The protective effects of Berberine and Hesperidin on inflammatory factor-stimulating cardiac fibroblasts

X.-H. YU¹, Y.-F. WANG², F.-Y. DAI², J.-H. ZHAO², P. LI²

¹Cardiovascular Medicine, Beijing University of Chinese Medicine, Beijing, China
²Cardiovascular Medicine, Beijing University of Chinese Medicine Third Affiliated Hospital, Beijing, China

Abstract. – OBJECTIVE: The previous work has shown that Berberine and Hesperidin have beneficial effects on cardiovascular diseases. However, the underlying mechanisms remain unknown. This study aimed to investigate the effect of Berberine and Hesperidin on inflammatory cytokine secretion, proliferation, differentiation, and collagen synthesis of cardiac fibroblasts stimulated by the transforming growth factor-β1 (TGF-β1), and the potential of these drugs to regulate the Notch1 signaling pathway.

PATIENTS AND METHODS: Neonatal rat primary cardiac fibroblasts were stimulated with 5 ng/mL TGF-β1 as model (TGF) group. In the Berberine (TGF+B) group cells were given TGF-β1, along with 1.25/2.5/5/10 mg/L Berberine, while the Hesperidin (TGF+H) group was treated with TGF-β1 and 12.5/25/50/100 µmmol/L Hesperidin. Cellular proliferation, differentiation, and collagen synthesis were evaluated. The role of the Notch1 signaling pathway in the protective effects of Berberine and Hesperidin was analyzed by using γ-secretase inhibitor (DAPT) to block the Notch1 pathway.

RESULTS: 5/10 mg/L Berberine intervention could noticeably decrease both TGF-β1 and IL-1β levels, 25/50/100 µmol/L Hesperidin could reduce IL-1β secretion from TGF-β1 stimulated cardiac fibroblasts. Both Berberine and Hesperidin decreased the expression of α-SMA and cell viability in a concentration-dependent manner; however, the apoptosis of cardiac fibroblasts was not influenced. 10 mg/L Berberine or at least 50 µmol/L Hesperidin could noticeably decrease MMP-1 expression, and at least 5 mg/L Berberine or 100 µmol/L Hesperidin could markedly reduce MMP-9 expression. Using DAPT to block Notch1 signaling could reverse the protective effects of Berberine and Hesperidin.

CONCLUSIONS: Berberine and Hesperidin can reduce the secretion of inflammatory cytokines, differentiation, and proliferation, and increase the collagen synthesis of cardiac fibroblasts stimulated by TGF-β1 via the Notch1 signaling pathway.

Key Words: Berberine, Hesperidin, TGF-β1, Notch1, Cardiac fibroblasts.

Introduction

The heart consists of myocardial and non-myocardial cells, the latter includes cardiac fibroblasts, vascular endothelial cells (VECs), and vascular smooth muscle cells (VSMCs). Cardiomyocytes account for approximately 80% of the heart’s weight but make up less than 30% of the total number of heart cells. Instead about half of the total heart cells are cardiac fibroblasts². Cardiac fibroblasts have two major functions. First, they connect myocardial cells to maintain the normal structure of the heart³,⁴. In addition, they secrete collagen and some cytokines⁵,⁶. The proliferation of cardiac fibroblasts can be activated by stimulation such as peroxide and inflammatory factors and cytokines can promote differentiation from fibroblasts to myofibroblasts leading to extracellular matrix (ECM) accumulation⁷,⁸. Hence, inhibition of cardiac fibroblast differentiation is a way to suppress the development of cardiac fibrotic remodeling in chronic heart failure (CHF)⁹.

Berberine is an alkaloid found in the herbs Coptis chinensis and Cortex phellodendri. Previous studies¹⁰-¹² provided strong evidence that Berberine has beneficial effects on hypertension, hyperlipidemia, hyperglycemia, and arrhythmia. One study¹³ showed that Berberine could regulate collagen synthesis and distribution, inhibiting the myocardial hypertrophy and ventricular remodeling in rats. Hesperidin is a main component of bergamot and has been shown to have antioxidant, anti-inflammatory, and anti-hyperlipidemia

Corresponding Author: Ping Li, MD; e-mail: dr_pingli@126.com
functions which closely associate with cardiovascular diseases\(^4\). However, the precise mechanism of their effects remains unknown.

The activation of the Notch signaling pathway requires the release of the Notch intracellular domain (NICD) and its subsequent translocation into the nucleus, triggering the activation of downstream target molecule Hairy and Enhancer Split-1 (Hes-1)\(^5\). Most studies\(^6\) of Notch signaling were related to the embryonic heart development and they showed that the knockdown of Notch or its ligands led to cardiac valve abnormalities in mice. Kyle and Aly\(^7\) reported that Notch signaling pathway was involved in the cellular differentiation of myofibroblasts. The over-expression of Notch1 promoted the differentiation of lung fibroblasts and epithelial cells into cardiac fibroblasts. However, the functions of the Notch signaling pathway in cardiac fibroblasts are largely unclear. Hence, in this study we examine the regulatory effects of Berberine and Hesperidin on inflammatory cytokine secretion, proliferation, differentiation, and collagen synthesis of cardiac fibroblasts stimulated by TGF-β1 and the Notch1 signaling pathway.

Materials and Methods

Isolation and Culture of Neonatal Rat Cardiac Fibroblasts

1-3-day old neonatal Sprague Dawley (SD) rats were purchased from Hua-Fukang (Beijing, China), and immersed in 75% alcohol. Tissue forceps were used to clip the tissue along the edge of the sternum, expose the heart, separate the ventricle, and cut off excess blood vessels. Ventricles were washed with phosphate-buffered saline (PBS) 2 times, then transferred to serum solution, and quickly cut into pieces. Ventricles were dissociated in 5mL 400 kU/L collagenase II (Sigma-Aldrich, St. Louis, MO, USA) in a 37°C water bath at 150 r/min for 20 min. The cells were collected through a sieve rotated at 1200 r/min for 5 min. Cardiac fibroblasts were separated using the differential adhesion method. The third generation of cells was used for experiments. The investigation was approved by the Ethics Committee of Beijing University of Chinese Medicine Third Affiliated Hospital.

Experimental Groups and Interventions

Rat cardiac fibroblasts were cultured in 6-well plates. TGF group (TGF) was treated with 5 ng/mL TGF-β1 (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Berberine group (TGF+B) was treated with 5 ng/mL TGF-β1 along with 1.25/2.5/5/10 µg/L Berberine (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Hesperidin group (TGF+H) received TGF-β1 and 12.5/25/50/100 µmol/L Hesperidin (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. γ-secretase inhibitors (DAPT; Sigma-Aldrich, St. Louis, MO, USA) were used to block Notch1 signaling. Cells without intervention were used as control (CTL) group.

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-1β and TGF-β1 levels were measured using an Enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems (Minneapolis, MN, USA) according to the manufacturer’s instructions. After adding stop solution to each well, the optical density (OD) was read at 450 nm immediately.

Cell Counting Kit (CCK-8) Assay

Rat cardiac fibroblasts were cultured in 96-well plates, 100 µl/well. Following previously described interventions in each group, 10 µl of CCK-8 solution (Solarbio, Beijing, China) was added to each well and cells were incubated at 37°C and 5% CO\(_2\) for 1 h. The optical density (OD) was read at 450 nm immediately.

Annexin V-FITC/PI Apoptosis Detection

Rat cardiac fibroblasts were cultured in 6-well plates. Following previously described interventions in each group; cells were washed with PBS 3 times and digested with trypsin (Sigma-Aldrich, St. Louis, MO, USA). Cells were suspended in 400 µL liquid suspension, and stained with 5 µl Annexin V-FITC (Sigma-Aldrich, St. Louis, MO, USA) and 10 µL PI (Sigma-Aldrich, St. Louis, MO, USA) by mixing in the dark at 4°C for 15 min and 5 min, respectively. Cellular apoptosis was evaluated by flow cytometry.

Western Blotting Analysis

Protein concentrations were measured using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Antibodies against α-SMA, MMP-1, MMP-9, NICD, and Hes1 (Cell Signaling Technology, Danvers, MA, USA) were used to identify specific proteins, which were evaluated by enhanced chemiluminescence (ECL) method.
Immunohistochemical Staining

Rat cardiac fibroblasts cultured in 6-wells plate were incubated with 3% hydrogen peroxide (H₂O₂) for 30 min. Antigen retrieval was performed for several hours, then blocked with 5% fetal bovine serum (FBS) and incubated with antibody against matrix metalloproteinase-1 (MMP-1) and matrix metalloproteinase-9 (MMP-9) (Abcam, Cambridge, MA, USA) at 4°C overnight. Then, secondary antibody and diaminobenzidine (DAB) were incubated respectively (Zhongshan Golden Bridge, Guangzhou, China). The coverslip was observed using magnification ×100.

Statistical Analysis

GraphPad Prism 6.0 was used for statistical analysis. The data are shown as mean ± standard deviation. A two-sided t-test was used to test individual differences. p < 0.05 was considered statistically significant.

Results

Effect of Berberine and Hesperidin on Inflammatory Cytokine Secretion from Cardiac Fibroblasts

To evaluate the effect of Berberine and Hesperidin on inflammatory cytokine secretion from cardiac fibroblasts, we examined the expression of TGF-β1 and IL-1β by enzyme-linked immunosorbent assay (ELISA). The results showed that in cardiac fibroblasts stimulated with 5 ng/mL TGF-β1, treatment with 5 mg/L or 10 mg/L Berberine markedly decreased secretion of both TGF-β1 and IL-1β (Figures 1A and B). The expression of IL-1β could be markedly down-regulated by Hesperidin treatments ranging from 25 µmol/L to 100 µmol/L (Figure 1C), but levels of TGF-β1 were not influenced by Hesperidin intervention (Figure 1D).

Effect of Berberine and Hesperidin on Proliferation, Apoptosis, and Differentiation of Cardiac Fibroblasts

The differentiation of cardiac fibroblasts was evaluated by Western blotting. The expression level of α-SMA protein increased when cardiac fibroblasts were stimulated with 5 ng/mL TGF-β1, and then gradually reduced with the addition of increasing Berberine concentration from 1.25 mg/L to 10 mg/L or increasing Hesperidin concentration from 12.5 µmol/L to 100 µmol/L (Figure 2A). These data demonstrated that both Berberine and Hesperidin could suppress the differentiation of cardiac fibroblasts into myofibroblasts. Cellular proliferation and apoptosis were determined using a CCK-8 assay and Annexin V/PI staining. We found that the cellular viability of TGF group was significantly higher than CTL group and that this enhanced viability was reversed by both Berberine and Hesperidin at different concentrations (Figures 2B, C). The total proportion of cardiac fibroblasts undergoing apoptosis at both early and late stage had no significant differences between any groups (Figures 2D, E).

Effect of Berberine and Hesperidin on Cardiac Fibroblast Collagen Synthesis

The up-regulation of collagen synthesis has shown to be an important indicator of cardiac fibrosis. Therefore, we observed the expression of Collagen I/III with Sirius red staining. Our results showed that Collagen I/III was expressed at low levels in each group making it difficult to draw comparisons between them. Previous studies have shown that high MMP1/9 expression levels were related to deteriorated fibrosis in pulmonary fibrosis and ventricular remodeling. Immunohistochemical staining showed that stimulation with 5 ng/mL TGF-β1 increased both MMP-1 and MMP-9 levels, while 10 mg/L Berberine or at least 50 µmol/L Hesperidin could noticeably decrease the MMP-1 expression (Figures 3A, B), and at least 5 mg/L Berberine or 100 µmol/L Hesperidin could markedly reduce MMP-9 expression (Figures 3C, D).

Effect of Notch1 Signaling Pathway on Berberine and Hesperidin Treatment for TGF-β1 Stimulated Cardiac Fibroblasts

To further investigate the underlying mechanism of Berberine and Hesperidin treatment on TGF-β1 stimulated cardiac fibroblasts, we utilized the γ-secretase inhibitor (DAPT) to block the Notch1 pathway. We found that the ability of Berberine and Hesperidin to decrease IL-1β secretion could be blocked by DAPT and that the ability of Berberine to decrease TGF-β1 secretion could be blocked always by DAPT (Figures 4A, B). The expression level of Notch intracellular domain (NICD) and the downstream target molecule Hes1 noticeably increased following 10 mg/L Berberine or 100 µmol/L Hesperidin treatment in TGF-β1 stimulated cardiac fibroblasts, and then markedly decreased following the additional DAPT treatment (Figures 4C, D). The inhibitory effect of Berberine and Hesperidin on...
The effect of Berberine and Hesperidin on cardiac fibroblasts

cellular differentiation was reversed by DAPT, as evidenced by an elevated α-SMA level (Figures 4C, D). The suppression of cell viability by Berberine and Hesperidin also could be rescued by DAPT (Figure 4E). To analyze the effect on cardiac fibrosis, we found that MMP-1 and MMP-9 levels were significantly up-regulated following DAPT treatment (Figures 4F, G).

**Discussion**

Cardiac fibroblasts can secrete a variety of biologically active substances to adjust their own production of collagen and the physiological function of surrounding myocardial cells. These active substances include pro-hypertrophy factors such as TGF-β1, endothelin-1 (ET-1), tumor necrosis factor-α (TNF-α), and anti-hypertrophy factors like NO and prostaglandin I$_2$ (PGI$_2$), which mediate the development of myocardial hypertrophy by a single action or interaction$^{22}$. In our work, we stimulated cardiac fibroblasts with TGF-β1 and investigated the effects of Berberine and Hesperidin on their differentiation. TGF-β1 has been shown to play an important role in cardiac fibrosis and decreased levels of TGF-β1 led to a reduced

---

**Figure 1.** Effect of Berberine and Hesperidin on inflammatory cytokines secretion of cardiac fibroblasts. Cardiac fibroblasts were stimulated with 5ng/mL TGF-β1 and gave different concentrations of A, B Berberine from 1.25 mg/L to 10mg/L; C, D Hesperidin from 12.5 umol/L to 100 umol/L. IL-1β, interleukin-1β; TGF-β1, transforming growth factor-β1; CTL, control group; TGF, model group; B, Berberine; H, Hesperidin. Data were shown as Mean ± SD. Comparing to CTL, #p<0.05, ##p<0.01, ###p<0.001; Comparing to TGF, *p<0.05, **p<0.01, ***p<0.001.
Figure 2. Effect of Berberine and Hesperidin on proliferation, apoptosis and differentiation of cardiac fibroblasts. Cardiac fibroblasts were stimulated with 5ng/mL TGF-β1 and gave different concentrations of Berberine and Hesperidin. 

A, Protein levels of α-SMA; B, C Cell viability using CCK-8 assay kit; D, E Cellular apoptosis by Annexin V/PI staining were analyzed. Data were shown as Mean ± SD. Comparing to CTL, #p<0.05; Comparing to TGF, *p<0.05, **p<0.01.
Figure 3. Effect of Berberine and Hesperidin on collagen synthesis of cardiac fibroblasts. Cardiac fibroblasts were stimulated with 5ng/mL TGF-β1 and gave different concentrations of Berberine and Hesperidin. A, B Immunohistochemical staining of MMP-1; C, D MMP-9 was executed. MMP, matrix metalloproteinases. (Magnification: ×100).
Figure 4. Effect of Notch1 signaling pathway on Berberine and Hesperidin treatment for TGF-β1 stimulated cardiac fibroblasts. Cardiac fibroblasts were stimulated with 5ng/mL TGF-β1 and gave 10mg/L Berberine or 100 umol/L Hesperidin and DAPT. A, IL-1β levels in each group; B, TGF-β1 levels in each group; C, D Protein levels of α-SMA, NICD, and Hes1; E, Cell viability using CCK-8 assay kit; F, G Immunohistochemical staining of MMP-1 and MMP-9. DAPT, γ-secretase inhibitors (magnification: ×100); NICD, Notch intracellular domain. Data were shown as Mean ± SD. Comparing to CTL, #p<0.05; Comparing to TGF+B10, *p<0.05, **p<0.01; Comparing to TGF+H100, &p<0.05.
expression of α-SMA and collagen I, as well as improved cardiac remodeling in rats with myocardial infarction.

Our results showed that Berberine could markedly reduce both TGF-β1 and α-SMA levels, indicating that the rate of differentiation from cardiac fibroblasts to myofibroblasts was reduced. Ai et al. reported that the protective effects of Berberine on Ang II-stimulated cardiac fibroblasts may be mediated by the activation of AMPK signaling and the downregulation of mTOR/p70S6K signaling. Li et al. found that in diabetic rats, Berberine elicited its anti-fibrotic effects by downregulating myocardial insulin-like growth factor-1 (IGF-1) receptor-regulated MMP-2/MMP-9 expression.

In our research, we found that Berberine treatment acted through the notch1 signaling pathway to mediate effects on TGF-β1 stimulated cardiac fibroblasts. Indeed, crosstalk between Notch1 signaling and TGF-β1 in the regulation of myocardial fibrosis had been previously reported. The activation of the Notch signaling could inhibit TGF-β1/Smad3 signaling to suppress myocardial fibrosis after MI. However, this is the first report showing that the mechanism of Berberine function involves Notch signaling.

The data showed that Berberine and Hesperidin did not affect apoptosis in TGF-β1 stimulated cardiac fibroblasts. Huang et al. reported that Berberine could improve cellular survival by suppressing excessive autophagy in cardiomyocytes in vitro and in vivo models of cardiac ischemia/reperfusion. In hepatocellular carcinoma HepG2 cells, Berberine induced apoptosis via down-regulating the NF-κB signaling.

A study presented that one-week pre-treatment with Hesperidin before left anterior descending coronary artery (LAD) occlusion in rats noticeably reduced myocyte apoptosis, inflammation, and cardiac dysfunction. Li et al. showed that Hesperidin had beneficial effects on hypoxia cardiomyocytes might through activating PI3K/Akt signaling pathway. These different results of Berberine and Hesperidin on cellular apoptosis may be due to different drug concentrations, different time of administration, and different cell types used in the various studies.

Conclusions

We demonstrated that Berberine and Hesperidin had anti-fibrotic effects on TGF-β1 and stimulated cardiac fibroblasts by regulating inflammatory cytokines secretion, proliferation, differentiation, as well as collagen synthesis of cardiac fibroblasts, potentially acting through the Notch1 signaling pathway. Thus, Berberine and Hesperidin could serve as a novel therapeutic strategy to treat cardiac fibrosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References


