Original Article

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Astraglaus polysaccharide protects diabetic cardiomyopathy by activating NRG1/ErbB pathway

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Summary **Diabetic cardiomyopathy (DCM) is one of the main cardiac complications among diabetic patients. According to previous studies, the pathogenesis of DCM is associated with oxidative stress, apoptosis and proliferation of local cardiac cells. It showed, NRG1 can improve the function of mitochondria, and thereby, increasing proliferation and decreasing apoptosis of cardiac muscle cell *via* ErbB/AKT signaling, also, exert antioxidative function. Besides, NRG1/ErbB pathway was impaired in the DCM model which suggested this signaling played key role in DCM. *Astraglaus polysaccharide* (APS), one of the active components of *Astragalus mongholicus*, showed striking antioxidative effect. Here, in this study, our data showed that APS can promote proliferation and decrease apoptosis in AGE-induced DCM cell model, besides, APS can decrease intracellular ROS level, increase activity of SOD, GSH-Px and lower level of MDA and NO in DCM cell model, indicating APS exerted antioxidative function in DCM model cells. Besides, western blot results revealed APS induced NRG1 expressing and the phosphorylation level of ErbB2/4. In addition, the elevated NRG1 promoted AKT and PI3k phosphorylation which indicated APS may exert its function by NRG1/ErbB and the downstream AKT/PI3K signaling. Canertinib is ErbB inhibitor. The effect of APS on proliferation, apoptosis, antioxidation and NRG1/ErbB pathway was partly abolished after the cells were co-treated with APS and canertinib. Taken together, these results suggested APS may display its protective function in DCM cells by activating NGR1/ErbB signaling pathway. And our study increased potential for prevention and therapy to DCM.**

Keywords: *Astraglaus polysaccharide* (APS), Diabetic cardiomyopathy, Antioxidation, NRG1/ErbB

1. Introduction

Diabetes mellitus (DM) is the most common metabolic disorders worldwide. In 2010, the diabetic patients were 285 million and, it will increase to 439 million by 2030 (*1,2*). Diabetic patients were characterized with persistent hyperglycemia which may lead to damage to various organs such as the heart (*1,2*). Therefore, DM may cause cardiovascular complications including coronary heart disease, hypertension and diabetic cardiomyopathy (DCM) which are responsible for 80% of mortality and morbidity for diabetic patients (*3-6*). The characterized pathology of DCM is distinct from hypertension and coronary artery diseases (*3-6*). According to the previous studies, the main reason for pathological change of DCM is microangiopathy, which can cause damage to cardiac structure and function, such as apoptosis of the cardiac cells (*3-6*). But, the pathogenesis of DCM has not been clearly understood.

It's showed that various biology processes are associated with DCM, such as cardiomyocyte apoptosis, oxidative stress which is believed to be the key reason induced DM to DCM (*7-10*). There is a balance between reactive nitrogen species and reactive oxygen species

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production in normal cells, while, when the cells were progressed to DCM, the balance was disrupted and the accumulated production can't cleaned in time which finally caused damage. Reactive oxygen species (ROS) are reactive chemical species and excess accumulated ROS can induce oxidative stress (*11-16*). Hence, inhibition of the oxidative stress and improvement of the antioxidative function are believed to be one of the important therapeutic strategies for DCM patients.

The studies showed that antioxidative natural products displayed potential therapeutic effect for DCM (*4,17-21*). Among these natural products, *Astragalus polysaccharides* (APS) is one of the main active extract from *Astragalus membranaceus* (*20,22-27*). Previous studies showed that, for diabetic cardiomyopathy in hamsters, APS can induce myocardial collagen deposition and improve cardiac function by activating ERK1/2 signal pathway (*23,28*). Besides, APS can improve cardiac glucose metabolism dysfunction by inducing expression of GLUT-4 and inhibiting PPAR expressing (*20,24,29*).

Here, in this study, we showed that, APS can promote proliferation and inhibit apoptosis in AGE-induced H9C2 DCM model cells. Besides, APS exerts antioxidative function. Further studies showed, APS exerts its promoting proliferation, suppressing apoptosis and antioxidative function by NRG1/ErbB and its downstream AKT/PI3K pathway in DCM model cells. In summary, our study proved APS exerts protective function in DCM model cells by NRG1/ErbB signal pathway which suggesting APS have great promising for DCM therapy.

2. Materials and Methods

2.1. *Cell culture and treatment*

H9C2 cells (purchased from Shanghai Cellular Research Institute, Shanghai, China) were cultured with Dulbecco's modified essential medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% antibiotic-antimycotic (Gibco, USA) and were cultured at 37°C in 5% CO_2 incubator. To establish DCM model, H9C2 cells were treated with 200 mg/L AGE-BSA and 30 mm/L glucose for 24 h, besides, the cells treated with 200 mg/L BSA and 5 mm/L glucose were used as normalized control group. For the experimentation, the model cells were treated with indicated APS (Tianjin Cinorch Pharmaceutical Company, China) or APS combined with NGR1/ErbB1 inhibitors Canertinib (Sigma, USA).

2.2. *CCK8 assay*

Cell proliferation ability was tested by CCK8 assay (Beyotime Biotechnology, China). The normalized control group or diabetes mellitus model group H9C2 cells in 96-well plates were treated with the indicated concentration APS for indicated time or APS combined with Canertinib, then the cells were incubated with CCK-8 solution at 37°C for 2 hours. Subsequently, the optical density (OD) at 450 nm wavelength was tested by Microplate reader (Biotek, USA).

2.3. *ROS assay*

The intracellular ROS production was detected by ROS assay Kit (Beyotime, China) and the measurement process was carried according to the protocol. Following the indicated treatment, the H9C2 cells in 96-well were washed 2 times with PBS and then incubated with 10 μmol/L 2, 7- dichlorodi-hydrofluorescein diacetate (DCFH-DA) for 20 minutes at 37°C. Followingly, the cells were washed three times with PBS and then the ROS-sensitive signal was examined with Microplate reader at excitation wavelength of 488 nm and emission wavelength of 525 nm.

2.4. *ELISA assay*

The activity of superoxide dismutase (SOD) and Glutathione peroxidase (GSH-Px) and level of malondialdehyde (MDA) and NO l in cell supernatant were tested by the respective ELISA kit (Jiancheng Bioenjineering Institute, China) according to the manufacture's protocol. The H9C2 cells were treated as indicated and the cell supernatant was collected. After the treatment process, the absorbance was measured by spectrophotometer.

2.5. *Apoptosis analysis*

Cell apoptosis was analyzed by flow cytometry. After indicated treatment, the cells were collected, washed with PBS solution and then resuspended in binding buffer. Then, then cells were stained with PI and Annexin V (Invitrogen, USA) for 15 minutes in dark at room temperature. Subsequently, the doubled stained cells were analyzed by flow cytometer (BD, USA).

2.6. *Western blot*

The protein samples were harvested by loading buffer. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). After been blocked with 5% non-fat milk TBST solution for 2 hours, the membranes were incubated overnight at 4°C with primary antibodies against NRG1 (Abcam, 1:1,000), p-ErbB2 (Abcam, 1:800), ErbB2 (Abcam, 1:1,000), p-ErbB4 (Abcam, 1:2,000), ErbB4 (Abcam,1:500), p-PI3K (Abcam,1:2,000), PI3K (Abcam, 1:1,000), p-AKT (Abcam,1:500), AKT (Abcam,1:500) and

GAPDH (Abcam, 1:10,000). Following with secondary antibody (BOSTER, 1:20,000) for 1 hour, the membranes were visualized by ECL kit (Thermo, USA) and the image was scanned and collected by ScanMaker 1000XL Plus instrument.

2.7. *Statistical analysis*

All data were presented as mean ± SD. Every experiment was replicated at least three times. Student's *t*-test and oneway ANOVA were used to perform statistical analysis. *P* value < 0.05 was considered to have statistical difference.

3. Results

3.1. *APS promotes proliferation and inhibits oxidative stress in DCM model H9C2 cells*

To identify the function of APS in DCM, firstly, we tried to get DCM model cells. H9C2 cells were incubated with 200 mg/L AGE-BSA and 30 mm/L glucose for 24 hours to obtain DCM model cells and the treated with 200 mg/L BSA and 5 mm/L glucose cells were used as normal control (NC). The DCM model cells were cultured with various concentrations of APS (0.1, 1.0, 10, 100 μg/mL) for indicated time (0, 12, 24, 48 and 72 hours). After the treatment, cell viabilities were tested by CCK-8 assay. The data showed, the APS can increase the cell viabilities dose-dependently and time-dependently (Figure 1A). Besides, compared with NC group cells, the intracellular ROS level was higher in DCM model cells (Figure 1B) which is consistence with the previous studies, while, APS decreased intracellular ROS level in DCM model cells dose-dependently and time-dependently (Figure 1B). DCM is closely associated with oxidative stress.

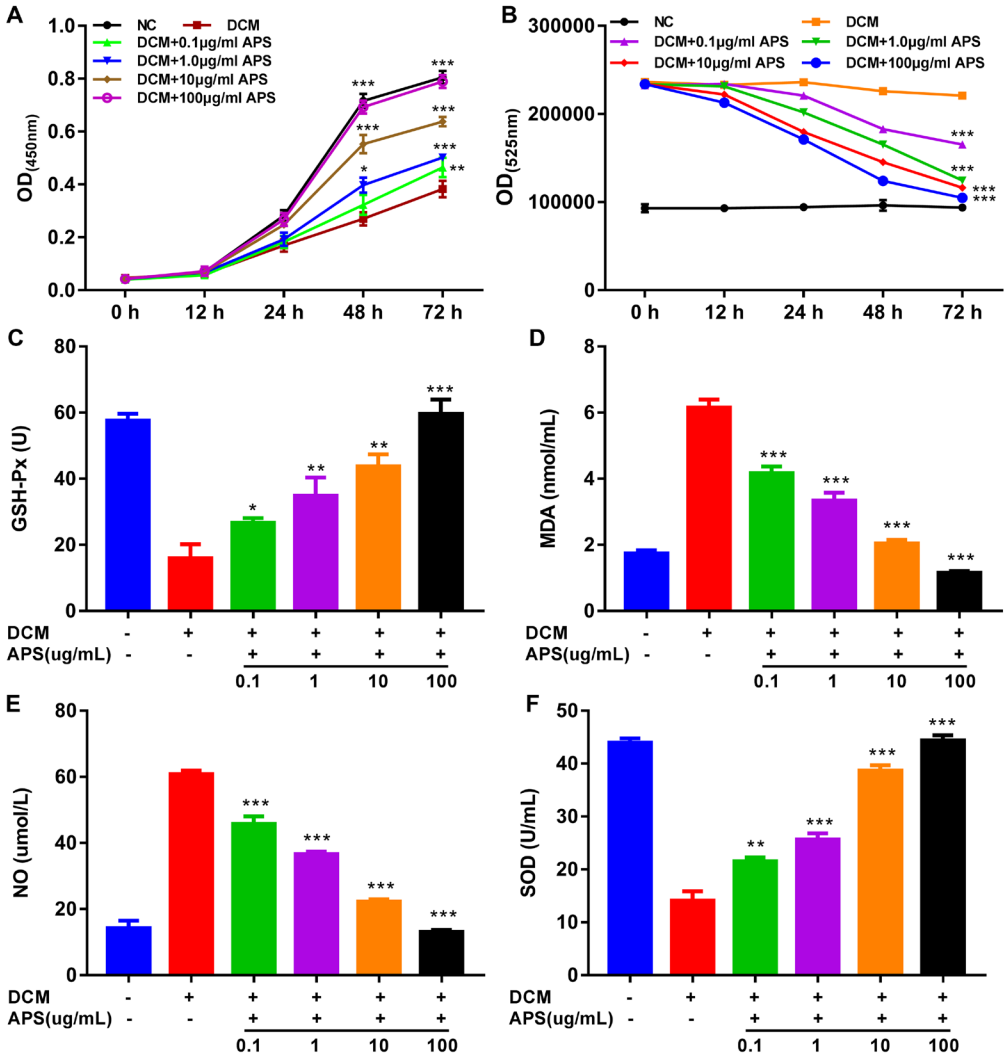

Figure 1. APS promotes proliferation and inhibits oxidative stress in DCM model H9C2 cells. (A and **B)** DCM model H9C2 cells were treated with indicated concentrations of APS for various time. CCK-8 assay was used to test the cell viability and intracellular ROS level was tested by DCF. NC (normal control), DCM (DCM model cells). **(C, D, E,** and **F)** The DCM model H9C2 cells were incubated with indicated concentrations APS for 48 hours. The activity of GSH-Px **(C)** and SOD **(F)** and the level of MDA **(D)** and NO **(E)** were examined by ELISA assay. Data was presented as Mean ± SD, *$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$ *vs*. DCM group cells.

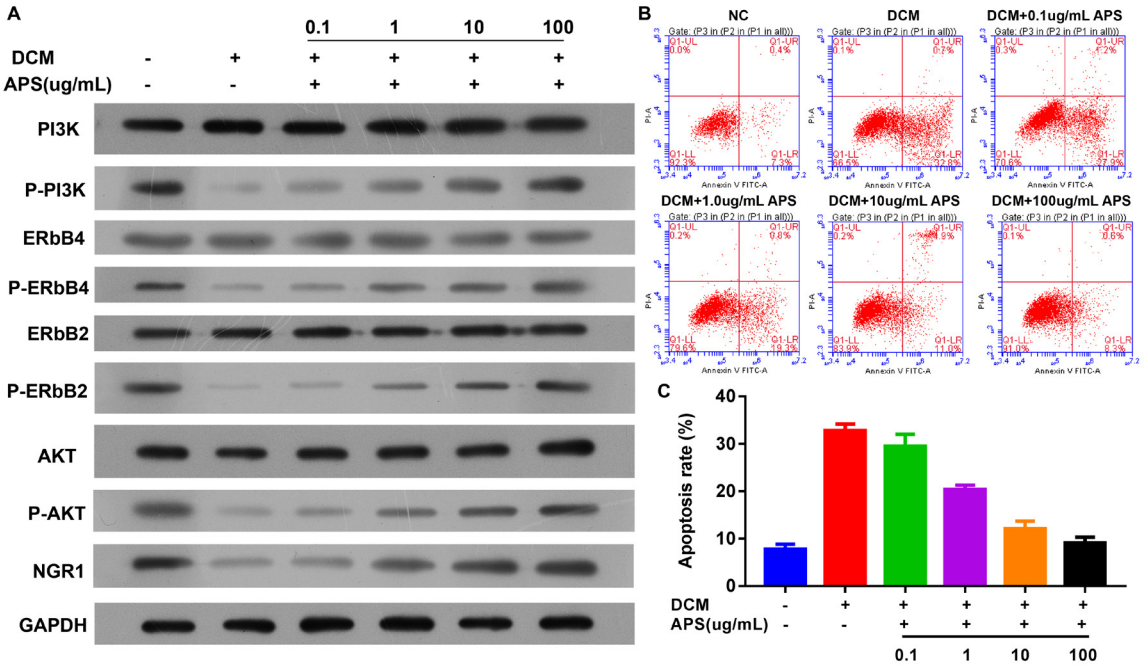

Figure 2. APS inhibits apoptosis and activates NRG1/ErbB pathway. (A) The effect of APS on NRG1/ErbB pathway (level of NRG1, ErbB2 and ErbB4 and phosphorylation level of ErbB2 and ErbB4) and downstream AKT/PI3K signaling (expression level of AKT, PI3K and phosphorylation level of AKT and PI3K0 in DCM model H9C2 cells is examined by western blot. GAPDH expression was used as internal control. **(B** and **C)** The function of APS on apoptosis in DCM model H9C2 cells is analyzed by flow cytometry. $^{**}p < 0.01$ *vs*. DCM group cells.

Activity of GSH-Px and SOD, and level of MDA and NO are markers of oxidative stress. Our data revealed, compared with the NC group cells, the activity of GSH-Px and SOD was decreased while MDA and NO levels were increased in DCM model cells (Figure 1C, 1D, 1F and 1E) which also proved DCM is correlated with oxidative stress. Conversely, APS inhibited activity of GSH-Px and SOD and increased the level of MDA and NO, indicating APS may exert therapeutic effect for DCM by antioxidative function.

3.2. *APS inhibits apoptosis and activates NRG1/ErbB pathway*

Previous studies showed, NRG1 can bind to ErbB receptor to downstream signaling effectors such as AKT/PI3K pathway, thereby, NRG1/ErbB signaling was involved in various biology processes. It proved that NRG1 increased oxidative capacity and improved mitochondrial function. Besides, NGR1/ErbB pathway was impaired in DCM suggesting this pathway may be involved in pathogenesis of DCM. Here, in this study, western blot results showed APS increased expression level of NGR1 in DCM model cells dose-dependently (Figure 2A). APS did not affect expression level of ErbB2 or ErbB4, whereas the phosphorylation level of ErbB2 and ErbB4 was increased (Figure 2A). In addition, APS-induced NRG1 activated downstream AKT/PI3K pathway which promoted phosphorylation of AKT and PI3K (Figure 2A). NRG1/ErbB and downstream AKT/PI3K pathway are involved in apoptosis. Besides, suppression on apoptosis cardiac cells is one of the therapeutic strategies for DCM. Our data proved, compared with NC group cells, apoptosis was higher in DCM model cells (Figure 2B and 2C); and, APS exerted inhibitory function on apoptosis in DCM model cells dose-dependently (Figure 2B and 2C). Our results suggested APS may exert inhibiting apoptosis by activating NRG1/ErbB pathway.

3.3. *Canertinib partly abolishes the function of APS on proliferation and antioxidation*

Our results suggested APS may exert its function by activating NRG1/ErbB pathway. Therefore, ErbB inhibitor (Canertinib) was used as the tool to explore the mechanism of APS in DCM model H9C2 cells. DCM model H9C2 cells were incubated with 100 μg/mL APS and/or Canertinib. Then, the cell viabilities were tested by CCK-8 assay. As previous showed, APS promoted proliferation whereas Canertinib can partly reverse this promotional function (Figure 3A). Besides, Canertinib can also partly abolish the inhibitory effect of APS on intracellular ROS level (Figure 3B). Previously, We have showed APS suppressed the activity of GSH-Px and SOD and increased the level of MDA and NO (Figure 1C, 1D, 1E and 1F). Here, the results showed Canertinib treatment can partly reverse the effect of APS on these oxidative stress markers (Figure 3C, 3D, 3E and 3F). Taken together, our data proved APS promoteed

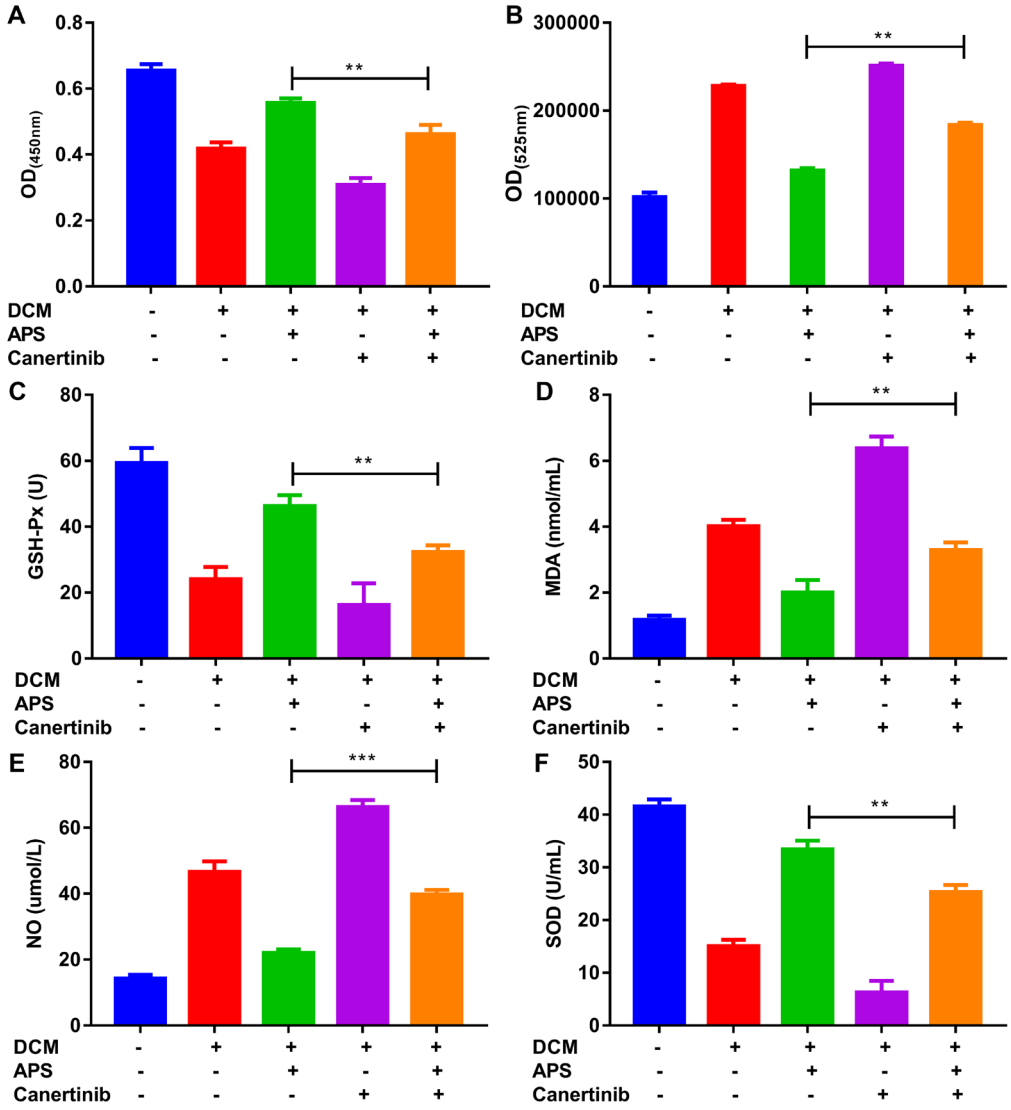

Figure 3. Canertinib partly abolishes the function of APS on proliferation and antioxidation. (A and **B)** DCM model H9C2 cells were incubated with 100 μg/mL APS and/or Canertinib for 48 hours. Then, the cell viabilities of the treated was tested by CCK-8 assay **(A)** and intracellular ROS level was tested by DCF **(B)**. **(C, D, E,** and **F)** The DCM model H9C2 cells were treated as A and B, then, the activity of GSH-Px **(C)** and SOD **(F)**, and the levels of MDA **(D)** and NO **(E)** were detected by ELISA assay. Data was presented as Mean ± SD, $*p < 0.05$, $**p < 0.01$ and *** $p < 0.001$, DCM + APS *vs*. DCM + APS + Canertinib.

proliferation and antioxidation in DCM model cells by activating ErbB.

3.4. *APS suppresses apoptosis in DCM model H9C2 cells by activating NRG1/ErbB pathway*

Previously, we showed APS inhibited apoptosis in DCM model H9C2 cells (Figure 2B). To explore whether ErbB was associated with the effect of APS on apoptosis in DCM model H9C2 cells, DCM model cells were treated with APS and/or canertinib, then, apoptosis was analyzed by flow cytometry. The analysis revealed, canertinib can partly reverse the inhibitory function of APS on apoptosis in DCM model cells (Figure 4A and 4B). This result proved ErbB was involved in the function of APS on apoptosis. APS-induced NRG1 can activate ErbB and the downstream AKT/PI3K signaling (Figure 2A), while canertinib can partly restore APS-induced NRG1 expression (Figure 4C). Further analyze showed canertinib also reversed APS-induced phosphorylation level of ErbB2/4, AKT and PI3K (Figure 4C). In summary, our data proved APS exerted its protective function for DCM model cells by activating NRG1/ErbB pathway.

4. Discussion

DCM is one of the most common complications of DM. DCM is responsible for almost 80% of the mortality of the diabetic patients. Initially, DCM was defined as the results of coronary artery disease and abnormal myocardial structure (*3-6*). However, recently, the studies revealed DCM is a result of the long persistent process of the metabolic effect of DM on myocardium.

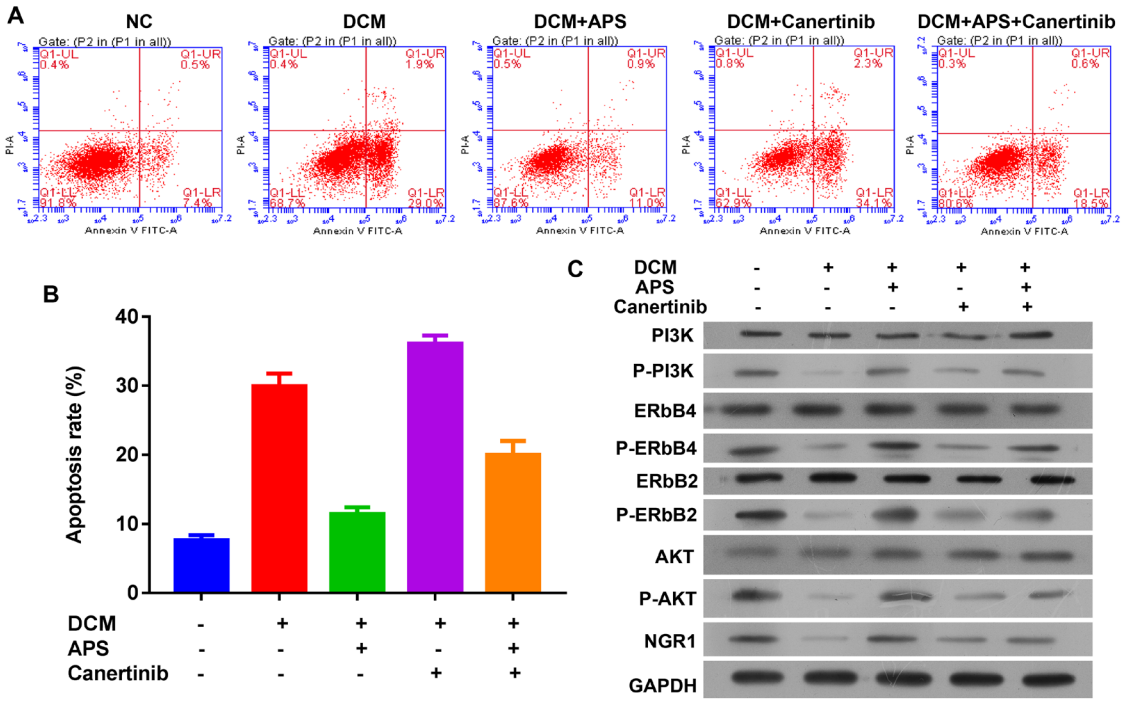

Figure 4. APS suppresses apoptosis in DCM model H9C2 cells by activating NRG1/ErbB pathway. (A and **B)** DCM model cells were incubated with APS and/or Canertinib for 48 hours, then, apoptosis was analyzed by flow cytometry. **(C)** DCM model cells were treated with APS and/or Canertinib. After 48 hours, the samples were harvested and the effect of canertinib on APS-activated NRG1/ErbB pathway (expression of NRG1, ErbB2, ErbB4 and phosphorylation level of ErbB2 and ErbB4) and downstream AKT/PI3K signaling (expression level of AKT, PI3K and phosphorylation level of AKT and PI3K) was tested by western blot. GAPDH expression was used as internal control.

And, the pathology of DCM is independent of hypertension and coronary. But, the pathogenesis of DCM is not fully understood right now. According to previous studies, multiple biological processes are involved in the pathogenesis and progression of DCM, such as cardiomyocyte apoptosis, endoplamic reticulum, autophagy, mitochondrial dysfunction (*7-10*). Among these various biology processes, oxidative stress is the most important process inducing DM to DCM (*11-16*). Reactive oxygen species (ROS) are the most common chemically reactive chemical species, it showed that over accumulated ROS can cause oxidative stress (*11-16*). Here, in this study, our data also showed, compared with the normal control (NC) cells, intracellular ROS level is higher, further studies displayed the activity of GSH-Px was decreased while the levels of MDA and NO were elevated in DCM model cells (Figure 1C, 1D, 1E and 1F). Our results proved the previous studies in turn. Therefore, inhibiting oxidative stress is believed to be an important strategy for the therapy of DCM. Previous studies revealed, NRG1/ErbB pathway is impaired in DCM cells which suggested NRG1/ErbB pathway may play an important role in DCM (*30-32*). Besides, NRG1/ErbB can improve glucose tolerance in healthy and diabetic rodents (*32*). And, also previous studies showed NRG1 can regulate myocyte oxidative capacity (*33,34*). Our data revealed, compared with the normal control cells, NRG1/ErbB pathway is inactive in DCM model cells (Figure 2A).

Recently, increasing evidences showed natural products exerted antioxidative effect in DCM. APS is one of the key active component of the traditional Chinese medical herb *Astragalus membranaceus* (*20,22-24,26,35*). Previously, it's showed that APS decreased apoptosis in high-glucose-induced H9C2 cells by regulating the function of mitochondria and inhibiting expressing of caspase (*23*). Present, we showed that, APS improved proliferation (Figure 1A) and decreased apoptosis (Figure 2B and 2C) in DCM model H9C2 cells. Further studies showed, firstly, APS lowered the intracellular ROS level in DCM model cells (Figure 1B). Secondly, APS can also increase the activity of GSH-Px and SOD and decreased the level of MDA and NO in DCM model H9C2 cells (Figure 1C, 1D, 1E and 1F). These results indicated APS may play protective role in DCM cells. Our results proved, compared with the normal control cells, NRG1/ErbB pathway was inactive in DCM model H9C2 cells (Figure 2A). And, APS activated the NRG1/ErbB pathway dose-dependently and time-dependently (Figure 2A), which suggested APS may exert its protective role in DCM model H9C2 cells by activating NRG1/ErbB pathway. Canertinib is the inhibitor of ErbB. Pretreatment of DCM model cells with APS and Canertinib can partly abolish the protective effect of APS in DCM model H9C2 cells by inhibiting NGR1/ErbB pathway. Canertinib can partly reverse APS-induced proliferation (Figure 3A). And, canertinib can also partly abrogate the function of APS

on apoptosis (Figure 4A), intracellular ROS level (Figure 3B) and the activity of GSH-Px and SOD and the levels of MDA and NO (Figure 3C, 3D, 3E and 3F). Taken together, our results proved APS exerted its protective role by activating NRG1/ErbB pathway.

In conclusion, our study proved: *i*) DCM is associated with proliferation, apoptosis, oxidative stress and inactive NRG1/ErbB pathway; *ii*) APS can increase proliferation, inhibit apoptosis and improve antioxidative function including reducing intracellular ROS level, elevating activity of GSH-Px and SOD and lowering the level of MDA and NO by activating NRG1/ErbB pathway. Our study broadened the mechanisms of DCM and increased potential for prevention and therapy to DCM.

Acknowledgements

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