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*Original Article*

# Protective effect of 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside on aldosterone-induced cardiomyocytes injury via interfering p53 signaling pathway

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**Abstract:** Objective: To observe the protective effect of 2,3,5,4'-Tetrahydroxystilbene-2-O-β-D-glucoside (TSG) on aldosterone-induced cardiomyocytes injury. Materials and methods: Primary cardiomyocytes were isolated from new born SD rats and aldosterone was used as a stimulant for cell injury. Cell viability was assayed by MTT method and LDH leakage was detected by related assay kit; cell apoptosis and protein expression was identified by flow cytometry and western blot, respectively. Results: TSG was able to increase cell viability as well as decrease the leakage of LDH in aldosterone-induced cardiomyocytes. Meanwhile, TSG decreased the percentage of aldosterone-induced apoptosis. Further research indicated that TSG decreased the level of bax and caspase-3 and increased the expression of bcl-2 in aldosterone-stimulated cardiomyocytes. The above phenomena may be related to p53 pathway for specific p53 inhibitor was able to decrease the level of bax and caspase-3 and increased the expression of bcl-2 in aldosterone-stimulated cardiomyocytes. Conclusion: TSG is able to protect aldosterone-induced cardiomyocytes injury by interfering p53 signaling pathway. These results provide a new evidence for the potential protective effects of TSG.

**Keywords:** 2,3,5,4'-Tetrahydroxystilbene-2-O-β-D-glucoside, aldosterone, cardiomyocytes, cardiovascular disease, p53 signaling pathway

## Introduction

Cardiovascular disease is one of the most frequent diseases both in developed countries and developing countries [1]. Heart failure is widely common cardiovascular disease in wrinkly and elderly people, which characterized by impaired systolic and/or diastolic function with high morbidity and mortality [2]. Cardiomyocytes loss including necrosis and apoptosis is a wide accepted concept that can result in heart failure [3]. The apoptotic process involves many factors, such as the sympathetic nervous system, renin-angiotensin system, reactive oxygen species and p53 pathway [4-6]. Evidences from laboratorial or clinical suggested that excessive aldosterone (ALD) plays a key role in the process of heart failure, and heart is also one of direct targets of ALD, which can provoke hypertrophy and apoptosis of cardiomyocytes [7-9].

2,3,5,4'-Tetrahydroxystilbene-2-O-β-D-glucoside (TSG) is one of the major bioactive constituents extracted from *polygonum multiflorum Thunb* [10] and demonstrates various pharmacologic activities including antioxidant, anti-inflammatory, and anti-atherosclerotic effects, improvement of memory and learning ability, neuroprotection, anti-aging, promotion of hair growth, and attenuation of human platelet aggregation [11-18]. However, little is known about the effect of TSG on ALD induced cardiomyocytes injury and the potential mechanisms. Hence, the aim of present study was to investigate the protective effect of TSG on ALD-induced apoptosis in cardiomyocytes which mainly focus on the anti-apoptosis effect and p53 pathway.

## Materials and methods

### *Chemicals and animals*

TSG was from National Food and Drug Testing Institute (Beijing, China). RPMI 1640 Medium and fetal bovine serum (FBS) were purchased

from Gibco (Grand Island, NY, USA). Penicillin and streptomycin were from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). MTT was purchased from Invitrogen (Carlsbad, CA, USA). Protein quantification kit was from Beyotime (Jiangsu, China). Caspase-3, Bax and Bcl-2 antibodies were from Abcam (Cambridge, MA, USA), p53 antibody was purchased from Beyotime (Jiangsu, China). GAPDH antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were purchased from the Zhongshan Company (Beijing, China).

Sprague-Dawley (SD) rats (Between 3-7 days old) were provided by the experimental animal center of Beijing. The animal experiment was according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised in 1996).

Primary cardiomyocytes were from new born SD rats according to previous methods [19]. The purity of cultured cardiomycoytes were 98% as evaluated by immunofluoresence staining using cardiac muscle sarcomeric a-actin antibody (Abcam, Cambridge, MA, USA) and showed in Supplementary Figure 1. Cardiomyocytes were kept in the 1% FBS for 12 h prior to the experiment, and then stimulated by ALD as indicated. In the TSG treatment experiments, Cardiomyocytes were exposed to ALD (10 μmol/L) for 12 h after pretreated with the TSG for 1 h.

*MTT assay*

The cardiomyocytes were exposed to different concentration of TSG for 12 h or with the different concentration of ALD for 12 h or ALD at 10 μmol/L for indicated time and the cells were subjected to ALD (10.0 μmol/L) treatment in the absence or presence of TSG. After indicated treatment, 20 μL of 5 mg/mL MTT solution was added into and incubated at 37°C for 3.5 h, then supernatant of each well was removed, and Formazan salt crystals were then dissolved with 150 mL dimethylsulfoxide for each well. The mixtures are determined at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

*LDH assay*

The cardiomyocytes were exposed to ALD (10.0 μmol/L) treatment in the absence or presence of different concentration of TSG for 12 h; and then the measurement of LDH activity was by using the relative LDH activity assay kit (Beyotime, Jiangsu, China) according to the instruction of the kit. LDH leakage rate was expressed as the percentage of the total LDH activity (the extracellular LDH activity plus the intracellular LDH activity), according to the following equation: % LDH release rate = (LDH activity in medium/total LDH activity) × %.

*Apoptosis assay*

The cardiomyocytes were exposed to ALD (10.0 μmol/L) treatment in the absence or presence of different concentration of TSG for 12 h; and the Apoptotic cells was identified by an Annexin V/PI apoptosis kit (Keygen Biotech, Nanjing, China). About $1\times10^6$ cells were harvested and re-suspended in 200 μL binding buffer. Next, the cells were incubated with 5 μL Annexin V-fluorescein isothiocyanate (FITC) and 5 μL PI (50 mg/mL) for 20 min in the dark and immediately analyzed by flow cytometry. Data from at least $1\times10^5$ cells of each sample were acquired and analyzed using Cell Quest software, version 7.5.3 (Becton Dickinson).

*Western blot*

The cardiomyocytes were exposed to ALD (10.0 μmol/L) treatment in the absence or presence of different concentration of TSG for 12 h; and the changes of protein level was detected by western blot. The cells were washed once in PBS and lysed on ice in lysis buffer (Beyotime, Jiangsu, China) after treatments were completed. The protein concentration was determined by a BCA protein assay kit. About 40 μg proteins were added to 10% SDS-polyacrylamide gel. After being electrophoresed, the proteins were transferred onto a PVDF membrane by using a Bio-Rad western blot analysis apparatus. The membrane was incubated with blocking buffer for 5 h at room temperature and then incubated overnight at 4°C with the primary polyclonal antibodies against p53 (1:800), bax (1:1000), caspase-3 (1:1500) and bcl-2 (1:3000), followed by incubation with corresponding secondary antibodies. Specific protein bands were visualized with an ECL advanced western blot analysis detection kit (Merck Millipore, Billerica, MA, USA).

*Statistical analysis*

The results are represented as means ± standard error of the mean (S.E.M.). Statistical analysis was performed by one-way ANOVA, and the

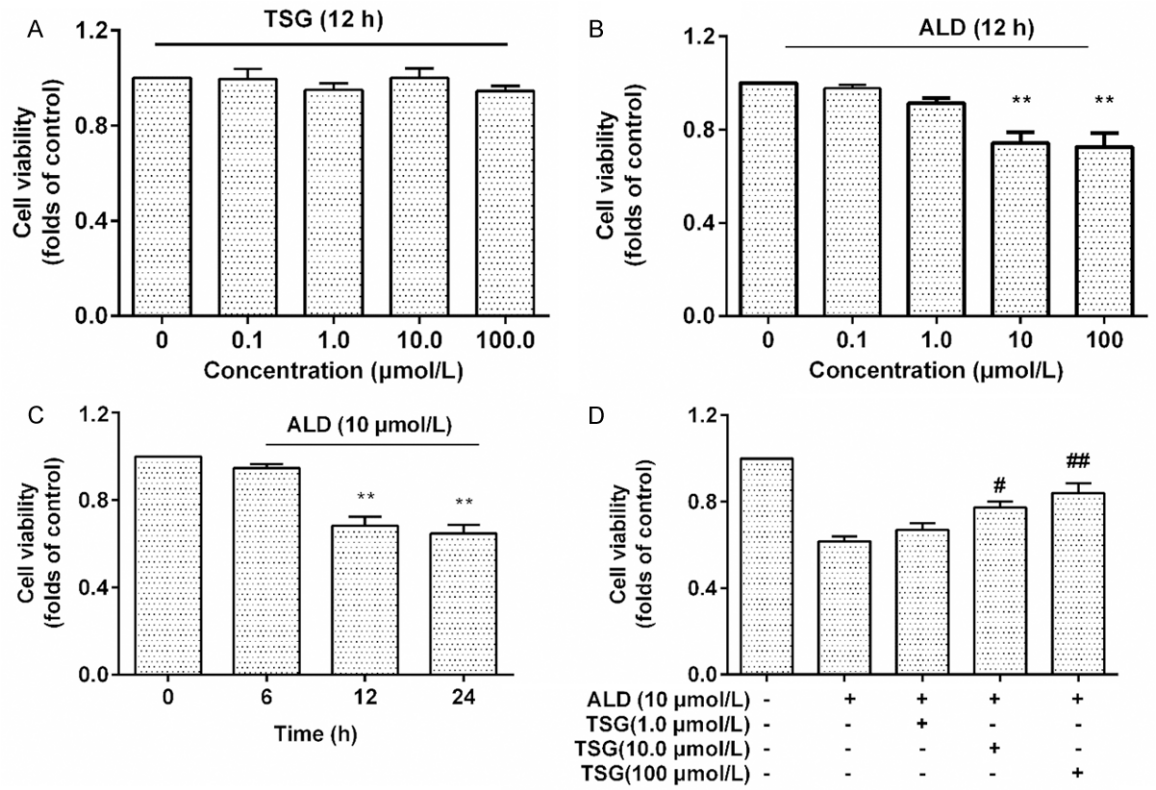



**Figure 1.** The effect of TSG treatment on cell viability in ALD-treated cardiomyocytes. A: The cells were incubated with the different concentrations of TSG for 12 h; B: The cells were incubated with the different concentrations of ALD for 12 h; C: The cells were incubated with the concentrations of ALD 10 μmmol/L for indicated time; D: Cells were subjected to ALD (10.0 μmol/L) treatment in the absence or presence of TSG. Results were from six independent experiments and expressed as mean ± S.E.M., $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ vs. ALD alone.

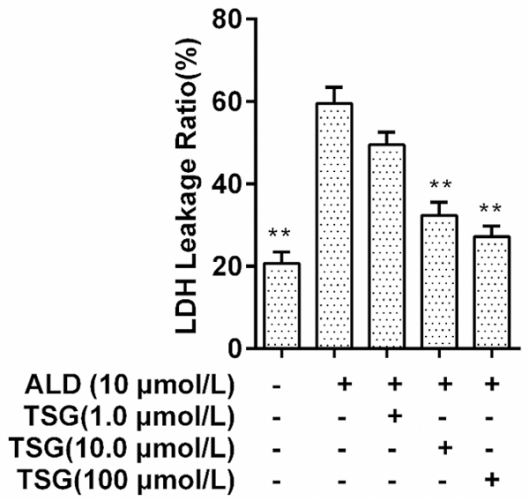



**Figure 2.** Inhibited effects of TSG on ALD-induced cardiomyocytes LDH release. The cells were pretreated with TSG (1.0, 10.0 or 100.0 μmol/L) for 1 h and then cells were stimulated with ALD (10.0 μmol/L) for 12 h. At the end of the incubation period, LDH release was detected. Results were from six independent experiments and expressed as mean ± S.E.M. **P < 0.01 compared with ALD alone.

Tukey-Kramer post test was used to locate any significant differences identified in the ANOVA. This was performed using GraphPad prism 5 (GraphPad Software, Inc. La Jolla, CA). A $p$ value of < 0.05 was considered statistically significant.

## Results

### *TSG increases the cell viability of ALD-treated cardiomyocytes*

**Figure 1A** showed that the concentration of TSG at 0.1-100.0 μmol/L has no significant cell viability changes in compared with the control group ($P > 0.05$). Next, the cells were subjected to ALD (0.1, 1.0, 10.0 and 100.0 μmol/L) for 12 h or ALD 10.0 μmol/L for 0-24 h. Compared with control group, when exposed to ALD 10.0 μmol/L for 12 h can significantly decrease the cell viability of cardiomyocytes (showed in **Figure 1B**, **1C**). In the following experiment, cells were subjected to ALD (10.0 μmol/L) treatment in the absence or presence of TSG (1.0,

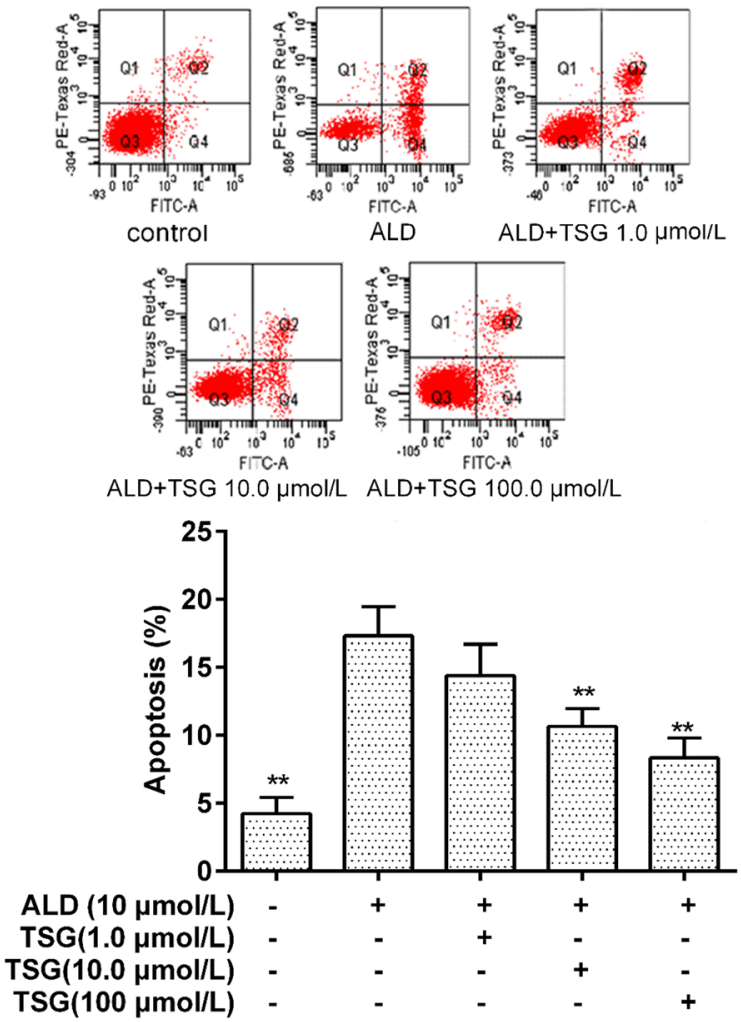



**Figure 3.** Impacts of TSG on ALD-induced cardiomyocytes apoptosis by annexin V/PI staining. The cells were pre-treated with TSG (1.0, 10.0 or 100.0 μmol/L) for 1 h and then were stimulated with ALD (10.0 μmol/L) for 12 h. At the end of the incubation period, the cells were then prepared for annexin V/PI staining. The data expressed as % apoptotic cardiomyocytes. Results were from 3 independent experiments and expressed as mean ± S.E.M., $^{**}P < 0.01$ compared with ALD alone.

10.0 and 100.0 μmol/L). ALD treatment significantly decreased cell viability ($P < 0.05$, compared with the control group), while TSG (10.0 and 100.0 μmol/L) treatment for 12 h resulted has a significant increase in cell viability, in a dose-dependent manner (**Figure 1D**, $P < 0.05$, compared with the ALD group).

*TSG decreases the leakage of LDH in ALD-induced cardiomyocytes*

LDH is served as a bio-maker of cellular membrane injury. **Figure 2** showed that there was a significant increase in LDH leakage ratio ($P < 0.01$ compared with the control group) in cells exposed to 10 μmol/L ALD alone for 12 h. However, the level of LDH leakage ratio was significantly reduced in cells pre-treated with TSG, especially for the concentration of TSG 10.0 and 100 μmol/L.

*TSG prevents the cell apoptosis induced by ALD treatment*

**Figure 3** showed that ALD treatment increased the percentage of apoptotic cells ($P < 0.05$, compared with control group). Compared with the ALD group, TSG treatment attenuated the cardiomyocytes apoptosis, in a dose-dependent manner ($P < 0.05$). These data showed that exposure of the cardiomyocytes to ALD induced cell death, however, pretreatment with TSG reversed this effect and protected the cardiomyocytes.

*TSG decreases the expression of cleaved-caspase-3, bax and bcl-2 in ALD-treated cardiomyocytes*

As shown in **Figure 4**, the protein expression of cleaved caspase-3, and bax in the ALD group was significantly upregulated, compared with that in the control group ($P < 0.05$). However, TSG attenuated the ALD-induced up regulation in the expression of caspase-3 and bax, compared with the ALD group ($P < 0.05$). In addition, conversely, ALD produced a significant decrease in the expression of Bcl-2 ($P < 0.05$, compared with control group). While, TSG treatment up-regulated the ratio of Bcl-2 ($P < 0.05$, compared with the ALD group).

*Protective effect of TSG on ALD-induced cardiomyocytes injury via interfering p53 signaling pathway*

**Figure 5** exhibited that pretreatment of TSG and p53 specific inhibitor pifithrin-α were able to decrease the expression of p53 protein and the percentage of apoptosis cells as well as

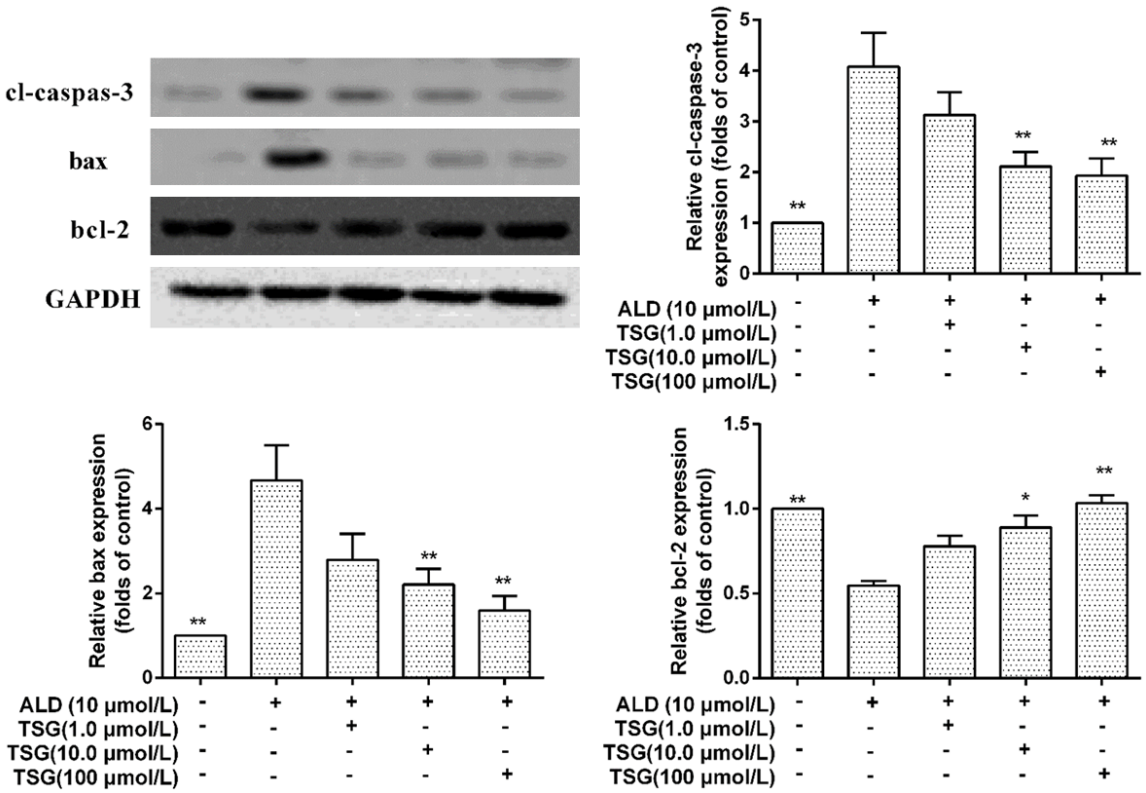



**Figure 4.** Regulation of TSG on ALD-induced bax, bcl-2 and cleaved-caspase-3 levels in cardiomyocytes. The cells were pre-treated with TSG (1.0, 10.0 or 100.0 μmol/L) for 1 h and then were stimulated with ALD (10.0 μmol/L) for 12 h. At the end of the incubation period, each protein level was analyzed by western blot. Levels of protein are shown as folds of control from 3 independent experiments and expressed as mean ± S.E.M. **P* < 0.05 OR ***P* < 0.01 compared with ALD alone.

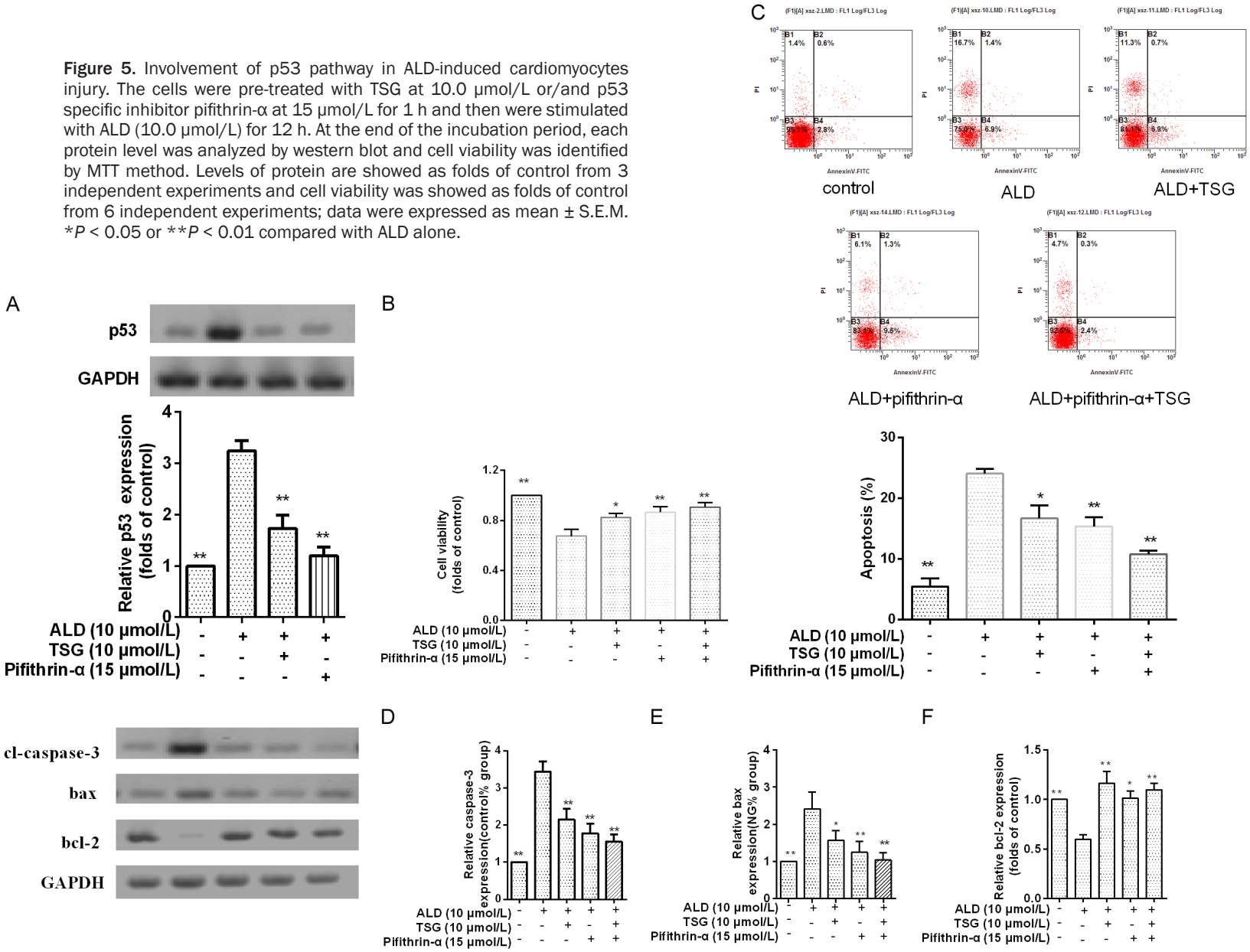
increase the cell viability in ALD-induced cardiomyocytes (*P* < 0.05, compared with ALD group, **Figure 5A-C**). Further research showed that TSG and p53 specific inhibitor pifithrin-α alone or in combination can down regulate ALD-induced the expression of caspase-3 and bax as well as up regulate the level of bcl-2 in compare with ALD group (**Figure 5D-F**).

## Discussion

In the recent study, we report the protective effect of TSG on the ALD induced cardiomyocytes injury for the first time. The protective effects relevant to decrease the leakage of LDH and prevent ALD induced apoptosis of cardiomyocytes through interfering p53 signaling pathway.

Traditional Chinese medicines (TCMs) and their ingredients have been used as the most important therapeutic agents for thousand years in China. TSG, a water-soluble constituent mainly from *Polygonum multiflorum Thunb*, is well-recognized for its cardiovascular activity. TSG was previously reported to protect the heart against I/R injury by reducing ROS generation and inhibiting cell apoptosis [20, 21]. While ALD is a mineralocorticoid hormone, and the function of ALD is regulate sodium and potassium transports in the epithelial cells of renal tubules [22-24]. While excess aldosterone not only causes the abnormal water homeostasis, but also increases the risk of cardiovascular events independent of hypertension [25, 26]. Research showed that ALD also plays a key role in the process of heart failure, and heart is one of direct targets of ALD, which can evoke hypertrophy and apoptosis of Cardiomyocytes [27-29]. In this investigate, we found a protective effect of TSG on ALD treated cardiomyocytes. As we all known that Cardiomyocytes loss including necrosis and apoptosis is one of the most important reason of heart failure, so the protective effect of TSG in ALD treated cardiomyocytes is of great important.

**Figure 5.** Involvement of p53 pathway in ALD-induced cardiomyocytes injury. The cells were pre-treated with TSG at 10.0 μmol/L or/and p53 specific inhibitor pifithrin-α at 15 μmol/L for 1 h and then were stimulated with ALD (10.0 μmol/L) for 12 h. At the end of the incubation period, each protein level was analyzed by western blot and cell viability was identified by MTT method. Levels of protein are showed as folds of control from 3 independent experiments and cell viability was showed as folds of control from 6 independent experiments; data were expressed as mean ± S.E.M. $*P < 0.05$ or $**P < 0.01$ compared with ALD alone.



Apoptosis, a form of programmed cell death, can occur in a wide range of physiological and pathological situations, which characterized by cell shrinkage, programmed DNA degradation, cytoplasmic cytochrome C release increased and activation of caspases and so on [30-32]. The tumor suppressor p53 plays a pivotal role in DNA damage-induced apoptosis. P53 aims at its transcriptional target pro-apoptotic bax ortranslocates to mitochondria to interact with anti-apoptotic bcl-2 in non-transcriptional way. Anti-apoptotic members bcl-2 can promote survival by inhibiting the function of the pro-apoptotic bax proteins [33, 34]. Our results showed that the ALD-induced p53 and bax elevation were significantly diminished in the present of TSG, which suggests that TSG pretreatment may depress p53 to inhibit the ALD-induced apoptosis. Furthermore, the activating of caspase-3 in the mitochondrial apoptotic pathway can lead to loss of ATP and reactive oxygen species generation [35]. The abnormal leakage of lactate dehydrogenase (LDH) is an index of plasma membrane damage and cell apoptosis [36]. In our research we found that TSG could decrease the leakage of LDH and down regulate the protein of caspase-3 in ALD-treated cardiomyocytes.

## Conclusion

TSG could effectively alleviate the plasma membrane damage induced by ALD; which was indicated by the release of LDH. TSG could decrease the level of bax and caspase-3 in ALD-treated cardiomyocytes and also increase the expression of bcl-2, and the above mechanism may be via interfering P53 patnway. This founding provides a new evidence for the potential effects of TSG in treating cardiovascular diseases.

## Disclosure of conflict of interest

None.

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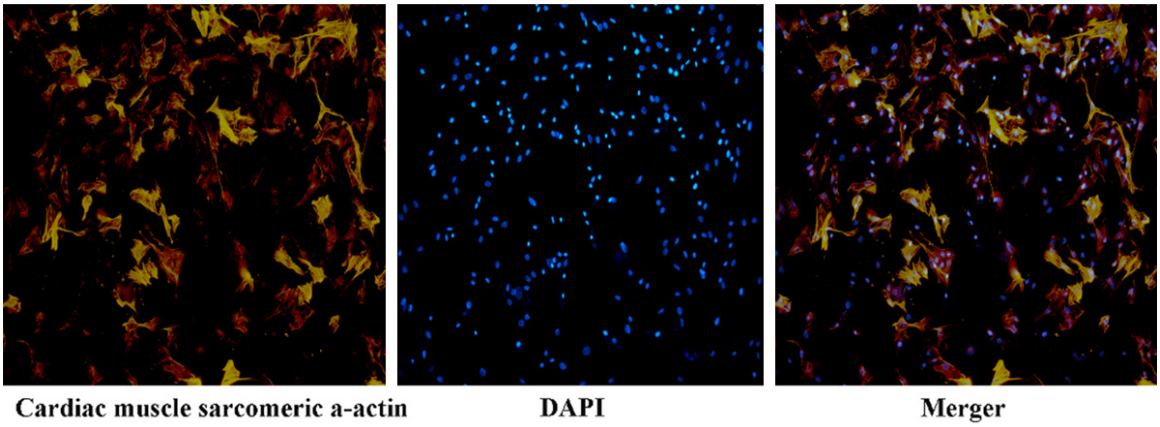


**Supplementary Figure 1.** Characterization of primary cultured rats' cardiomyocytes derived from the male Sprague-Dawley rats. The cells were stained with cardiac muscle sarcomeric a-actin and labeled with the cy3 conjugated antibody. The cells were observed at magnification of 200× under a fluorescence microscope.