Substance P prevents doxorubicin-induced cardiomyocyte injury by regulating apoptosis and autophagy: *In vitro* and *in vivo* evidence

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Abstract. The function of substance P (SP) in myocardial ischemia is well understood, but its effects on congestive heart failure are unclear. The present study aimed to use *in vitro* and *in vivo* approaches to investigate the effects of SP on doxorubicin-induced cardiomyocyte injury. Pathological changes, apoptosis, cardiomyocyte ultrastructure and molecular mechanisms were evaluated *in vitro* and *in vivo*. The effects of SP on cell viability of H9c2 myocardial cells were evaluated using the Cell Counting Kit-8 and flow cytometry. B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), Beclin-1 and microtubule-associated protein 1A/1B-light chain 3 (LC3) were detected by western blotting. Heart failure in rats was established by intraperitoneal injection of doxorubicin. The *in vitro* data demonstrated that SP at concentrations of 1 μg/ml inhibited doxorubicin-induced apoptosis of H9c2 cells. Administration of doxorubicin reduced Bcl-2, Beclin-1 and LC3 expression levels in H9c2 cells, while having no effect on Bax levels. Administration of SP to these doxorubicin-treated cells did not affect Bcl-2 or Bax expression, but further reduced Beclin-1 while inhibiting the reduction in LC3 expression. *In vivo*, food intake was significantly increased in rats in the SP group compared with the model group. Cardiomyocytes in the heart-failure group underwent dysfunctional autophagy as ascertained by transmission electron microscopy. Compared with the heart-failure group, these pathological changes, including loss of striations and vacuolation, were inhibited by SP treatment, which promoted Bax expression, reduced Beclin-1 expression and inhibited the reduction in LC3 expression. Taken together, SP reduced cardiomyocyte apoptosis in doxorubicin-induced cardiomyocyte injury, likely by promoting autophagy, which suggested that SP is a potential therapeutic target for doxorubicin-induced heart failure.

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Key words: substance P, heart failure, autophagy, apoptosis

Introduction

Doxorubicin is one of the most widely used chemotherapeutic drugs (1), which acts primarily by triggering apoptosis via inhibition of topoisomerase activity and generation of reactive oxygen species (ROS) (2). Cardiotoxicity and congestive heart failure (CHF) are prominent side effects of doxorubicin because of its toxic effects on cardiomyocytes (3) and this limits its clinical use. Notably, doxorubicin is widely used to produce animal and cellular models of heart failure (4,5). Apoptosis is known to be one of the key pathological processes of CHF (6); therefore, a strategy to prevent cardiomyocyte apoptosis could effectively delay and treat CHF (7).

Substance P (SP) belongs to the tachykinin family of sensory neuropeptides, which serves an important role in activating tachykinin receptor 1. SP functions in the repair of sensory injury, regulation of smooth muscle contraction and modulation of inflammation/immune responses (8-12). SP is mainly expressed in the central nervous system and peripheral afferent sensory neurons, especially in C-fibers (13). In addition to neurons, SP is also found in heart tissue (14). Expression of SP has been reported to markedly increase following ischemic injury, which SP has previously been shown to limit (15,16). In addition, SP is hypothesized to promote inflammation and cardiac hypertrophy in myocarditis (17,18). SP may also be involved in heart failure caused by hypertension or stress by promoting expression of MMPs (19). However, the effects of SP on doxorubicin-induced heart failure remain to be elucidated.

Autophagy is one type of cellular degradation, which functions to remove unnecessary or damaged components (20,21). Inappropriate autophagy is associated with several diseases, including cardiac and neurodegenerative diseases (22). The present study used cellular and animal experiments to investigate the effects of SP on cardiomyocyte injury caused by doxorubicin. Additionally, the potential mechanisms involving autophagy were investigated. The data demonstrated that SP limited doxorubicin-induced cardiomyocyte injury, probably by regulating apoptosis and autophagy. These results have

implications for the prevention of doxorubicin-induced heart failure.

Materials and methods

Ethics statement. The animal protocols performed in the present study were approved by the Ethics Committee of People's Hospital Affiliated to Nanchang University (Nanchang, China; approval no. 2019-037). After the experiments, animals were anesthetized by 5% isoflurane, followed by decapitation.

Cell culture and treatments. H9c2 myocardial cells were purchased from BeNa Culture Collection (Beijing Beina Chunglian Institute of Biotechnology; cat. no. BNCC295057) and were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in an incubator with 5% CO_2 at 37˚C. The effects of SP on the viability of H9c2 cells were determined using the Cell Counting Kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology). The protective effects of SP on doxorubicin-induced cell injury were evaluated as follows: After cells had adhered to the substrate, culture medium was discarded and the cells were washed with 1X phosphate buffered saline (PBS). Doxorubicin (2 μM; cat. no. D107159; Shanghai Aladdin Biochemical Technology Co., Ltd.; model group) or 2 μM doxorubicin + 1 μg/ml exogenous SP (cat. no. HY-P0201A; MedChemExpress) medium (SP group) were then added to paired wells; after 24 h at 37˚C, flow cytometry and western blotting were performed. The cells in the control group did not receive any treatment.

CCK-8 assay. After H9c2 cells had completely adhered to the well, the supernatant was discarded and different concentrations of SP (0, 0.1, 0.5, 1, 5 and 10 μg/ml) suspended in fresh culture medium were added to the cells. The cells were cultured in an incubator at 37˚C for 24 h and cell viability was assessed to screen for safe concentrations of SP. Briefly, following treatment, 10 μl CCK-8 solution was added to each well and incubated at 37˚C for 1.5 h. Optical density was measured at a 450 nm using a microplate reader to calculate cell viability at each SP concentration and 1 μg/ml SP was selected to investigate its effect on doxorubicin-induced cardiomyocyte injury.

Flow cytometry. Following treatment for 24 h, cells were collected, washed with PBS and centrifuged at 897 x g for 3 min at 4˚C. Annexin V-fluorescein isothiocyanate (3 μl) and propidium iodide (5 μl) were added to the cells according to the instructions of the assay kit (cat. no. C1062S; Beyotime Institute of Biotechnology). After gentle mixing, the cells were incubated at room temperature for 10 min in the dark and apoptosis was measured by flow cytometry (NovoCyte® 2060R; ACEA Biosciences, Inc.) and analyzed using FlowJo 7.6 (FlowJo, LLC), The percentage of early + late apoptotic cells were counted.

Preparation of a heart-failure model and treatments. A total of 18 male Sprague Dawley rats (age, 2 months; weight, 200±20 g) were purchased from Hunan Slake Jingda Experimental Animal Co., Ltd. [license no. SCXK (Xiang) 2019-0004]. The animals were housed in a specific pathogen-free condition that was automatically maintained at a temperature of 23±2˚C, a relative humidity of 45-65%, and with a controlled 12 h light/dark cycle and free to access to food and water. The animals were divided into three groups (n=6/group): i) Control group; ii) model group; and iii) SP treatment group. The heart-failure rat model was prepared as previously described (23). Briefly, doxorubicin was injected intraperitoneally once every 3 days (3 mg/kg) with a cumulative total of 15 mg/kg. The injections were completed within 2 weeks to establish the rat myocardial injury model. SP was injected via the caudal vein at a dose of 6.7 μg/kg once every 4 days as previously described (24). The rats in the control and model groups were injected with the same amount of saline. An electrocardiogram (ECG) was used to monitor heart function after treatment and heart rate was automatically recorded. Thereafter, the rats were decapitated following anesthesia (5% isoflurane). Myocardial tissue was collected and fixed in 4% paraformaldehyde at 4˚C overnight for determination of pathological changes.

Hematoxylin and eosin (H&E) staining. Fixed tissue was washed with running water for several hours, serially dehydrated in 70, 80 and 90% ethanol, a mixture of ethanol and xylene for 15 min, and then xylene for 30 min. The tissues were subsequently immersed in a mixture of xylene and paraffin for 15 min, and then in paraffin for 50-60 min. The paraffin-embedded tissues were then sectioned (10 μm). After warming, dewaxing and rehydrating, the sections were stained with hematoxylin (3%) and eosin (3%) for 5 min at room temperature. The sections were observed under a light microscope (BX53, Olympus Corporation).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. After dewaxing (xylene treatment for 10 min) and rehydrating in a gradient ethanol series at room temperature, 50 μg/ml proteinase K was added and the sections were incubated at 37˚C for 30 min. The tissues were then washed with PBS three times (5 min/wash). The PBS was removed and TUNEL solution (5 μl/ml; Beyotime Institute of Biotechnology) was added to each slide and incubated at 45˚C for 2 h in the dark, followed by DAPI (5 μg/ml) staining at room temperature for 5 min. The liquid on the slide was dried using absorbent paper and the slide was sealed and observed under a fluorescence microscope.

Transmission electron microscopy (TEM). Myocardial tissues were placed in 2.5% glutaraldehyde at 4˚C for 4 h followed by fixation with 1% osmium tetroxide for 1.5 h. and washed three times with pre-cooled PBS. After dehydration with a gradient ethanol series and acetone, the tissues were incubated in epoxy resin overnight at room temperature prior to sectioning (2 nm slices). After that, the slices were stained with 2% uranyl acetate and 0.5% lead citrate for 5 min at room temperature. Autophagic ultrastructure was observed by transmission electron microscopy (HT7700; Hitachi High-Technologies Corporation; magnification, x8,000).

Western blotting. Myocardial tissue was ground into powder in liquid nitrogen. Proteins were extracted from tissues and

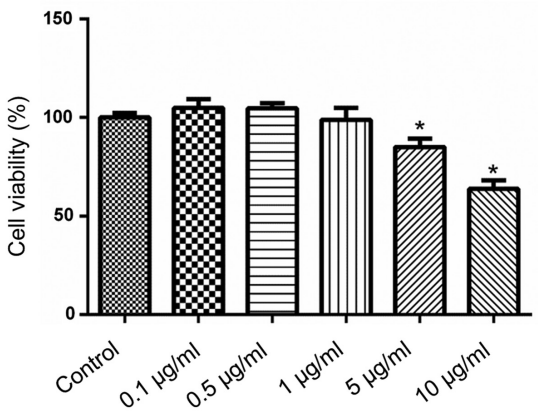

Figure 1. Effects of SP on the viability of H9c2 cells. SP at concentrations ranging from 0.1 to 1 μg/ml did not affect the viability of H9c2 cells. By contrast, 5 or 10 μg/ml SP reduced cell viability. *P<0.05 vs. control cells (n=6 repeats/group). SP, substance P.

H9c2 cells using a protein isolation kit (cat. no. 28-9425-44; Cytiva) and protein concentration was determined by the bicinchoninic acid method. The proteins (25 μg/lane) were denatured and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis for 2 h (12% gel), followed by transfer to a nitrocellulose membrane (300 mA was applied for 80 min) as described previously (25,26). The membranes were blocked in 5% skimmed milk for 2 h at room temperature. Thereafter, the membrane was incubated with primary antibodies at 4˚C overnight, and then after washing, the membrane was incubated with a secondary antibody (horseradish peroxidase-labeled goat anti-rabbit IgG; 1:100; cat. no. ab6721; Abcam) at room temperature for 2 h. Dye solution from an enhanced chemiluminescence kit (cat. no. RPN2133; Cytiva) was added to the membrane and staining was visualized using a gel imaging system (Bio-Rad Laboratories, Inc.). The gray value was analyzed by Quantity One software (version 4.62; Bio-Rad Laboratories, Inc.). The primary antibodies included mouse monoclonal anti-β-actin (1:2,000; cat. no. TA-09; OriGene Technologies, Inc.), mouse anti-B-cell lymphoma 2 (Bcl-2; 1:500; cat. no. ab692; Abcam), rabbit anti-Bcl-2-associated X protein (Bax; 1:500; cat. no. A0207; ABclonal Biotech Co., Ltd.), rabbit anti-Beclin-1 (1:1,000; cat. no. ab62557; Abcam) and rabbit anti-microtubule-associated protein 1A/1B-light chain 3 (LC3-II; 1:500; cat. no. bs-8878R; BIOSS).

Statistical analysis. All data were expressed as the mean ± standard deviation with six repeats in both the animal and cell culture experiments. Statistical analysis was carried out with GraphPad Prism 7 (GraphPad Software, Inc.) using one-way ANOVA followed by the Bonferroni test. P<0.05 was considered to indicate a statistically significant difference.

Results

SP inhibits doxorubicin-induced apoptosis of H9c2 cells. H9c2 myocardial cells were treated with different concentrations of SP for 24 h. Viability of H9c2 cells was detected using the CCK-8 method. SP at concentrations ranging between 0.1 and 1.0 μg/ml did not affect H9c2 cell viability (Fig. 1). By contrast, 5 or 10 μg/ml SP significantly reduced cell viability compared with untreated controls.

Using the results of the CCK-8 assay, 1 μg/ml SP was selected to investigate the effect of SP on doxorubicin-induced injury of H9c2 cells. The apoptosis of H9c2 cells treated with doxorubicin was significantly higher compared with that detected in the control H9c2 cells, whereas SP significantly reduced this effect (Fig. 2).

Effects of SP on the expression levels of Bax, Bcl-2, Beclin-1 and LC3 in doxorubicin-treated H9c2 cells. In order to further explore the specific effects of SP on apoptosis and autophagy, the protein expression levels of Bcl-2, Bax, Beclin-1 and LC3 were monitored in H9c2 cells. As shown in Fig. 3, Bcl-2 expression in doxorubicin-treated H9c2 cells was significantly lower compared with that in the control group, whereas the addition of SP to the doxorubicin-treated cells had no effect on Bcl-2 expression. Bax expression was comparable in all three groups, which indicated that Bax was unaffected by doxorubicin and SP.

Compared with in the control H9c2 cells, doxorubicin treatment reduced the expression levels of Beclin-1 and LC3. SP further reduced Beclin-1 expression, but inhibited the doxorubicin-induced decrease in LC3 (Fig. 3). These data suggested that SP might rescue doxorubicin-induced autophagy dysfunction.

SP increases food intake in rats with heart failure. Of the 12 rats used in the present study, none died during the experiments. Compared with the control rats, the food intake and body weight of the rats in the heart-failure group were significantly decreased (Fig. 4). Administration of SP to the heart-failure rats was associated with no significant change in body weight for 12 days; however, food intake rose on days 10-12.

Subsequently, a six-lead ECG was used to monitor heart function and representative traces are shown in Fig. 5. Heart rate was also quantified in the three groups. Compared with in the control group, heart rate in the heart-failure group was significantly decreased, whereas SP treatment inhibited this decrease (Fig. 5).

Histological observation of rat myocardium. The results of H&E staining of rat myocardial tissue are shown in Fig. 6. Compared with in the control group, the cardiomyocytes in the doxorubicin-induced heart-failure group were arranged in a disordered pattern and were loosely connected. SP treatment ameliorated these pathological changes caused by doxorubicin.

Compared with in the control group, cardiomyocytes in the heart-failure group exhibited a loss of striations, vacuolation with damaged organelle, indicating dysfunction of autophagy. By contrast, SP reduced the loss of striations, as well as vacuolation (Fig. 7).

SP reduces cardiomyocyte apoptosis in rats with heart failure. TUNEL staining was used to determine the level of apoptosis in myocardial tissue. Apoptosis in myocardial tissue from the heart-failure group was significantly higher compared with that in the control group, whereas this effect was inhibited by SP treatment (Fig. 8).

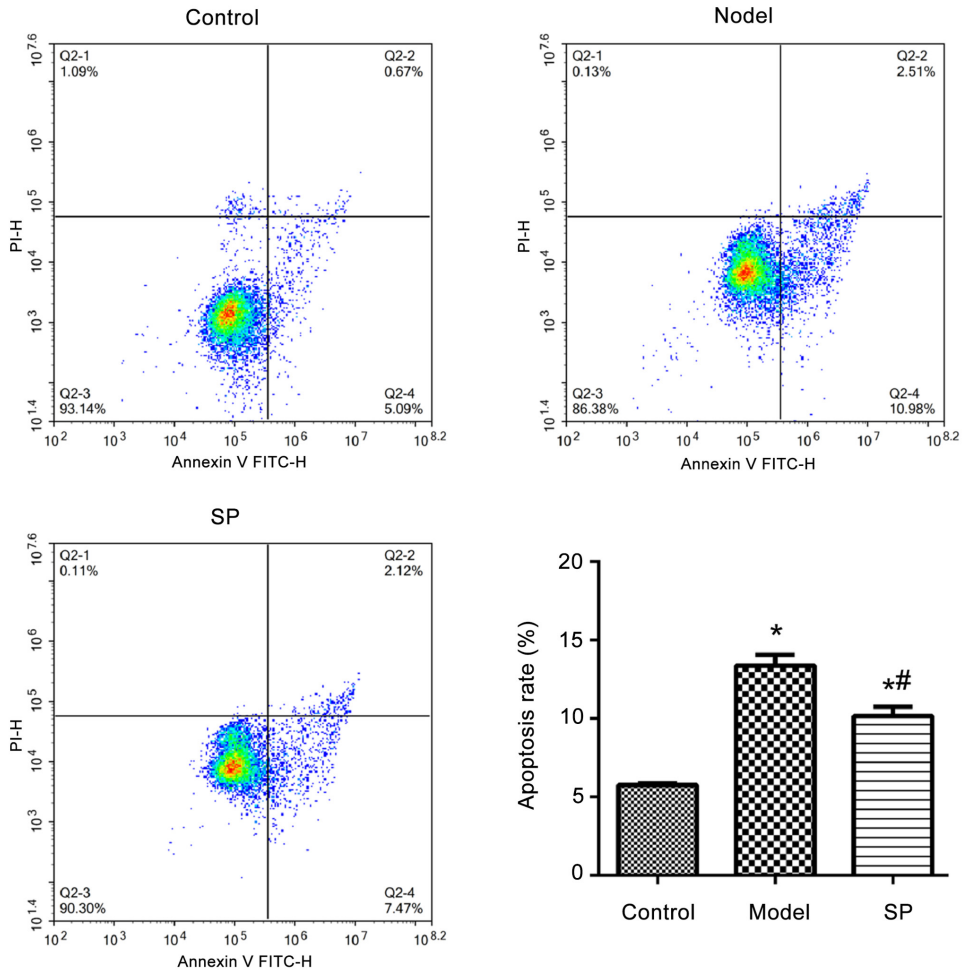

Figure 2. SP inhibits doxorubicin-induced apoptosis of H9c2 cells. Apoptosis of H9c2 cells in the doxorubicin-treated group was higher compared with that in control H9c2 cells, whereas SP attenuated doxorubicin-induced apoptosis. *P<0.05 vs. control cells; #P<0.05 vs. model cells (n=6 repeats/group). SP, substance P.

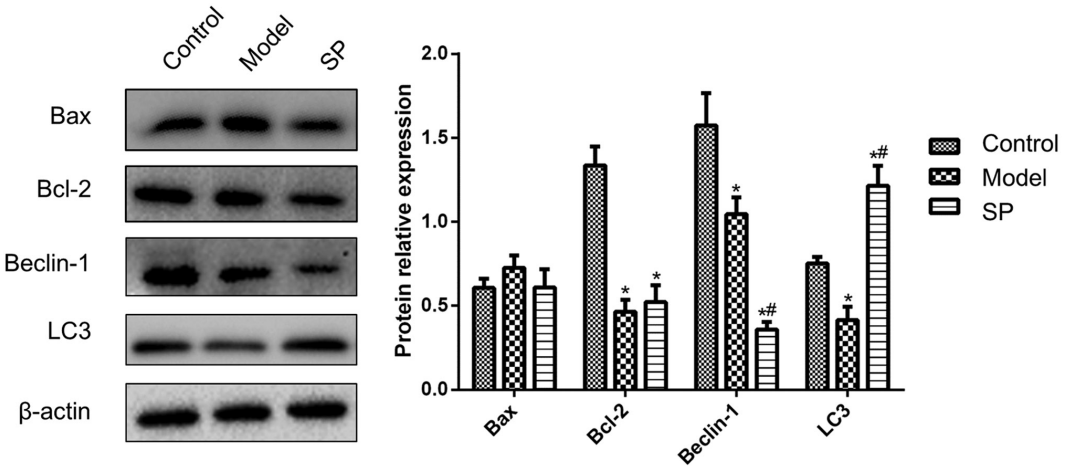

Figure 3. Effects of SP on the expression levels of Bax, Bcl-2, Beclin-1 and LC3 in H9c2 cells. Bcl-2 expression in the doxorubicin-treated group was significantly lower than in the control group, whereas SP did not influence Bcl-2 expression in the doxorubicin-treated group. Doxorubicin reduced the expression levels of Beclin-1 and LC3 in H9c2 cells, whereas SP further reduced Beclin-1 expression, but inhibited the doxorubicin-induced decrease in LC3 levels. *P<0.05 vs. control cells; #P<0.05 vs. model cells (n=6 repeats in each group). SP, substance P; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; LC3, microtubule-associated protein 1A/1B-light chain 3.

Effects of SP on the expression levels of Bax, Bcl-2, Beclin-1 and LC3 in heart-failure rats. Compared with in the control group, there was a significant decrease in the protein expression levels of Bcl-2, Beclin-1 and LC3, but not Bax, in the heart-failure group (Fig. 9). SP inhibited these changes in LC3, although Beclin-1 expression was further reduced.

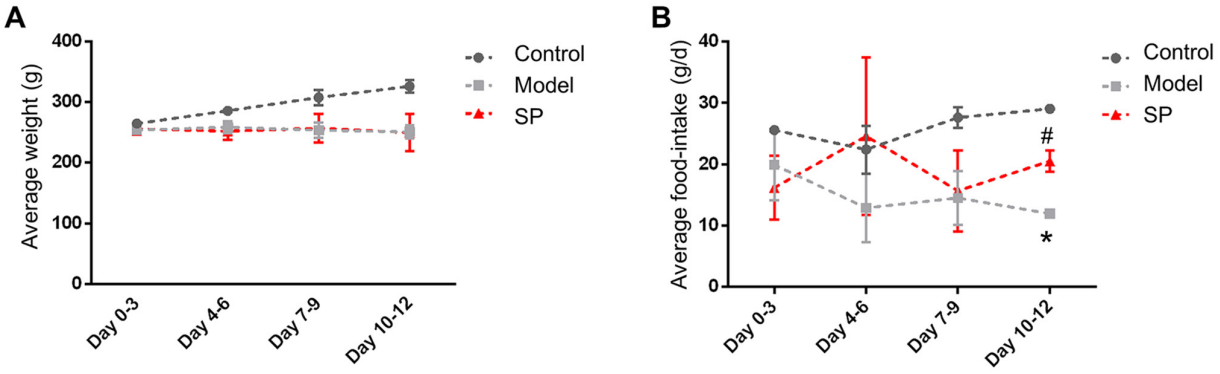

Figure 4. SP increases food intake in rats with heart failure. (A) Rat weight and (B) food intake. Food intake and weight of the rats decreased in the heart-failure group. Administration of SP increased food intake on days 4-12. *P<0.05 vs. control group; #P<0.05 vs. model group (n=6 repeats/group). SP, substance P.

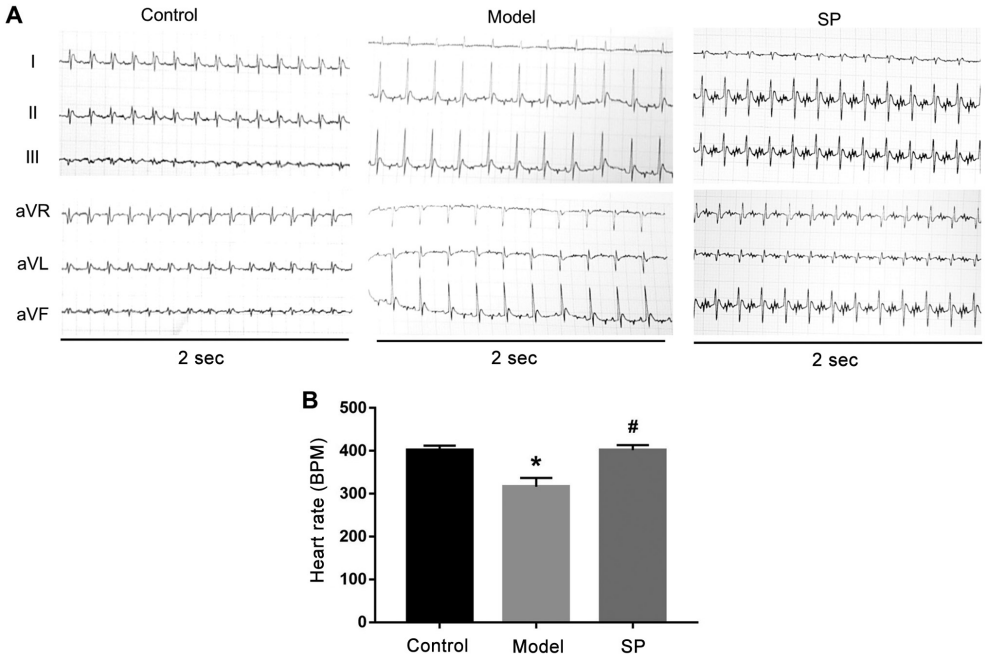

Figure 5. SP ameliorates the impaired heart rate in rats with heart failure. (A) representative ECG traces. (B) Heart rate. *P<0.05 vs. control group; #P<0.05 vs. model group (n=6 repeats/group). SP, substance P; BPM, beats per minute; ECG, electrocardiogram.

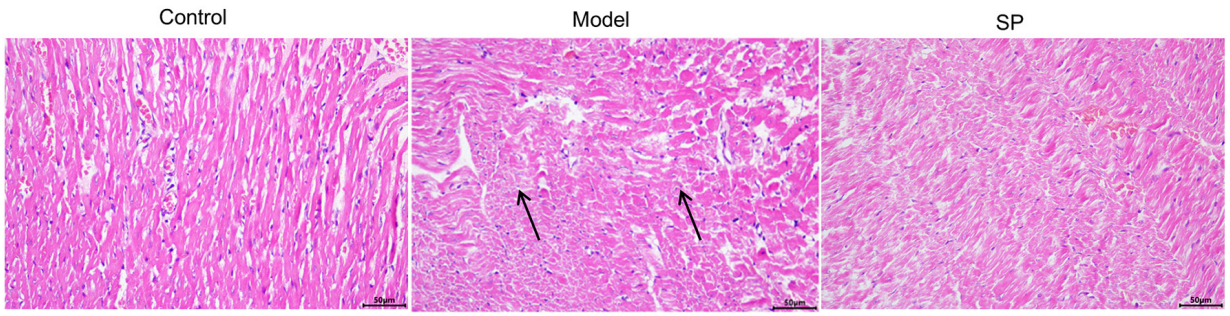

Figure 6. Pathological changes in myocardial tissues. Myocardial fibers were disordered and loose (arrows), and there were more gaps between muscle cells in the heart-failure group. SP treatment remedied the pathological changes caused by doxorubicin. Magnification, x200. SP, substance P.

Additionally, SP increased Bax expression, while it did not affect Bcl-2 expression compared with the model group. These results indicated that SP promoted autophagy, while reducing apoptosis.

Discussion

In the present study, the rate of apoptosis of H9c2 cells was increased following doxorubicin treatment. Pathological changes

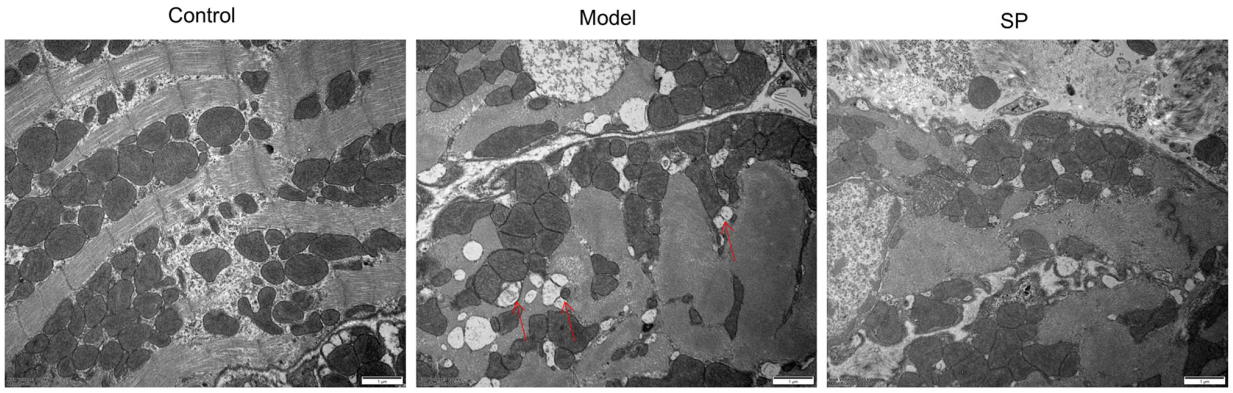

Figure 7. Ultrastructure of cardiomyocytes in the different groups. Cardiomyocytes in the heart-failure model lost striations, became vacuolated and exhibited obvious dysfunction of autophagy. SP promoted autophagy of cardiomyocytes in doxorubicin-induced heart failure. The arrow indicates the accumulated autophagic vacuole. Scale bar, 1 μm. SP, substance P.

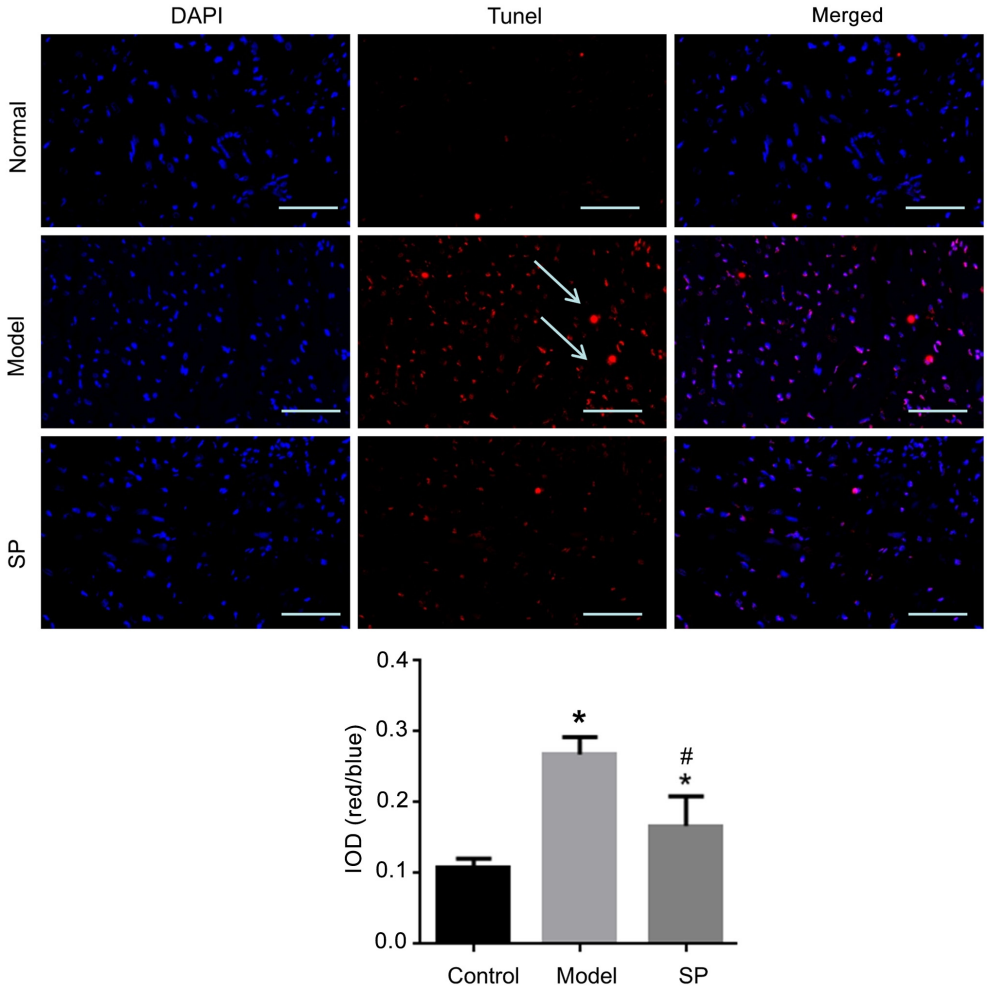

Figure 8. SP reduces cardiomyocyte apoptosis in rats with heart failure. The level of apoptosis in myocardial tissue of the heart-failure group was significantly higher compared with that in the control group, whereas the rate of apoptosis in the SP treatment group was lower compared with in the heart-failure group. The arrow indicates apoptotic cells. Scale bar, 100 µm. *P<0.05 vs. control group; #P<0.05 vs. model group (n=6 repeats/group). SP, substance P; IOD, integrated optical density.

of myocardial tissue in doxorubicin-treated rats were observed. By contrast, SP protected against doxorubicin-induced injury of H9c2 cells and heart tissue. The present study provided evidence that SP limited apoptosis and triggered autophagy, which potentially has implications for therapeutic applications.

Doxorubicin is a common chemotherapeutic drug, which acts primarily through the induction of apoptosis by inhibiting topoisomerase activity and generating ROS (1). Cardiomyocytes are susceptible to doxorubicin; therefore, cardiotoxicity is a prominent side effect (3), and doxorubicin is widely used to

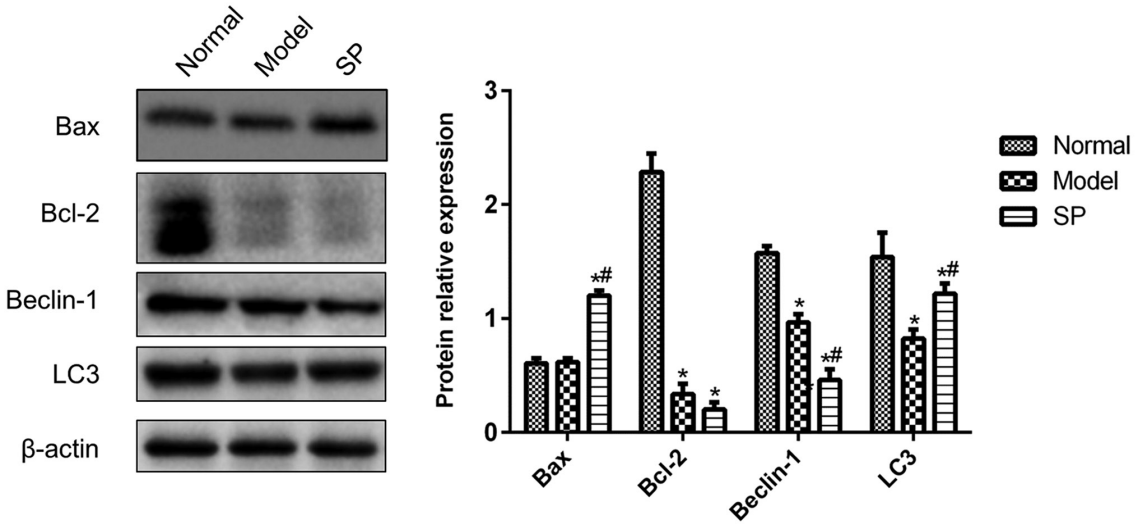

Figure 9. Effects of SP on the expression levels of Bax, Bcl-2, Beclin-1 and LC3 in heart failure rats. The expression levels of Bcl-2, Beclin-1 and LC3 were reduced in the heart-failure model. SP promoted the expression levels of Bax and LC3, and inhibited Beclin-1 expression. *P<0.05 vs. control group; #P<0.05 vs. model group (n=6 repeats/group). SP, substance P; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; LC3, microtubule-associated protein 1A/1B-light chain 3.

produce animal and cellular models of heart failure (4,5). The present study found that doxorubicin triggered cardiotoxicity, as evidenced by an increase in apoptosis and apoptosis-related protein (Bcl-2) expression. Notably, it was found that a safe concentration of SP prevented doxorubicin-induced apoptosis of H9c2 cells. These data suggested that SP at specific concentrations may prevent doxorubicin-induced cardiotoxicity.

The present study demonstrated that 1 μg/ml SP had no significant effect on the viability of H9c2 cells, but reduced doxorubicin-induced apoptosis of H9c2 cells. In addition, the experimental results demonstrated that doxorubicin could reduce the expression levels of Beclin-1 and LC3 in H9c2 cells, whereas SP further reduced the expression of Beclin-1 but increased the expression of LC3. As the mammalian ortholog of the yeast Atg6 gene, Beclin-1 is an essential mediator of autophagy. In addition, it has also been reported that Beclin 1 may have a proapoptotic role (27). By contrast, LC3 can be used to indicate autophagy (28). Therefore, the present study indicated that SP may promote autophagy and reduce apoptosis.

In the apoptosis cascade, Bcl-2 and Bax balance each other in controlling release of cytochrome c from mitochondria to determine cell death (29). Therefore, the expression levels of Bcl-2 and Bax are considered to be representative of apoptosis (30). The present study detected Bcl-2 and Bax expression in H9c2 cells and heart tissue that had experienced doxorubicin-induced injury. It was observed that doxorubicin reduced Bcl-2 expression *in vitro* and *in vivo*, whereas it did not affect Bax, which implicated apoptosis in doxorubicin-induced injury of cardiomyocytes (31,32). Notably, SP could reverse the effect of doxorubicin on the Bcl-2/Bax ratio. These data further support the conclusion that SP can prevent doxorubicin-induced apoptosis of cardiomyocytes.

The present study also investigated the effects of SP on food intake and body weight in rats with heart failure. The data demonstrated that SP could increase food intake following heart failure. Reduction of heart rate is an important functional index of heart failure (33). The present study used an ECG to monitor heart function. Heart rate was reduced in model group, indicating the heart function was impaired. By contrast, SP could reduce the impairment. The results of H&E staining also suggested that SP could repair doxorubicin-induced morphological changes in heart tissue. Taken together, these data support the conclusion that SP can block doxorubicin-induced heart malfunction.

Autophagy is considered to be an important mechanism of organelle and protein turnover in cells (20). Autophagy can prevent apoptosis in cases of mild external stimulation, and the activation of apoptosis-related caspase-3 can block autophagy (34-36). The present findings indicated that SP could reduce doxorubicin-induced cardiomyocyte apoptosis by increasing autophagy in myocardial tissue, which suggested a potential for SP in the treatment of heart failure.

The data in the present study contradicted some earlier publications (37,38) in which SP antagonists inhibited doxorubicin-induced cardiomyocyte apoptosis and triple-negative breast cancer chemoresistance. These discrepancies might be caused in part by bidirectional regulation of SP signaling pathways, as discussed in a previous review in which SP was shown to have beneficial as well as detrimental effects on heart failure (39). The duration and dose of SP might also control its various functions. The present study revealed that high doses of SP were detrimental to H9c2 cells, whereas low doses exerted protection against doxorubicin-induced cardiomyocyte injury. This observation might have important implications for clinical applications of SP and warrants further investigation.

The present study demonstrated a mechanism by which SP reduced cardiomyocyte apoptosis in doxorubicin-induced cardiomyocyte injury, potentially by promoting autophagy. However, the effects of SP on doxorubicin-induced oxidative stress deserve future investigation. Additionally, the direct relationship between apoptosis and autophagy still requires investigation.

In conclusion, the present study indicated that SP reduced cardiomyocyte apoptosis, potentially by promoting autophagy, in a rat model of doxorubicin-induced heart failure, which indicated that SP might be a potential therapeutic substance for heart failure.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FXC, QW, QLL, JF and LP performed the experiments and analyzed the data. FC and JH designed the study, wrote the manuscript and confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Ethics Committee of People's Hospital Affiliated to Nanchang University (Nanchang, China; approval no. 2019-037).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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