 cells were harvested and resuspended in the 1X binding buffer from the Annexin V/FITC kit (Beyotime, Shanghai, China). Then, 5 µL of Annexin V/FITC and 10 µL propidium iodide were added to probe the H9c2 cells. The cells were then analyzed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) to evaluate apoptosis.

### Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from H9c2 cells with the RNAeasy Mini Kit (QIAGEN, Gaithersburg, MD), and the RNA was reverse-transcribed into cDNA with the QuantiTect Reverse Transcription Kit (QIAGEN). The QuantiTect SYBR Green PCR Kit (QIAGEN) was used to measure TNF-α and IL-6 expression with GAPDH as the internal control. The qRT-PCR conditions were 95 °C for 10 min, 40 cycles of 95 °C for 10 s, and 60 °C for 60 s. Primers are shown in Table 1.

**Table 1:** Primers used for PCR

Gene	Forward	Reverse
TNF-α	5'- ACTGAACTTCGG	5'- GCTTGGTGGTTT
	GGTGATTG-3'	GCTACGAC-3'
IL-6	5'- GAGCATTGGAAG	5'- GCTTGGTGGTTT
	TTGGGGTA-3'	GCTACGAC-3'
GAPDH	5'- AGCCACATCGCT	5'- GCCCAATACGAC
	CAGACAC-3'	CAAATCC-3'

### Enzyme-linked immunosorbent assay (ELISA)

H9c2 cells were lysed in RIPA buffer (Shanghai Bicolor BioScience & Technology Co., Shanghai, China), the lysed cells were centrifuged at 12000 g, and the supernatants were collected. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) levels were measured using commercial ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

### Western blot

Protein concentrations of H9c2 cell supernatants were determined with the Bicinchoninic acid Protein Assay kit (Pierce, Rockford, IL, USA). Samples (40 µg) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dry milk. The membranes were then probed with antibodies to Bax and Bcl-2 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p65 and p-p65 (1:2500; Santa Cruz Biotechnology), IκBα and p-IκBα (1:3000; Santa Cruz Biotechnology), nuclear erythroid-related factor 2 (Nrf2), HO-1, and GAPDH (1:4000; Santa Cruz Biotechnology). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:5000; Kangcheng Inc., Shanghai, China) and

detection was performed using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) and a bio-image analysis system (Bio-Rad, Baltimore, MD, USA).

### Statistical analysis

Experiments were performed in triplicate at the least and data were presented as mean  $\pm$  standard deviation. Statistics between two groups were analyzed using the Student's t-test in GraphPad Prism 7 (GraphPad Inc., San Diego, CA, USA). One-way Analysis of Variance was used to analyze differences between multiple groups. A  $p$  value  $< 0.05$  was considered statistically significant.

## RESULTS

### Forsythiaside A attenuated H<sub>2</sub>O<sub>2</sub>-induced apoptosis in H9c2 cells

To establish an *in vitro* model of heart failure, H9c2 cells were incubated with H<sub>2</sub>O<sub>2</sub> and then analyzed by flow cytometry, which showed that apoptosis of H9c2 cells increased upon incubation with H<sub>2</sub>O<sub>2</sub> (Figure 1 A). Western blot and qRT-PCR analyses showed that Bax expression increased and Bcl-2 expression decreased upon incubation with H<sub>2</sub>O<sub>2</sub> (Figure 1B). However, pretreatment of H9c2 cells with forsythiaside A reduced H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis in a dose-dependent manner (Figure 1 A). Moreover, the increased Bax and decreased Bcl-2 expression in H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells was reversed upon pretreatment with forsythiaside A (Figure 1B). These data suggest that forsythiaside A has an anti-apoptotic effect on H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells.

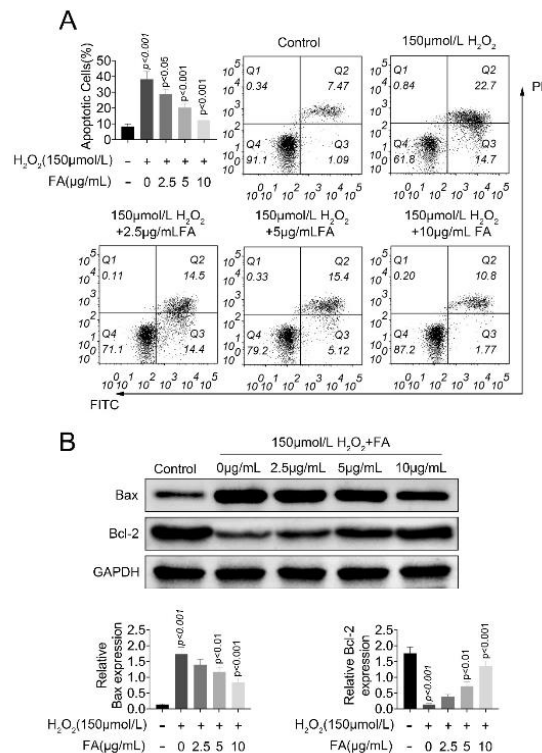
### Forsythiaside A attenuated H<sub>2</sub>O<sub>2</sub>-induced inflammation in H9c2 cells

TNF- $\alpha$  and IL-6 mRNA and protein expression was upregulated in H9c2 cells upon incubation with H<sub>2</sub>O<sub>2</sub>, however treatment with forsythiaside A attenuated this H<sub>2</sub>O<sub>2</sub>-induced increase in TNF- $\alpha$  and IL-6 expression (Figure 2 A and B) indicating that forsythiaside A plays an anti-inflammatory role in H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells.

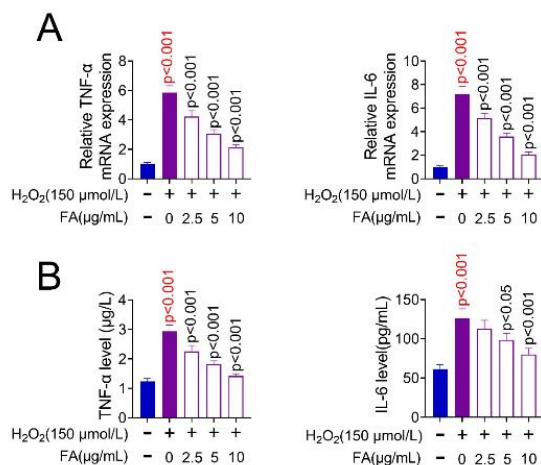
### Forsythiaside A attenuated H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in H9c2 cells

Upon incubation with H<sub>2</sub>O<sub>2</sub>, the MDA level increased and the SOD and GSH-Px levels decreased in H9c2 cells, however the increase in MDA and the decreases in SOD and GSH-Px were reversed by treatment with forsythiaside A in a dose-dependent manner (Figure 3). These

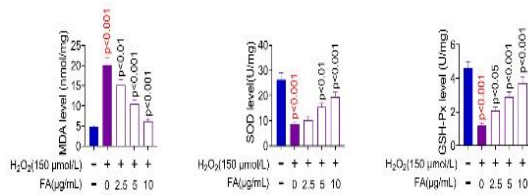
data demonstrate the anti-oxidant effect of forsythiaside A in H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells.



**Figure 1:** Forsythiaside A attenuated H<sub>2</sub>O<sub>2</sub>-induced apoptosis in H9c2 cells. (A) Forsythiaside A suppressed H<sub>2</sub>O<sub>2</sub>-induced H9c2 cell apoptosis. (B) Forsythiaside A attenuated the H<sub>2</sub>O<sub>2</sub>-induced increase of Bax expression and decrease of Bcl-2 expression in H9c2 cells



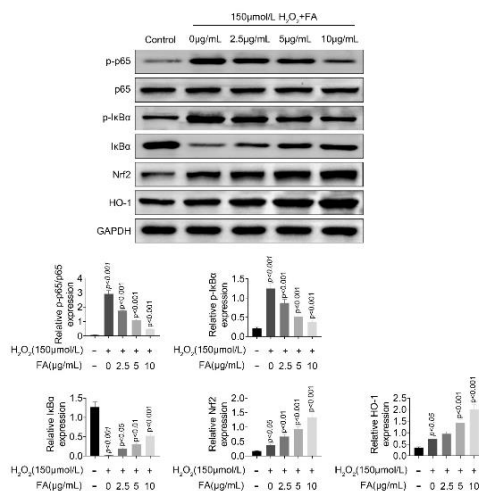
**Figure 2:** Forsythiaside A attenuated H<sub>2</sub>O<sub>2</sub>-induced inflammation in H9c2 cells. (A) Forsythiaside A attenuated the H<sub>2</sub>O<sub>2</sub>-induced increase in TNF- $\alpha$  and IL-6 mRNA expression in H9c2 cells. (B) Forsythiaside A attenuated the H<sub>2</sub>O<sub>2</sub>-induced increase in TNF- $\alpha$  and IL-6 protein expression in H9c2 cells



**Figure 3:** Forsythiaside A attenuated  $H_2O_2$ -induced oxidative stress in H9c2. Forsythiaside A attenuated the  $H_2O_2$ -induced increase in MDA expression and decreases in SOD and GSH-Px expression in H9c2 cells

### Forsythiaside A suppressed activation of NF- $\kappa$ B and promoted activation of Nrf2/HO-1

p65 protein expression in H9c2 cells was not affected by incubation with  $H_2O_2$  or forsythiaside A (Figure 4), however incubation with  $H_2O_2$  induced p-p65 expression, and this  $H_2O_2$ -induced increase of p-p65 in H9c2 cells was attenuated upon incubation with forsythiaside A (Figure 4). In addition, p-I $\kappa$ B $\alpha$  expression increased and I $\kappa$ B $\alpha$  expression decreased upon incubation with  $H_2O_2$ , and these  $H_2O_2$ -induced changes in gene expression were reversed upon incubation with forsythiaside A (Figure 4). These data indicate that forsythiaside A suppressed activation of NF- $\kappa$ B in  $H_2O_2$ -induced H9c2 cells. Protein expression of Nrf2 and HO-1 was upregulated in  $H_2O_2$ -stimulated H9c2 cells that were treated with forsythiaside A (Figure 4).



**Figure 4:** Forsythiaside A suppressed activation of NF- $\kappa$ B and promoted activation of Nrf2/HO-1 in  $H_2O_2$ -induced H9c2 cells. Forsythiaside A attenuated the  $H_2O_2$ -induced decrease in I $\kappa$ B $\alpha$  expression and increases in p-p65 and p-I $\kappa$ B $\alpha$  expression in H9c2 cells. Nrf2 and HO-1 protein expression was upregulated in  $H_2O_2$ -induced H9c2 cells upon incubation with forsythiaside A

## DISCUSSION

Oxidative and inflammatory biomarkers are upregulated in chronic heart failure suggesting their potential in the prognosis of heart failure [4]. Strategies to suppress oxidative stress and inflammation have been shown to ameliorate myocardial injury in heart failure [8]. Forsythiaside A has attracted increasing attention due to its anti-inflammatory and antioxidant properties [5]. The protective effects of forsythiaside A on heart failure was investigated in this study.

Apoptosis of cardiomyocytes occurs in a variety of cardiovascular diseases. It has been reported that apoptotic cells around infarct tissue play a role in heart remodeling and that inhibition of cell apoptosis significantly improves left ventricular remodeling and chronic heart failure [9]. Therefore, oxidative stress-induced apoptosis is considered a therapeutic target for heart failure [9]. In this study, incubation with  $H_2O_2$  induced apoptosis, enhanced Bax expression, and reduced Bcl-2 expression in H9c2 cells. A previous study showed that forsythiaside A protected NIH/3T3 fibroblast cells against ultraviolet radiation-induced cell apoptosis [10]. The results of this study confirmed that pretreatment with forsythiaside A decreased Bax expression and increased Bcl-2 expression to suppress apoptosis of  $H_2O_2$ -induced H9c2 cells.

Heart failure is a systemic pro-inflammatory state characterized by high levels of inflammatory cytokines, such as TNF- $\alpha$  and IL-6 [11]. Excessive levels of TNF- $\alpha$  and IL-6 are regarded as poor prognostic markers in heart failure [12]. Forsythiaside A was shown to reduce levels of TNF- $\alpha$  and IL-6 to ameliorate  $CuSO_4$ -induced inflammation in zebrafish [13]. Forsythiaside A repressed the  $H_2O_2$ -induced increases in TNF- $\alpha$  and IL-6 expression in H9c2 cells in a dose-dependent manner. Prolonged activation of the NF- $\kappa$ B pathway stimulates chronic inflammation with enhanced expression of TNF- $\alpha$  and IL-6 resulting in endoplasmic reticulum stress and cell death, thereby promoting heart failure progression [14]. Protein expression of p-p65 was upregulated in  $H_2O_2$ -stimulated H9c2 cells suggesting that the NF- $\kappa$ B pathway was activated. Forsythiaside A was shown to decrease NF- $\kappa$ B expression to alleviate virus-induced lung tissue damage [15]. In this study, forsythiaside A reduced p-p65 expression in  $H_2O_2$ -induced H9c2 cells. Therefore, we propose that forsythiaside A exerts its anti-inflammatory effect against heart failure through inactivation of the NF- $\kappa$ B pathway.

Oxidative stress, which is implicated in the pathophysiology of heart failure, induces cell apoptosis and inflammation of cardiomyocytes [16]. The level of MDA increased and levels of SOD and GSH-Px decreased in H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells suggesting that H<sub>2</sub>O<sub>2</sub> induced oxidative stress in H9c2 cells. Forsythiaside A attenuated the H<sub>2</sub>O<sub>2</sub>-induced increase in MDA and decreases in SOD and GSH-Px in H9c2 cells in a dose-dependent manner, indicating that forsythiaside A has an anti-oxidant effect against heart failure. Nrf2 is transferred into the nucleus to bind with anti-oxidant response elements of target genes, upregulates HO-1 to reduce oxidative stress, and protects against cardiovascular diseases, including heart failure [17]. Forsythiaside A was shown to increase levels of Nrf2 and HO-1, which exerted anti-oxidant effects in lipopolysaccharide-stimulated microglial cells [18]. In this study, forsythiaside A also promoted protein expression of Nrf2 and HO-1 in H<sub>2</sub>O<sub>2</sub>-induced H9c2 cells, which most likely exert anti-oxidant effects against heart failure.

## CONCLUSION

Forsythiaside A reduces apoptosis of H<sub>2</sub>O<sub>2</sub>-stimulated H9c2 cells, downregulates TNF- $\alpha$  and IL-6 expressions via inactivation of NF- $\kappa$ B pathway, and suppresses oxidative stress via activation of Nrf2/HO-1 pathway. These results indicate that forsythiaside A may be useful for the management of heart failure.

## DECLARATIONS

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Runqin Li and Dengfeng Ma designed the study and supervised the data collection. Zhihua Fu analyzed and interpreted the data. Xiaoxuan Zheng and Wenxiu Li prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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