



Cardioprotective Effect of Spinosin against Doxorubicin Induced Inflammatory and Hypertrophy via Nrf2 Signaling

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ABSTRACT

Pathological cardiac remodeling can lead to heart failure and cardiac hypertrophy. *Zizyphus jujuba* var spinosa seeds contain SPN, a C-glycoside flavonoid used in traditional medicine. A growing body of evidence indicates they have pharmacological potential against a wide range of diseases. Spinosin (SPN), however, has not been examined in relation to doxorubicin (Dox) induced cardiac injuries. To find out if SPN can reverse Dox-induced cardiomyopathies, we conducted this study. Results showed that Dox treatment led to bigger cardiomyocytes, more inflammatory markers, and more cytokines. SPN treatment, however, reduced Dox-induced enlargement, attenuated hypertrophic markers, and attenuated the expression of inflammatory markers. Moreover, we saw increased levels of oxidative stress-related proteins NRF2, HO-1, and NQO-1. SPN protects against oxidative stress and inflammation-induced cardiomyopathies, according to our study.

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INTRODUCTION

The most dangerous disease in the world is cancer, which is usually treated with doxorubicin (Dox) (Rivankar, 2014). In addition to causing cardiomyopathy, Dox also causes congestive heart failure (Huang *et al.*, 2017; Mouli *et al.*, 2015). In treating cancer patients with Dox, the biggest challenge is minimizing its cardiotoxic effects without compromising its antitumor capabilities. Dox's cardiotoxicity remains a mystery. As underlying mechanisms, mitochondrial dysfunction, increased ROS production, defective iron handling, and contractile failure have been suggested (Alzahrani *et al.*, 2021; Cheng *et al.*, 2020). Dox also alters some signaling involved in regulating genes for vital processes, including metabolism and cell survival. In an effort to gain a better understanding of Dox-induced cardiac apoptosis, researchers have looked into the signaling pathways (Imam *et al.*, 2018; Zhang *et al.*, 2019). In this pathophysiology, oxidative stress plays a role in intracellular signaling and apoptosis.

Through Nrf2 signaling via the Nfk pathway, this study tests whether spinosin (SPN) can protect against cardiac hypertrophy caused by Dox in cardiac cells.

Traditional medicine has used SPN, a C-glycoside flavonoid derived from the seeds of *Zizyphus jujuba* var spinosa, as a tranquilizer. SPN has been reported to have anxiolytic and hypnotic effects (Wang *et al.*, 2012). SPN improves scopolamine- or amyloid beta oligomer-induced memory impairment in mice (Lee *et al.*, 2016; Wang *et al.*, 2000). SPN also penetrates the blood-brain barrier. However, it's not clear whether SPN affects heart disease or improves it. SPN may be a promising therapeutic agent for cardiac disorders (Liu *et al.*, 2015; Wang *et al.*, 2000). Heart damage caused by Dox. With these factors in mind, the current study was designed to see if SPN can ameliorate Dox induced cardiomyopathies, and if so, what the mechanism is. SPN was found to significantly reduce Dox-induced cardiac hypertrophy, oxidative stress, and inflammation. SPN may be useful as a treatment for hypertension-induced heart damage because of its cardio protective properties.

MATERIALS AND METHODS

Cell culture

In Dulbecco's modified essential medium (DMEM, ThermoFisher Scientific, Waltham, MA, USA), we cultured H9c2 cardio myoblasts from the ATCC (VA, USA). H9c2 cells were treated with 100 mM (EC50) of SPN for 2 h before doxorubicin was added. We collected

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the cell lysate after 24 h.

MTT assay

H9c2 were cultured at 37°C with 10% FBS in DMEM. After 24 h, HUVECs were treated with Dox and/or SPN at the indicated concentrations. The MTT assay (Sigama, MO USA) was used to measure the proliferation of the cells.

Western blotting

We followed previously reported Western blotting procedures. Protein samples were separated on SDS-PAGE and then immunoblotted onto PVDF membranes. We developed the PBS-washed membranes and visualized the protein bands with a chemiluminescence substrate (Molcule-on Zwiterland). Samples were scanned with a LI-COR 3600-00-C-Digit Blot Scanner (AbuZahra *et al.*, 2021).

Actin staining

We stained the cells with actin staining for the analysis of hypertrophic effects on H9c2 cells, as we described in previous report (Rajendran *et al.*, 2020).

Immunostaining

Following treatment, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature. In the next step, cells were permeabilized with 0.1% TritonX100 in PBS for 3–5 min, washed with PBS, and then incubated overnight with primary antibodies of Nrf2. Incubation with the primary antibodies was followed by three PBS washes and 1 h at RT in the dark with secondary antibodies (Lin *et al.*, 2019).

ROS accumulation measurements

DCFH2-DA fluorescence dye was used to amount of intracellular ROS accumulation (Ismail *et al.*, 2021). We seeded 1×10^7 cells/mL in a 6-well plate, then treated with SPN (for 2 h) followed by Dox treatment. Afterward, DCFH2-DA was supplementary to the culture medium for 30 min at 37°C. Using fluorescence microscopy, we examined the dichlorofluorescein (DCF) fluorescence intensity inside cells. By comparing the fluorescence intensity of treated cells and vehicle-treated cells, we measured ROS levels.

Tunel assay

As described earlier, we used the apoptosis detection TUNEL kit to analyze. In fluoro microscopy at 454 nm, the nuclei of the TUNEL-positive cells were visible. We measured the images using microscopy (200X magnification) (Hu *et al.*, 2019).

Statistical analysis

This study analyzed the data using an analysis of variance (one-way analysis of variance) and compared the controls using Tukey's post-hoc test. The results were considered significant at $p < 0.05$.

RESULTS

SPN protect cell death from Dox induced toxicity

MTT was used to test Dox and SPN for cytotoxicity. Figure 1A and B shows that treatment of HUVECs with SPN didn't have any cytotoxic effects up to 100 μM /mL for 24 and 48 h. I used a SPN concentration of 100 μM /mL in the experiments. Figure 1C shows that Dox (0.5 and 1 μM) significantly reduced cell viability up to 65%, but SPN dose-dependently protected the Dox-induced induction of cell death. It's clear from these results that Dox is protective against Dox when exposed to cardiac toxicity.

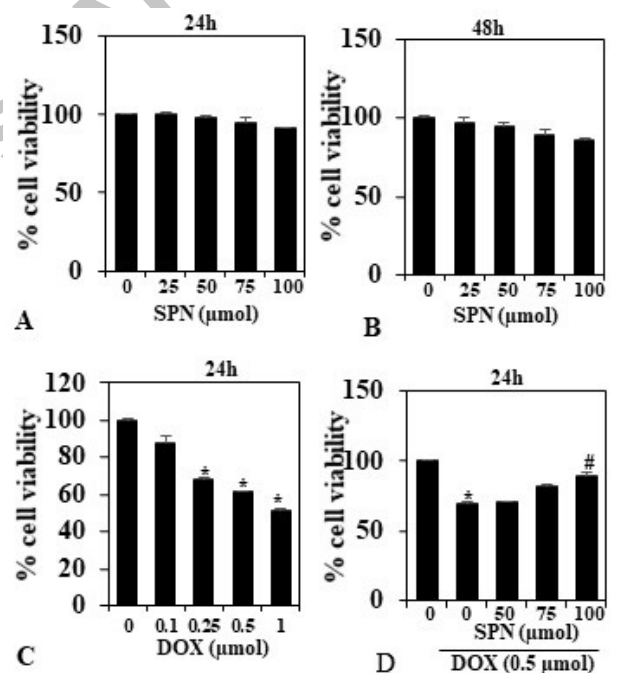


Fig. 1. SPN inhibits cell death in Dox induced cardiac cells. (A and B) Cell growth effect of SPN indicated time and dose by MTT. (C) Dox cell toxicity assay by MTT. (D) Cell growth effect of SPN on Dox induced H9c2 cells. $P < 0.05$ significantly different from the control group.

Impact of SPN on Dox-induced hypertrophy

By western blotting analysis, we first evaluated whether SPN could attenuate the expression of ANP and BNP. As shown in Figure 2A, treatment with Dox induces

the expression of these proteins; however, treatment with SPN dose dependently reduces their expression. The expression of calcineurin, p-GATA-4, and GATA-4 (Fig. 2B) was assessed as well as the activation of hypertrophic signaling cascades related proteins. SPN also attenuated these signaling mediators, similar to the results above. Secondly, we determined whether SPN could reduce expansion of cardiomyocytes, in cardiac hypertrophy. Interesting, the results showed that Dox significantly enlarged cardiomyocytes, yet treatment with SPN significantly lowered the Dox-induced enlargement.

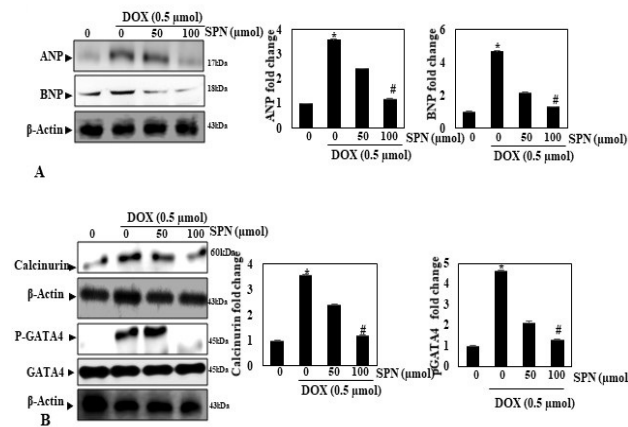


Fig. 2. SPN inhibits the cardiac abnormalities in Dox induced cardiac cells. (A) Cardiac abnormalities marker ANP and BNP expression analyzed by western blot. (B) Effect of SPN on calcineurin and phosphorylation of GATA4 in Dox induced H9c2 cells. $P < 0.05$ significantly different from the control group.

SPN and Dox-induced cardiac inflammation

Furthermore, in view of the fact that inflammatory responses play a key role in the Dox-induced remodeling of the heart, we investigated whether SPN could dampen these inflammatory responses. We measured inflammatory cytokines like TNF- α , IL-6, and COX-2, as well as NF- κ B expression (Fig. 3A). These inflammatory markers were significantly reduced by SPN.

Effects of SPN on Dox-induced cytoskeletal changes

The role of desmin and microtubule cytoskeletal alterations in cardiac hypertrophy and failure has been described in earlier experiments (Pai *et al.*, 2018; Shibu *et al.*, 2018). In the current study, we found that Dox increased cell size in H9c2 cardiomyoblasts within 24 h, as shown by the actin filament staining (Fig. 3B). F-actin staining showed SPN-treated cells reduced Dox-induced cellular hypertrophy after pretreatment. So, SPN inhibits Dox-induced cardiac hypertrophy in H9c2 cells.

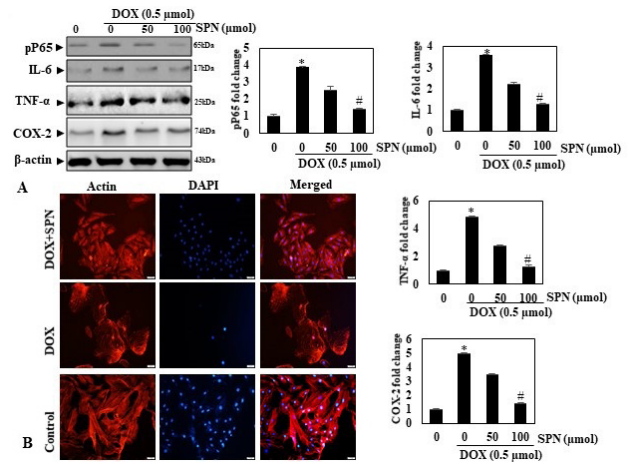


Fig. 3. SPN on pro-inflammatory cytokine expression Dox induced cardiac cells. (A) Expression of pP65, IL-6, TNF- α and COX-2 by western blot. (B) Actin filament changes control and treatment groups. $P < 0.05$ significantly different from the control group.

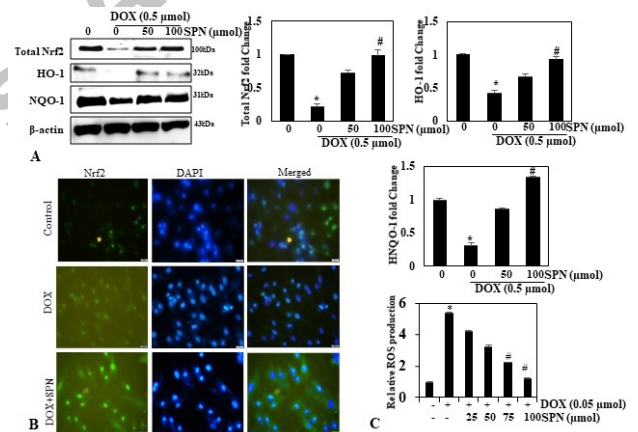


Fig. 4. SPN on Nrf2 signaling. (A) WB showing expression of total Nrf2, HO-1 and NQO-1 control and treatment groups. (B) Effect of SPN on Nrf2 nuclear translocation. (C) ROS formation. $P < 0.05$ significantly different from the control group.

Effect of SPN on Dox induced oxidative stress related proteins

Lastly, we examined the expression of oxidative stress-related proteins, such as Nrf2, HO-1, and NQO-1, in response to Dox induced cardiac remodeling (Fig. 4A). As can be seen, these oxidative stress markers were reduced after Dox treatment; however, SPN treatment upregulated their expression in a dose-dependent way. In Figure 4B visualized the nuclear localization of Nrf2 in H9c2 with SPN using fluorescence microscopy. According to immunofluorescence images, as seen by the high

Nrf2 staining in SPN-treated cells, SPN increased Nrf2 nuclear aggregation. ROS level significantly elevated in Dox treated cells in fig 4C, whereas SPN with Dox cells dramatically reduce this elevated level of ROS.

SPN inhibit apoptosis in Dox induced H9c2 cells

We looked at Western blots to see if SPN activates Bcl-2 and Bcl-xL and caspase-3 in H9c2s in Dox treated cells. In Dox with SPN therapy, Bcl-2 and Bcl-xL levels increased and caspase-3 levels decreased (Fig. 5A). SPN activated Bcl-2 and Bcl-xL and suppressed apoptosis. To investigate how SPN prevents Dox-induced cell death, we treated endothelial cells with SPN and/or Dox for 24 h with DNA fragmentation measured with TUNEL (Fig. 5B). The fluorescence microscopy images showed an increase in the number of TUNEL-positive cells after Dox treatment alone, whereas SPN treatment reduced TUNEL-positive cells significantly. SPN treatment clearly prevents Dox-induced apoptosis in H9c2 cells.

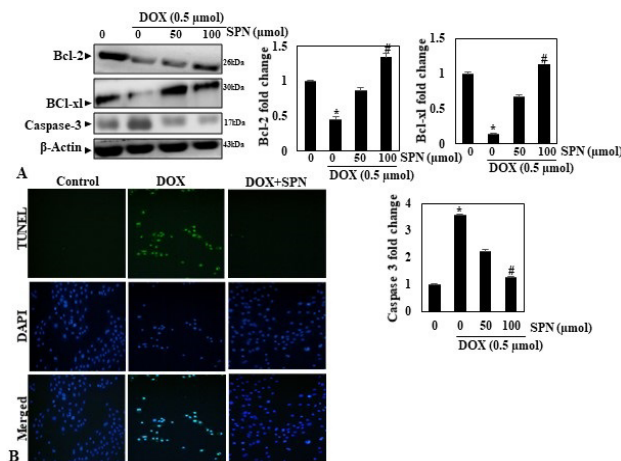


Fig. 5. Effect of SPN on Dox induced apoptosis. (A) Cell survival protein Bcl2 and BclxL and apoptotic marker caspase-3 analyzed by WB. (B) Apoptosis was analyzed by TUNEL assay. P<0.05 significantly different from the control group.

DISCUSSION

This study examined the cardio-protective role of SPN against Dox-induced cardiac injury. Researchers are trying to understand the pathophysiology behind cardiac injuries. There is a lot of consensus that a better understanding of this complex mechanism might lead to new avenues of prevention and/or treatment for hypertension-induced cardiomyopathies (Barik *et al.*, 2021; Lin *et al.*, 2021a, b). It is widely accepted that contractile dysfunction in cardiomyocytes is the result of a

combination of factors including hypertrophy in the heart, oxidative stress, and inflammatory responses. In other words, attenuating these underlying responses might help preserve the contractile function of the cardiomyocytes, which might cardiomyocytes. This even prevents cardiac dysfunction. Hypertrophy of cardiomyocytes often causes heart failure. Sympathetic hyperactivity and Dox-induced hypertension can result from an imbalance between pro- and anti-inflammatory cytokines (Pecoraro *et al.*, 2016; Quagliarello *et al.*, 2021; Singla *et al.*, 2019). Western blot analysis of myocardial cells in the Dox group revealed elevated COX-2, TNF- α , and IL-6 cytokines. A significant increase in myocardial inflammation accompanied the elevated inflammatory cytokines. Many anti-inflammatory drugs have been reported to protect against Dox-induced myocardial damage, suggesting inflammation plays a big part. SPN has been found to have significant anti-inflammatory potential, and it restored the normal inflammatory cytokine balance (Lee *et al.*, 2016; Xu *et al.*, 2019). SPN blocks doxorubicin-induced cytokine depletion. Several pathological events cause the myocardium to hypertrophy, including myocardial infarction, hypertension, and adrenal over-activity. Cell size increases, protein synthesis increases, and ANP, BNP, calcineurin and phosphorylation of GATA4 fetal cardiac genes get stimulated (Lin *et al.*, 2019). Current findings confirm that Dox induces a cardiac hypertrophic response, correlated with increases in ANP and BNP, markers of hypertrophy. In H9c2 cells treated with SPN, there was a significant decrease in levels of the above-mentioned proteins, which prevented hypertrophy induced by Dox. Therefore, SPN has a protective effect on cardiac hypertrophy.

Nrf2 is a transcription factor that regulates a bunch of cyto protective genes in the heart. Through attenuating oxidative stress, activating Nrf2 has been shown to protect against pathological cardiac remodeling. Further studies showed that SPN increased Nrf2 levels, indicating its ability to amplify Nrf2 pathway, thereby suppressing oxidative stress, which is critical for HF development. So, it's possible to envision that SPN might play a role in restoring the redox balance, which then orchestrates events that help with heart remodeling. Inflammatory responses play an important role in Dox-induced cardiac remodeling. Interestingly, the SPN reduces the expression of these inflammatory cytokines COX-2, IL-6, TNF- α and NF- κ B by a various dose- reliant on mechanism. Bcl-2 and Bcl-xL inhibit cytochrome c release and protect cardiac cells from oxidative stress and apoptosis (Chen *et al.*, 2017). Rather, caspase-3 executes the apoptotic program and cleaves PARP when the cell dies (Chen *et al.*, 2017). In our study, we found that SPN treatment induced upregulation of Bcl2

and Bcl-xL, and suppressed Dox-induced downregulation of Bcl2. However, SPN suppressed caspase activation.

Combined, our study shows that SPN protects against Dox-induced cardiomyopathies. *In vitro* and animal testing still needs to be done to define and illuminate the rationale behind its use in the clinic.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Statement of conflict of interest

The authors have declared no conflict of interest.

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