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Original Research Article

Tanshinone IIA inhibits exosome-induced cardiomyocyte pyroptosis through NLRP3/caspase 1 pathway

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Abstract

Purpose: *To investigate the effect of Salvia miltiorrhiza, a traditional Chinese medicinal plant, on exosome-induced cardiomyocyte pyroptosis.*
Methods: *Pyroptosis was induced in human AC cells using exosomes. Then, the effect of Danshen (dried roots of S. miltiorrhiza) on exosome-induced pyroptosis was determined using flow cytometry. The expressions of pro-inflammatory cytokines were measured by enzyme-linked immunosorbent assay (ELISA), while protein levels of cytokines were assayed by Western blotting.*
Results: *Tanshinone IIA (Tan IIA), the bioactive molecule in Danshen, inhibited cardiomyocyte pyroptosis by significantly reducing the expressions of proinflammatory cytokines ($p < 0.001$). Thus, Tan IIA reduced pyroptosis induced by cardiomyocyte-derived exosome via inhibition of the expression of NLRP3 inflammasome in human AC cells.*
Conclusion: *This study has identified a potential mechanism through which Danshen functions to prevent cardiac diseases. It involves, at least in part, the inhibition of pyroptosis in cardiomyocytes. Thus, tanshinone IIA may be a pharmacologically beneficial cardioprotective compound, especially when used against heart failure.*

Keywords: *Heart failure, Exosomes, Tanshinone IIA, NLRP3 inflammasome, Caspase 1, Pyroptosis*

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INTRODUCTION

Heart failure (HF) is elicited in myocardial death which is triggered by processes such as apoptosis, pyroptosis, necrosis and autophagy and excessive activation of the neuroendocrine system [1]. Recent research has highlighted the role of pyroptosis in progression of several cardiovascular disorders, e.g., HF [2]. Pyroptosis is a form of inflammation-induced cell death. An early step in the initiation of pyroptosis entails activation of inflammasomes. The NLRP3 inflammasome has been reported to be associated with several cardiovascular diseases [3]. In recent years, exosomes have been recognized as important extracellular factors that contribute to cardiovascular diseases, including HF which is the terminal stage of these diseases [4]. It has been reported that cardiomyocytes regulate the microenvironment via secretion or uptake of exosomes. For instance, studies have demonstrated that the viability and hypertrophy

of cardiomyocytes are deeply affected by cardiac fibroblast-derived exosomes [5-7]. Nevertheless, the exact effect of exosomes on the progression of pyroptosis in cardiomyocytes has not been elucidated.

Salvia miltiorrhiza (Danshen) is a traditional Chinese medicinal plant. Increasing experimental evidence have revealed that Danshen exerts desirable and positive effects by preventing death and ROS accumulation in cardiomyocytes [8]. The purpose of this research was to study the influence of Tan IIA on serum exosome-mediated cardiomyocyte pyroptosis, and also to elucidate the underlying mechanism.

METHODS

Cell culture

Human AC cells were plated in DMEM (Hyclone) spiked with 10 % FBS and penicillin-streptomycin (1 %; Solarbio) at 37 °C in a 5 % CO_2 incubator.

Plasmid construction

For NLRP3 overexpression, the CDS of NLRP3 (NM_004895.4) was constructed into pCDNA3.1(+) vector using primers containing Hind III and EcoR I restriction enzyme cutting sites. The relevant primers are shown in Table 1.

Cell transfection

The AC16 cells were trypsinized and counted, and a cell suspension containing 1×10^6 cells per milliliter was made, 2 mL of which was inoculated in 6-well plates under the conditions of 5 % CO_2 and 37 °C, followed by a 12-h incubation. Thereafter, transfection with control, vector or NLRP3 using Lipofectamine 2000 (11668-019, Invitrogen) was done.

Clinical samples

Fifteen (15) subjects were involved in the study. They comprised 9 severe burn patients and 6 healthy volunteers. Their ages ranged from 25 to 46 years, and the population had 10 males and 5 females. The inclusion and exclusion criteria for severity of burns were based on a previous report [9]. Venous blood from different subjects were collected using tubes without anticoagulant. The blood samples were centrifuged at the speed of 3500 rpm at room temperature for 8 min, to obtain sera. This study received approval from the ethical authority of our institution, and it met the criteria stipulated in the Declaration of Helsinki [9].

Extraction and identification of serum exosomes

Blood samples from severe burn patients and corresponding control were centrifuged for ½ h at 4 °C at 10,000 g, and the sera were taken up in 5 mL ultra-high speed centrifugal tubes containing PBS. The samples were then centrifuged twice for 2 h at 4 °C at 17,000 g. Then, the sediments were taken up in PBS and kept frozen at -80 °C. The exosome CD biomarkers (ab92726), CD81(ab109201) and TSG101(ab125011) were used as indexes for identification of serum-derived exosomes.

PKH-67 tracer exosomes

Exosomes obtained from the serum samples of burn patients were co-cultured with AC16 cells. Endocytosis of exosomes by AC16 cells was traced using commercial kit (UR52303, Umibio). All steps used were consistent with kit instructions. Images were collected using a laser-scanning microscope.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNAs were obtained with TRIzol (1596-026, Invitrogen) in line with instructions on the kit manual. Commercial cDNA Synthesis kit (Fermentas) was used for reverse-transcription of RNA to cDNA in a reaction done on ABI 7300 RT-PCR instrument (ABI-7300, Applied Biosystems). The relative level of mRNA was determined using the $2^{-\triangle\triangle Ct}$ method, with GAPDH as internal control. Table 2 shows the primers used.

Table 1: Primers used for NLRP3 overexpression

	Forward	Reverse
NLRP3	5’-CCCAAGCTTATGAAGATGGCAAGCACCC-3’ (Hind III)	5’-CGGAATTCCTACCAAGAAGGCTCAAAGACGAC-3’ (EcoRI)

Table 2: Primer sequences used in PCR

Variable	Forward	Reverse
NLRP3	5'-TTCGGAGATTGTGGTTGGG-3'	5'-TCAGGGAATGGCTGGTGC-3'
GAPDH	5' AATCCCATCACCATCTTC 3'	5'-AGGCTGTTGTCATACTTC-3'

Western blot assay

Total protein was extracted using RIPA buffer, followed by protein quantification with BSA method. Then, equal amounts of protein (25-µg portions) were resolved using SDS-PAGE, followed by transfer onto PVDF membranes. Membrane blocking was done by incubation with 5 % non-fat milk for 60 min. Thereafter, incubation with 1º immunoglobulins for CD81 (1:1000, Ab109201), Pro-Caspase-1 (1:2000, #2225), TSG101 (1:1000, Ab125011), CD9 (1:1000, Ab92726), COX-2 (1 : 500, Ab15191, Abcam), BMP-2 (1 : 500, Ab14933, Abcam) NLRP3 (1:1000, Ab263899), and GSDMD-N (1:1000, Ab215203), active Caspase-1 (1:2000, #4199) and GAPDH (1 : 1000, #5174) was done for 12 h at 4º C, followed by incubation with goat anti-rabbit HRP-labeled 2º immunoglobulins (1 : 10000; ZB-2301, ZSGB-BIO, China) for 60 min at 37°C.

Cell pyroptosis assay

Following treatments, the cells were resuspended in PBS and incubated with active caspase-1 (1: 30; EL900443, EterLife). Then, after rinsing thrice, they were subsequently incubated with 3 µM propidium iodide solution (PI, P3566; Invitrogen) for 15 min in the dark. Pyroptosis rates were determined using flow cytometry (BD Biosciences).

ELISA

Secretion concentrations of interleukins 18 and 1β were measured with corresponding commercial ELISA kits.

Statistics

Data were analyzed using GraphPad Prism 7.0. Results are presented as mean ± SD. Comparison was done with one-way ANOVA. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Serum exosomes enhanced pyroptosis of human cardiomyocytes

To investigate the influence of serum exosomes on pyroptosis, exosomes were isolated from patients with third degree burns using ultracentrifugation. First, the integrity of the exosomes was confirmed using transmission electron microscopy (TEM). The results are presented in Figure 1 A. The serum levels of exosome markers (CD9, CD81 and TSG101) are shown in Figure 1 B. Functional integrity of the exosomes was confirmed by checking the endocytosis of exosomes labeled with PKH-67 dye when co-cultured with AC16, a human cardiomyocyte cell line (Figure 1 C).

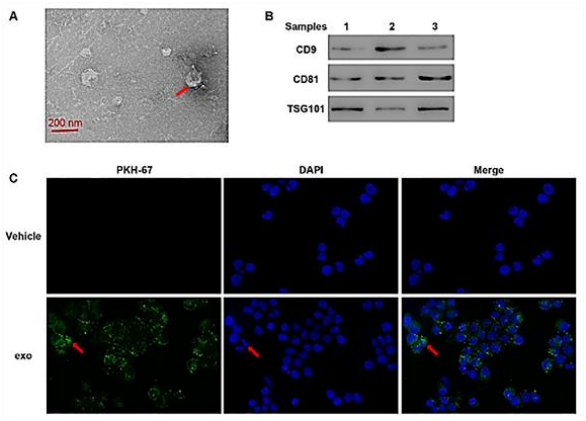

Figure 1: Isolation and characteristics of exosomes from sera of burn patients. Serum exosomes were isolated from third degree burn patients using ultracentrifugation. (A) Exosome content and purity, as evaluated using Transmission Electron Microscopy. (B) Expression levels of the indicated exosome markers, as measured using Western blotting. (C) Results of functional characterization of the exosomes via measurement of endocytosis of PKH-67 dye-labeled exosomes in AC16 cells

Following co-culturing of exosomes with AC16 cells, active caspase-1 level was quantified via Fluorescence Activated Cell Sorting (FACS). The serum exosomes increased active caspase-1 level within 12 h of co-culture. Moreover, the protein level of caspase-1 was increased in a time-dependent manner up to 48 h post co-culture (Figure 2 A). Likewise, IL-1β and IL-18 levels were markedly upregulated upon co-culturing with serum exosomes in the cells within 12 h (Figure 2 B and C), and the increases continued in a time-based fashion until 48 h following co-culturing (Figure 2 B and C). Results that were obtained from Western blot suggested that protein levels of active Caspase-1, NLRP3 and GSDMD-N were upregulated by serum-derived exosomes in a time-dependent manner (Figure 2 D). In all, these results indicate that serum exosomes promoted pyroptosis in cardiomyocytes.

Tan IIA inhibited serum exosome-induced cardiomyocyte pyroptosis

The effects of the bioactive compounds of Danshen i.e., CTN, Tan IIA, SAA and SalB on serum exosome-mediated pyroptosis in AC16 cells were determined. In this section, the serum-derived exosomes and the bioactive compounds of Danshen were used to co-culture human AC

cells. As shown in Figure 3, treatment of the cells with CTN, Tan IIA, SAA and SalB led to significant reduction of pyroptosis in AC6 cells. More importantly, Tan IIA displayed the most robust inhibition of serum exosome-induced pyroptosis among all the compounds that inhibited pyroptosis.

Tan IIA dose-dependently inhibited cardiomyocyte pyroptosis

To further test the influence of Tan IIA in pyroptosis, AC16 cells co-cultured with serum exosomes were treated with varying doses of Tan IIA, and the effects of the treatments on different factors contributing to pyroptosis were evaluated. It was found that active caspase-1 expression increased dose-dependently in Tan IIA-treated cells (Figure 4 A). Likewise, IL-1β and IL-18 expressions showed dose-dependent responses in cells treated with different concentrations of Tan IIA (Figure 4 B and C). Furthermore, like the active Capase-1, the expression of NLPR3 and GSDMD-N was dose-dependently reduced in AC16 cells treated with Tan IIA. These results collectively demonstrate the effectiveness of Tan IIA in inhibiting serum exosome-mediated pyroptosis in cardiomyocytes.

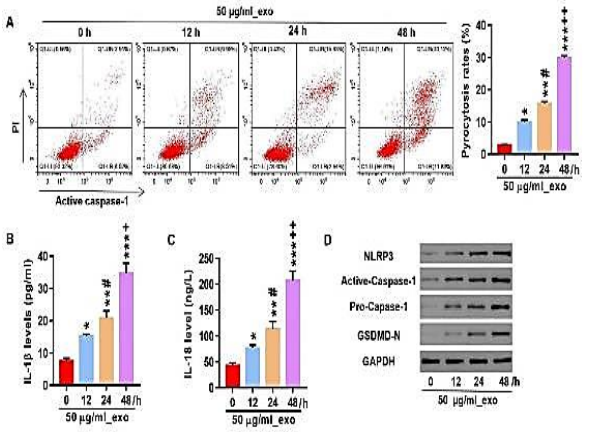

Figure 2: Serum exosomes induced pyroptosis in cardiomyocytes. The AC16 cells were exposed to serum exosomes for the indicated times. **(A)** Rate of pyroptosis of cardiomyocytes after treatment with serum-derived exosomes for 12, 24 and 48h, as determined using flow cytometry. **(B & C)** Cytokine expression levels in cardiomyocytes after treatment with serum-derived exosome for 12, 24 and 48 h, as measured with ELISA. **(D)** IL-1β and IL-18 proteins, as assayed with immunoblotting. *P < 0.05, < 0.01**, < 0.001***, vs untreated; #p < 0.05 vs 12 h; +p < 0.05, vs 24 h; ++p < 0.01, vs 24 h

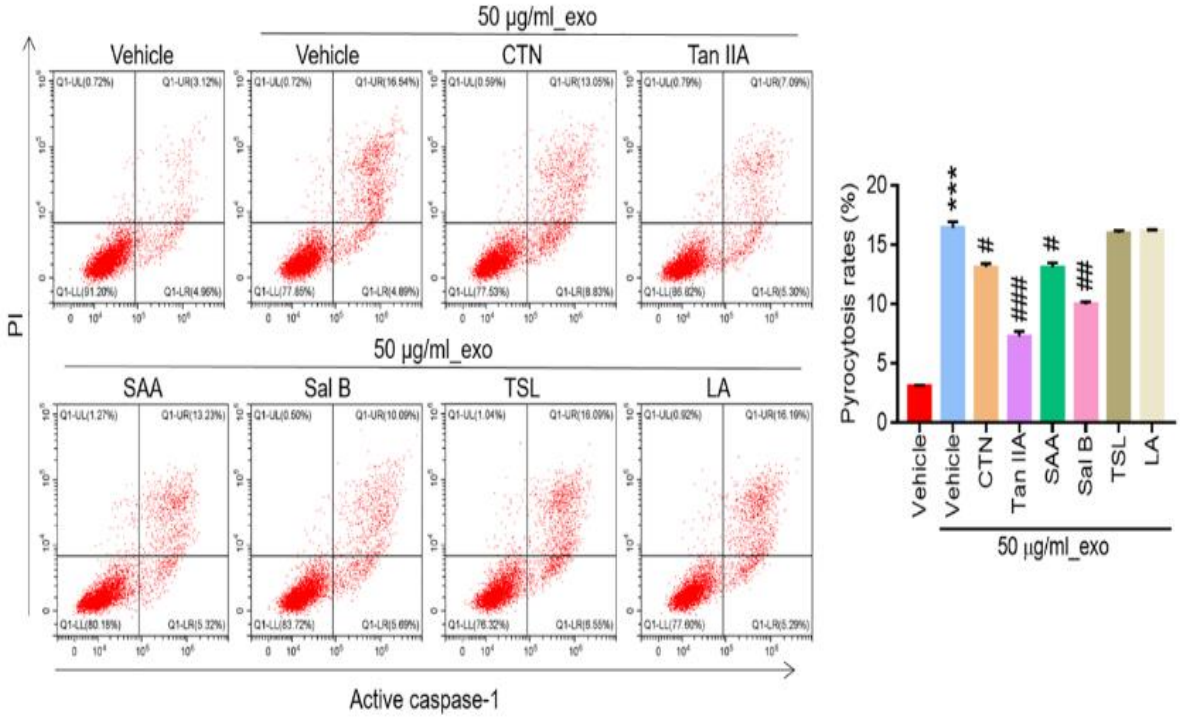

Figure 3: Effects of different Danshen monomers on pyroptosis in cardiomyocytes. The AC16 cells were treated with serum exosomes (50 μg/mL) along with vehicle or 10 μM of the indicated Danshen monomers for 24 h. Pyroptosis was assessed via quantifying the population of cells that expressed active Caspase-1, using FACS. ***P < 0.001, vs vehicle; #p < 0.05, ##p < 0.01, ###p < 0.001, vs 50 μg/mL exo + vehicle

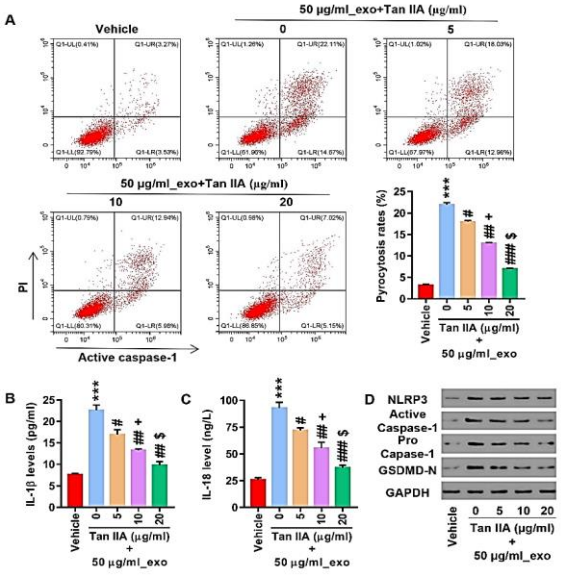

Figure 4: Inhibitory effect of Tan IIA on serum exosome-induced pyroptosis in cardiomyocytes. AC16 cells were exposed to serum exosomes (50 μg/mL) alone, or together with Tan IIA at indicated concentrations. **(A)** Using FACS**,** pyroptosis was assessed by quantifying cells that expressed active Caspase-1. **(B & C)** Expression levels of IL-1β and IL-18, as assayed with ELISA, and (**D**) their protein expressions, as assayed uing immnoblotting. ***P < 0.001, vs vehicle; #p < 0.05, ##p < 0.01, ###p < 0.001, vs 50 μg/mL exo without Tan IIA; +p < 0.05, vs 50 μg/mL exo + 5 μmol/L Tan IIA; \$$p$ < 0.05, vs 50 μg/mL exo+10 μmol/L Tan IIA

Tan IIA inhibited NLRP3-induced pyroptosis of human cardiomyocytes

The regulatory effect of Tan IIA on NLPR3 inflammasome-mediated pyroptosis in human cardiomyocytes was investigated. Overexpression of NLRP3 was induced using Lentivirus, as shown in Figures 5 A and B. Tan IIA treatment significantly decreased the accumulation of IL-1β and IL-18 in oeNLRP3-transfected cells (Figure 5 C). As shown in Figure 5 D, overexpression of NLRP3 enhanced the pyroptosis of human AC cells. However, treatment of the cells with Tan IIA significantly lowered the level of active Caspase-1 and reduced pyroptosis in cells overexpressing NLRP3. Furthermore, Tan IIA treatment markedly diminished the expressions of active Caspase-1 and GSDMD-N in cells overexpressing NLRP3 (Figure 5 E).

Flow cytometry was used to determine pyroptosis in oeNLRP3-transfected AC cells co-cultured with Tan IIA and serum exosomes. As expected, Tan IIA effectively blocked active Caspase-1 and pyroptosis in cells treated with serum exosomes (Figure 4F). However, treatment of cells overexpressing NLRP3 with Tan IIA significantly elevated the level of active Caspase-1 and increased pyroptosis (Figure 4F).

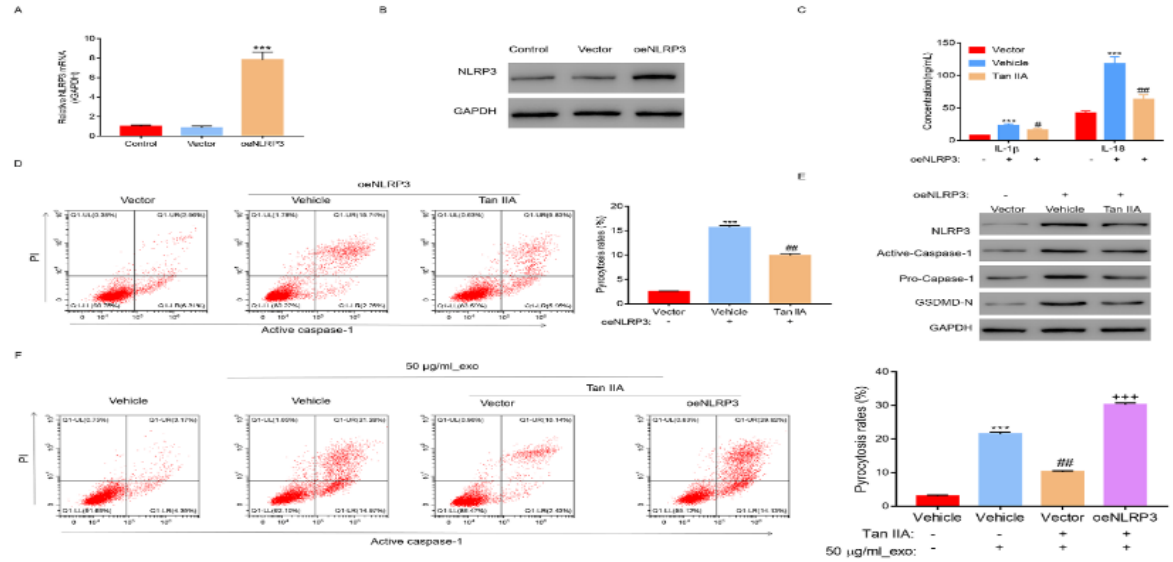

Figure 4: Tan IIA inhibited pyroptosis in cardiomyocytes through regulation of NLRP3 expression. NLRP3 was overexpressed in AC16 cells. **(A & B)** NLRP3 mRNA expression, as measured using RT-qPCR (A), and NLRP3 protein expression (B), as measured using Western blot assay. **(C)** Expression levels of IL-1β and IL-18, as assayed with ELISA. (**D-F)** Pyroptosis in AC16 cells overexpressing NLRP3 following treatment with Tan IIA 10 μM) or vehicle, in terms of cells expressing active Caspase-1, as quantified using FACS; (**D and E**) Protein expression levels f Caspase-1, as determined using Western blot assay. **(F)** Overexpression of NLRP3 abolished the effect of Tan IIA (10 μM) on human AC16 cells in the presence of serum exosomes (50 μg/mL)**.** ***P < 0.001 vs vehicle; ##p < 0.01 vs 50 μg/mL exo + vehicle; +++p < 0.001 vs 50 μg/mL exo + Tan IIA + vector

These results collectively indicate that Tan IIA suppressed pyroptosis by lowering the expression of NLRP3.

DISCUSSION

Heart failure is a major health challenge in developed countries. This highlights the importance of developing new therapies that would slow down the progression of the disease. Danshen is a widely studied traditional Chinese medicine that has produced a variety of medicinal benefits. These include anti-inflammation [10], anti-oxidation [10], and anti-thrombosis [11]. These benefits contribute to cardiovascular protection. It has been reported that Danshen contains over 200 bioactive compounds [12]. Studies have demonstrated that many of these compounds exert anti-inflammatory effects, although via different mechanisms. For instance, a study has shown that salvianolic acid B produced anti-inflammatory effect through suppression of TNF-α-induced NF-κB activation in aortic endothelial cells [13]. Moreover, Tan IIA showed anti-inflammatory function in endothelial progenitor cells [14], and cryptotanshinone exhibited anti-inflammatory activity [15].

In the present study, it was revealed that Danshen inhibited serum exosome-induced pyroptosis in cardiomyocytes. Thus, it may provide protection against HF. Moreover, several bioactive components of Danshen effectively inhibited pyroptosis, with Tan IIA being the most potent anti-pyroptotic compound. Nonetheless, it was also found that the other components such as CTN, SAA and Sal B significantly blocked pyroptosis in cardiomyocytes. Future studies will determine whether these compounds have biological relevance in preventing heart disease.

The present data has shown that Tan IIA inhibited serum exosome-induced pyroptosis by blocking the expression of NLRP3 inflammasome. Elevated levels of circulating proinflammatory biomarkers have been correlated with the severity of HF [16]. This suggests that the suppressive influence of Tan IIA on expressions of proinflammatory cytokines may be be directly involved in arresting the progression of HF.

The NLRP3 inflammasome has emerged as an important factor involved in regulation of inflammation under different pathological conditions, including cardiovascular diseases [3]. It promotes cell death through pyroptosis. The loss of cardiomyocytes through pyroptosis has been shown to reduce contractile reserves, leading to HF [17]. Thus, this study has shed new light on the mechanism through which Danshen blocks pyroptosis in exosome-induced cardiomyocyte pyroptosis, thereby indicating that it may be beneficial in the cure and prevention of cardiovascular diseases. This study was performed in a cervical cancer cell line. However, it is possible that Tan IIA may play context-dependent roles in provision of health benefits for different diseases.

CONCLUSION

The present study has demonstrated that Tan IIA might be the main bioactive component of Danshen involved in suppressing pyroptosis in human cardiomyocytes. This indicates the role of Danshen as a potential agent in the treatment for HF.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

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

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. Shun Xu and Meng-Han Wang conceived, designed, and wrote the manuscript. Shun Xu, Meng-Han

Wang, Yu Chen, Zao-Li Shen, performed the experiments. Qing Jia and Ai-Li Wang did analysis and interpretation of data. All authors read and approved the final manuscript.

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