

## Original Article

# Stilbene glycosides suppress oxidized low-density lipoprotein-induced macrophage Raw264.7 cell apoptosis in vitro

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Received September 5, 2016; Accepted October 28, 2016; Epub May 1, 2017; Published May 15, 2017

**Abstract:** Objective: Elevated oxidized low-density lipoprotein (OxLDL) level associates with severity of coronary syndromes and stilbene glycosides (TSG) could reverse OxLDL-induced cell dysfunction. This study was to investigate the effect of TSG on mouse macrophage Raw264.7 cells apoptosis by OxLDL. Materials and methods: Mouse macrophage Raw264.7 cell line was maintained with OxLDL to establish cell apoptosis. TSG was administrated to OxLDL-induced apoptotic cells to explore effect of it on cell apoptosis by OxLDL. Apoptotic related proteins and signaling pathways, cell viability, and apoptosis rates were detected using appropriated methods. Differences among treatments were analyzed. Results: OxLDL-induced cytotoxicity in macrophage Raw264.7 cells via promoted apoptosis in a dose-dependent manner. Apoptotic related proteins including activated Caspase-3, -9, Bax/Bcl-2 ratio, as well as NF- $\kappa$ B and P13K signaling pathways were upregulated by OxLDL. TSG treatment, on the contrary, reversed OxLDL-induced cell apoptosis and upregulation of apoptotic related proteins and signaling pathways, in a dose-dependent manner. Conclusions: TSG intervention could reverse OxLDL-induced cytotoxicity by cell apoptosis, upregulation of apoptotic related proteins and signaling pathways, *in vitro*. Results in this study revealed TSG might be used as a potential therapy for coronary diseases.

**Keywords:** OxLDL, TSG, coronary artery diseases, cell apoptosis

## Introduction

Oxidized low-density lipoprotein (OxLDL) plays a crucial role in pathogenesis of coronary artery diseases (CAD) including atherosclerosis [1, 2]. Reportedly, elevated OxLDL level in human plasma associates with severity of coronary syndromes, and used as a predictor for acute coronary heart disease (CHD) events [3, 4]. Elevated OxLDL levels induced alteration of metabolism, apoptosis of endothelial cells, and inflammatory responses *in vivo* and *in vitro*, showing OxLDL holds a prominent place in maintaining body's normal metabolism and health [5, 6].

Various drugs including glucosides had been reported to be protective against OxLDL-induced cell cytotoxicity, such as delphinidin-3-glucoside [7, 8], phenolics of grape juice [9] and other extracts from plants rich in anthocyanins and flavones [10, 11], which exhibit capacity of antioxidation thus inhibiting elevated

OxLDL-induced reactive oxygen species (ROS) formation, cell cytotoxicity and dysfunction of cells including macrophages and endothelial cells, *in vivo* and *in vitro* [12-14].

Stilbene glycosides (2,3,5,4'-tetrahydroxy stilbene-2- $\beta$ -D-glycoside, TSG) was a botanical extract ingredients extracted from traditional Chinese medicine *Polygonum multiflorum* [15]. Numerous evidences had been showing the protective effect of TSG on cell proliferation [16], rats atherosclerosis [17], animal learning and memory abilities [18], Alzheimer disease [19], as well as its antioxidation [20]. However, there was rare study focusing on the TSG inhibitory effect of TSG on OxLDL-induced cell cytotoxicity and dysfunction.

To investigate the effect of TSG on OxLDL-induced cell cytotoxicity and the mechanism related to it, we treated mouse macrophage Raw264.7 cells with serial dose OxLDL for cell

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cytotoxicity. Then, TSG was administrated to OxLDL-induced apoptotic cells to explore effect of it on cell apoptosis by OxLDL. Apoptotic related proteins and signaling pathways, cell viability, and apoptosis rates were detected. The effect of TSG on OxLDL-induced cell cytotoxicity and cell function changes among treatments were analyzed. The potential using TSG as the therapy for CAD or CHD would be discussed.

### Materials and methods

#### *Cell line, cell culture conditions and cell treatment*

Mouse macrophage-like cell line RAW264.7 (ATCC, TIB 71, Manassas, VA, USA) was incubated in DMEM with 0.5% FBS (HyClone Laboratories, Logan, UT, USA) at 37°C, supplemented with penicillin-streptomycin. For cell treatment, cells were cultured in 96-well plates and treated with increased doses of OxLDL (Biomedical Technologies, Stoughton, MA, USA) 0, 20, 80, and 150 µg/mL, or TSG (Sigma-Aldrich, St. Louis, MO, USA) 10, 20, 50, 100 and 200 µM. The optimal conditions including doses and time points by OxLDL induced cell cytotoxicity were selected according to the cell viabilities.

#### *CCK-8 cell viability assay*

Cell Counting Kit 8 (CCK-8) assay (Sigma) was conducted for cell viability of RAW264.7 cells. RAW264.7 cells maintained in OxLDL and TSG for 0, 12, 24, and 48 h were allowed to make cells attached to the 96-well plate wall. Then, cells were additionally incubated in CCK-8 solution for 2 h. The optical density at 450 nm absorbance (A450) by a microplate spectrophotometer (Bio-Rad Labs, Sunnyvale, CA) was detected. For each experiment, three triplicates were performed and averaged value was calculated.

#### *Cell apoptosis assay*

To investigate cytotoxicity or protective capability by OxLDL or TSG, *in vitro*, cell apoptosis test were performed on RAW264.7 cells. Cells seeded on 24-well plates with OxLDL or TSG were maintained for 24 h. Then cell cultures were darkly supplemented with Annexin V-FITC and PI (propidium iodide, Clontech, Mountain View,

CA, USA) for 30 min and flow cytometer (BD FACS Calibur Flow Cytometer; BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze cell apoptosis.

#### *Western blotting analysis*

Cultured cells seeded into 6-well plates were harvested, and lysed. Cell lysates were quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). A total of 15 µl cell lysates was separated by 10% SDS-PAGE gels and transferred to PVDF membranes (Invitrogen Corp., Carlsbad, CA, USA). Then PVDF membranes were then blocked with 5% skimmed milk, incubated with the primary antibodies against Caspase 3 (1:1000 in dilution; Abcam Inc., Cambridge, MA, USA), Caspase 9 (1:1000 in dilution; Abcam), Bax (1:500 in dilution; Abcam), Bcl-2 (1:1000 in dilution; Cell Signaling Technology, CST, Danvers, MA, USA), phospho-p85 P13K (p-P13K; 1:1000 in dilution; CST) and P13K (dilution, 1:1000; CST), NF-κB (1:500 in dilution; Abcam) and GAPDH (1:2000 in dilution; CST) at 4°C overnight. Membranes were washed and incubated with infrared fluorescent dye-labeled secondary antibodies (1:10000 in dilution; Biyuntian Biotech Co., Ltd., Shanghai, China) for 1 hour. The signal intensities of polypeptide bands were quantified by an Odyssey infrared imager (NIR) scanner (Li-Cor Biosciences, Lincoln, NE, USA).

#### *Statistical analysis*

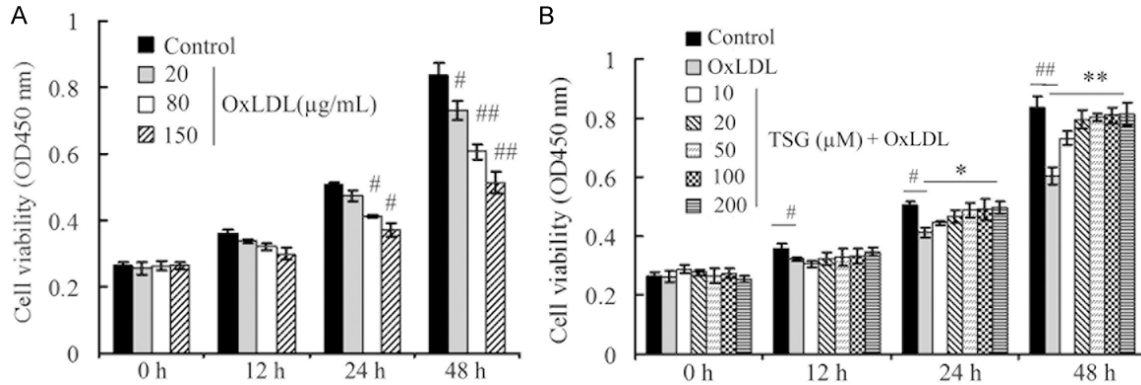
Statistical analysis was performed using SPSS version 18.0 (Chicago, IL, USA). All quantitative data are presented as mean ± standard deviations (SD). Significance between and among groups were performed by t test and ANOVA. P < 0.05 and P < 0.01 was regarded as significant and very significant, respectively.

### Results

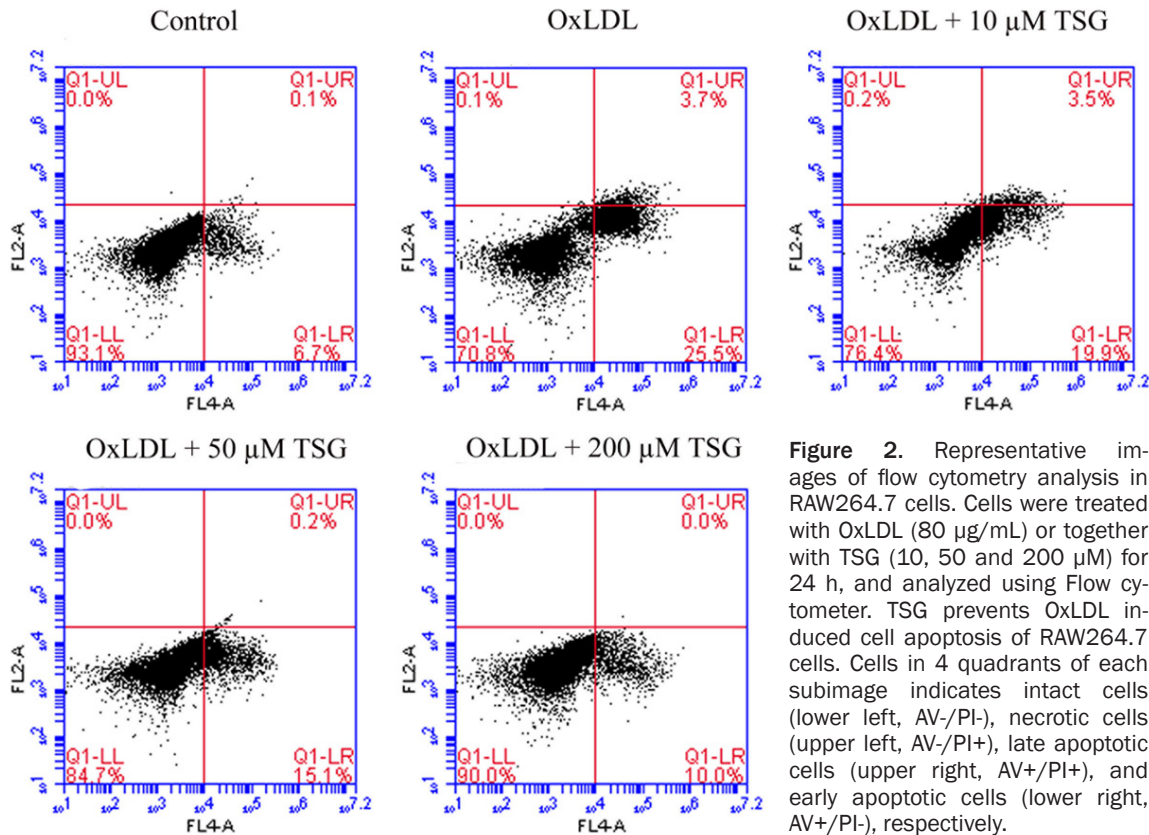
#### *OxLDL induces cytotoxicity on RAW264.7 cells*

For selection of optimal condition of OxLDL cytotoxicity by CCK-8 assay, cells were treated with OxLDL of 20, 80 and 150 µg/mL and maintained for 12, 24, and 48 h. CCK-8 assay showed OxLDL induced an inhibition on cell viability of RAW264.7 cells, in dose-dependent manner (**Figure 1A**). Cells treated with OxLDL

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**Figure 1.** TSG suppresses OxLDL-induced cytotoxicity on RAW264.7 cells. Cell viability was tested using CCK-8 assay. Cell cultures were treated with OxLDL alone (A) and together with TSG (B). #, and ## indicates significant level at  $P < 0.05$  and  $P < 0.01$ , vs. Control, respectively. \*, and \*\* indicates significant level at  $P < 0.05$  and  $P < 0.01$ , vs. OxLDL, respectively.



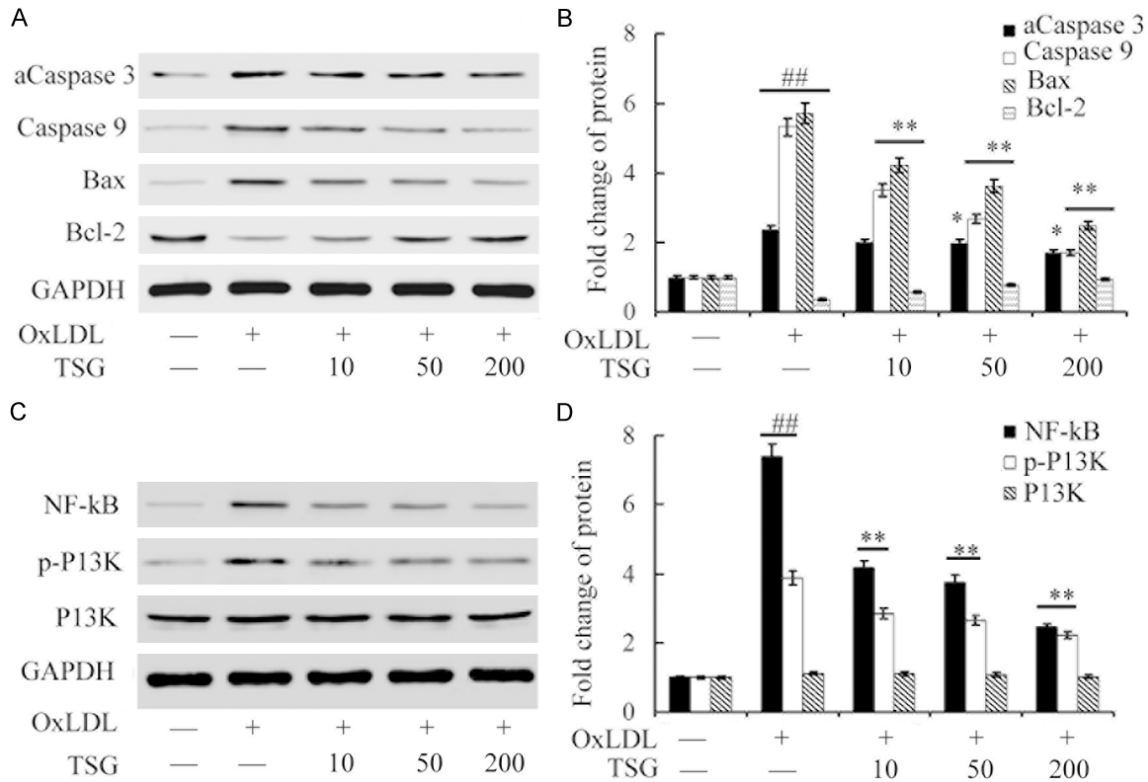
**Figure 2.** Representative images of flow cytometry analysis in RAW264.7 cells. Cells were treated with OxLDL (80 µg/mL) or together with TSG (10, 50 and 200 µM) for 24 h, and analyzed using Flow cytometer. TSG prevents OxLDL induced cell apoptosis of RAW264.7 cells. Cells in 4 quadrants of each subimage indicates intact cells (lower left, AV-/PI-), necrotic cells (upper left, AV-/PI+), late apoptotic cells (upper right, AV+/PI+), and early apoptotic cells (lower right, AV+/PI-), respectively.

showed a relative lower OD450 nm value than cells treated in the same conditions. Moreover, cells maintained in OxLDL of 80 and 150 µg/mL showed significant inhibition by cell viability both at 24 h and 48 h (**Figure 1A**). Then the optimal conditions for OxLDL induced cytotoxicity of RAW264.7 cells, *in vitro*, were selected as 80 µg/mL at 24 h.

### TSG suppresses OxLDL induced cytotoxicity on RAW264.7 cells

Next, we tested the protective ability of TSG against RAW264.7 cell cytotoxicity by OxLDL. TSG was administrated at serial doses of 10, 20, 50, 100 and 200 µM and cell viabilities by CCK-8 assay were detected. Results showed

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**Figure 3.** TSG reverses OxLD-induced dysregulation of apoptotic related proteins and signaling pathways. Lysates from cells treated with OxLDL (80  $\mu\text{g}/\text{mL}$ ) or together with TSG (10, 50 and 200  $\mu\text{M}$ ) were prepared for Western blotting analysis. A and B: TSG reverses OxLD-induced dysregulation of apoptotic related proteins. C and D: TSG inhibits OxLD-enhanced expression of NF- $\kappa\text{B}$  and P13K signaling pathways. #, and ## indicates significant level at  $P < 0.05$  and  $P < 0.01$ , vs. Control, respectively. \*, and \*\* indicates significant level at  $P < 0.05$  and  $P < 0.01$ , vs. OxLDL, respectively.

cells cultures treated with TSG revealed increments of cell viabilities by CCK-8 assay, compared with cells treated with OxLDL alone (Figure 1B). Moreover, cells treated with higher dose of TSG showed relative higher increase in cell viability of cells treated with OxLDL. Thus we conclude that TSG intervention resulted in a dose-dependent inhibition to OxLDL induced cytotoxicity on RAW264.7 cell.

### TSG prevents OxLDL induced cell apoptosis of RAW264.7 cells

Since TSG prevents inhibition of RAW264.7 cell viability by OxLDL, we supposed there might be a similar behavior of cell apoptosis. Then, cells incubated in three 3 interrupted doses of 10, 50, and 200  $\mu\text{M}$  were prepared for cell apoptosis assay by flow cytometer analysis. Cells treated with 80  $\mu\text{g}/\text{mL}$  OxLDL for 24 h showed obvious increase in ratios of both early and late apoptotic cells, vs. cells of Control (Figure 2). Cells maintained with TSG, however, showed

evident decline in ratios of both early and late apoptotic cells, compared to cells treated with OxLDL alone. These results showed that TSG administration could prevent OxLDL-induced cell apoptosis, in a dose-dependent TSG.

### Effect of TSG and OxLDL administration on apoptotic proteins

Expressions of cell apoptotic related proteins including activated Caspase-3, -9, Bax, and Bcl-2 were detected by Western blotting analysis. The expressions of proteins were significantly up- or down-regulated by OxLDL interference, and TSG management (Figure 3A and 3B). Western blotting analysis showed activated Caspase-3, Caspase-9, and Bax proteins were significantly upregulated, and Bcl-2 protein was obviously downregulated by OxLDL. TSG management, on the contrary, significantly reversed OxLDL-induced activated Caspase-3, Caspase-9, and Bax expression, and canceled



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OxLDL-inhibited Bcl-2. In addition, these reversions on protein expression by TSG against OxLDL were dose-dependent.

### *Effect of TSG and OxLDL administration on NF- $\kappa$ B and P13K signaling pathways*

Since NF- $\kappa$ B and P13K signaling pathways associated to cell apoptosis [21, 22], we determined the effect of TSG and OxLDL administration on NF- $\kappa$ B, p-P13K and total P13K proteins. Cells treated with OxLDL alone showed significant increase in NF- $\kappa$ B and p-P13K expression, compared to control (**Figure 3C and 3D**). Cells treated with OxLDL together with 3 doses of TSG 10, 50, and 200  $\mu$ M inverse revealed remarkable decrease in NF- $\kappa$ B and p-P13K expression, compared cells treated with OxLDL alone. Also, these reversions on NF- $\kappa$ B and p-P13K expression by TSG against OxLDL were TSG dose-dependent.

### **Discussion**

TSG is a kind of phenolics with antioxidant capacity as well as other phenolics including, glycosides such as delphinidin-3-glucoside, and anthocyanins and flavonols as phenolics of grape juice [9-11]. This present study studied the antioxidant capacity of TSG on OxLDL in mouse macrophage Raw264.7 cell line, *in vitro*. The results showed that TSG could inhibit OxLDL-induced cell viability and cell apoptosis by suppressing expression of apoptosis related proteins and signaling pathways.

As reported, OxLDL induces ROS formation [13], upregulation of microRNA-29b [23], accumulation of hypoxia-inducible factor (HIF)-1 $\alpha$  [24], and other factors associating with cell apoptosis. In this study, we determined that OxLDL administration induced cell apoptosis in a dose-dependent way via inhibiting cell viability, upregulating expression of activated Caspase-3, -9, Bax/Bcl-2 ratio, as well as NF- $\kappa$ B and P13K signaling pathways, revealing OxLDL-induced cell cytotoxicity in macrophage Raw264.7 cells.

TSG administration showed it reversed all the dysfunction in macrophage Raw264.7 cells by OxLDL in a dose dependent manner. Specifically, TSG administration canceled OxLDL-inhibited cell viability, inhibited OxLDL-triggered cell apo-

ptosis, suppressed OxLDL-elevated expression of activated Caspase-3, -9, Bax/Bcl-2 ratio, NF- $\kappa$ B, and p-P13K. Similarly, Jin *et al.* [7] revealed that management of delphinidin-3-glucoside, one bioactive compound known as anthocyanins ameliorating oxidative stresses, attenuated OxLDL-induced cell apoptosis, expression of Bax/Bcl-2 and aCaspase-3. This demonstrated the protective effect delphinidin-3-glucoside against OxLDL-induced cell cytotoxicity in primary human umbilical vein endothelial cells. Additionally, there some explores showing dietary polyphenols could prevent cardiovascular disease by regulating endothelial function [25-27]. Accordingly, we concluded there was a protective effect of TSG against OxLDL-induced cell cytotoxicity in macrophage Raw264.7 cells.

Cell apoptosis is a complicated process involving in numerous stimulation factors such as genes and signaling pathways [28-31]. For instance, Bcl-2 and Bax are important factors of cell apoptosis and elevation of Bax and inhibition of Bcl-2 benefit to cell apoptosis [28, 29, 32, 33]. Moreover, NF- $\kappa$ B/Bcl-2 signaling and PI3K/Akt signaling pathway are a key pathway essential for cell apoptosis [28, 29]. Inhibition of PI3K/Akt signaling pathway contributed to cell apoptosis [34]. Accordingly, we speculated that OxLDL might regulate activation status of p-P13K and NF- $\kappa$ B signaling pathways. To test this speculation, we detected and determined that OxLDL conferred to upregulation of Bax/Bcl-2 ratio, NF- $\kappa$ B and p-PI3K. Moreover, all the dysregulations were attenuated by TSG by dose-dependent manner, demonstrating both OxLDL-induced and TSG-suppressed apoptosis were mediated by NF- $\kappa$ B and p-PI3K signaling pathways.

### **Conclusion**

In this study, we obtained mouse macrophage Raw264.7 cell apoptosis by OxLDL administration. Further detection using Western blotting analysis showed expression of apoptosis related proteins, including activated Caspase-3, -9, and Bax/Bcl-2, and p-P13K and NF- $\kappa$ B signaling pathways were induced by OxLDL, and all reversed by TSG, in dose-dependent manner, *in vitro*. These results might reveal TSG can be used as a potential therapy for OxLDL-triggered diseases including coronary diseases. However, the mechanism and the association between

TSG and apoptosis related signaling pathways showed be digged and annotated by more experimental tests.

### Disclosure of conflict of interest

None.

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