

## Anti-proliferative and pro-apoptotic effects of tectorigenin on hepatic stellate cells

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### Abstract

**AIM:** To investigate the effect of tectorigenin on proliferation and apoptosis of hepatic stellate cells (HSC)-T6 cells.

**METHODS:** HSC-T6 cells were incubated with tectorigenin at different concentrations, and their proliferation was assessed by bromodeoxyuridine incorporation assay. Apoptosis was detected by flow cytometry assay with Hoechst 33342 staining. Also, generation of reactive oxygen species (ROS), intracellular  $[Ca^{2+}]_i$ , potential of mitochondrial membrane, activities of cytochrome c and caspase-9 and -3 were investigated to explore a conceivable apoptotic pathway.

**RESULTS:** Tectorigenin suppressed the proliferation of HSC-T6 cells and induced apoptosis of HSC-T6 cells in a time- and dose-dependent manner. Tectorigenin at the concentration of 100  $\mu$ g/mL greatly inhibited the

viability of HSC-T6 cells and induced the condensation of chromatin and fragmentation of nuclei. When treated for 48 h, the percentage of cell growth and apoptosis reached  $46.3\% \pm 2.37\%$  ( $P = 0.004$ ) and  $50.67\% \pm 3.24\%$  ( $P = 0.003$ ), respectively. Furthermore, tectorigenin-induced apoptosis of HSC-T6 cells was associated with the generation of ROS, increased intracellular  $[Ca^{2+}]_i$ , loss of mitochondrial membrane potential, translocation of cytochrome c, and activation of caspase-9 and -3.

**CONCLUSION:** Tectorigenin inhibits proliferation of HSC-T6 cells and induces apoptosis of HSC-T6 cells.

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**Key words:** Tectorigenin; Apoptosis; Hepatic stellate cells; Hepatic fibrosis; Mitochondria; Proliferation

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### INTRODUCTION

Hepatic fibrosis, a common wound-healing response to diseases such as chronic hepatitis and alcoholic liver damage, is a result of destruction in architecture of the liver parenchyma and an imbalance between fibrogenesis

and fibrolysis forming scars or fibrous tissues. Hepatic stellate cells (HSC), the major cells in hepatic fibrosis, are responsible for the development of fibrosis<sup>[1,2]</sup>. Activation and proliferation of HSC are the key to fibrogenesis while apoptosis of HSC is associated with the resolution of fibrosis. So, HSC have attracted increasing attention due to their essential role in liver fibrosis. With a better understanding of their biological properties, inhibiting the activation and proliferation of HSC and inducing apoptosis of activated HSC have been proposed as potential anti-fibrosis strategies.

In traditional Chinese medicine, *Iris tectorum* (*I. tectorum*) has been used in treatment of liver injury for a long time. It has been shown that tectorigenin, an important bioactive compound isolated from *I. tectorum*, has antioxidant, anti-inflammatory, and anticancer activities<sup>[3-5]</sup>. Experiments on animal models have also demonstrated that tectorigenin exhibits a hepatoprotective effect on CCl<sub>4</sub>-induced or *t*-BHP-induced hepatic injury in rats<sup>[6,7]</sup>. Since hepatic fibrosis is a common wound-healing response to liver injury, we studied whether tectorigenin has anti-fibrosis potentials and exhibits its hepatoprotective effect by playing a role in liver fibrosis.

The present study was therefore performed to investigate the effects of tectorigenin on proliferation and apoptosis-related events of activated HSC-T6 cells and disclose its possible mechanism underlying apoptosis of HSC-T6 cells.

## MATERIALS AND METHODS

### Materials

Tectorigenin was isolated from *I. tectorum* with a purity of over 98% as confirmed by high-performance liquid chromatography (HPLC) analysis.

### Cell culture

HSC-T6 cells, an immortalized rat hepatic stellate cell line, exhibit an activated HSC phenotype<sup>[8]</sup>. The cells, purchased from Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China), were cultured in Dulbecco's-modified Eagle's medium (DMEM; Gibco, NY, USA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 12% new bovine serum (Hangzhou Sijiqing Co., Ltd., Hangzhou, China), in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub> at 37°C. L02 cells (a human hepatocyte cell line), purchased from Xiangya Central Experiment Laboratory, Central South University, China, were cultured in DMEM medium (Gibco, NY, USA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (Hangzhou Sijiqing Co., Ltd., Hangzhou, China), in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub> at 37°C.

### Cell viability assay

Cells were plated in 96-well plates at a density of  $5 \times 10^5$  cells/well and grown for 24 h. Tectorigenin at different concentrations was added to the cells while only DMSO

(solvent) was added as a negative control. After growing for 12, 24, and 48 h, cell viability was evaluated by the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Amresco, OH, USA)<sup>[9]</sup>.

### Bromodeoxyuridine uptake

HSC-T6 cells were incubated for 48 h with 20, 40, 60 and 100 µg/mL tectorigenin, respectively. Two hours before the cells were harvested, bromodeoxyuridine (BrdU; GenMed Sciences Inc., USA) was added. The cells were fixed in 4% paraformaldehyde and stained with Hoechst 33342 following the manufacturer's protocol<sup>[10]</sup>.

### Morphological observation of nuclear change

HSC-T6 cells were incubated for 48 h with tectorigenin at 20, 40, 60 and 100 µg/mL, respectively. Nuclear morphological change was assessed using Hoechst 33342 staining<sup>[11]</sup>. In brief, cells were fixed in 4% paraformaldehyde for 10 min, washed three times with pre-chilled PBS and exposed to 5 µg/mL of Hoechst 33342 at 37°C in dark for 15 min. Samples were observed under a fluorescent microscope (Nikon UFX-II, Japan). Cells showing cytoplasmic and nuclear shrinkage, chromatin condensation or fragmentation, were defined as apoptotic cells.

### Flow cytometry analysis

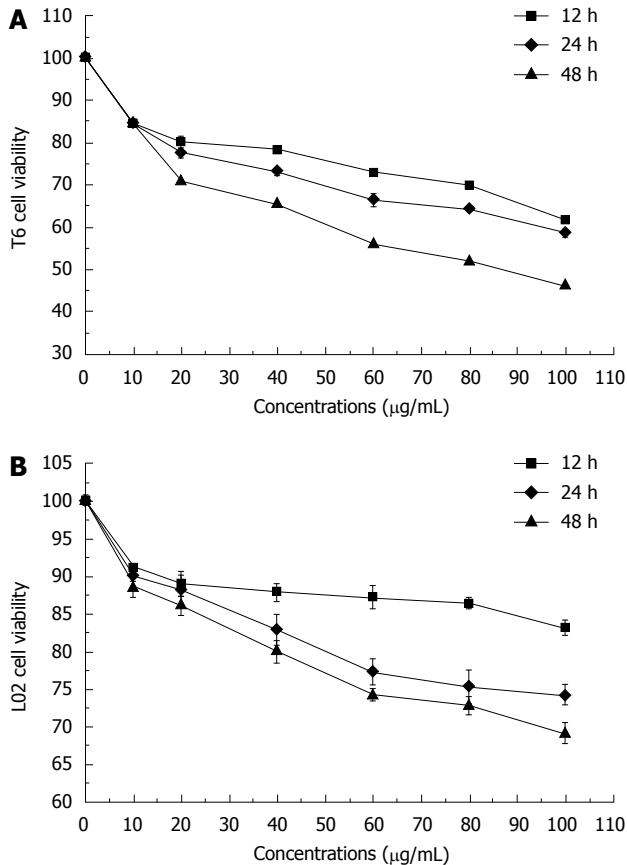
To quantify apoptotic cells, HSC-T6 cells were harvested after exposed to tectorigenin for 24 and 48 h, respectively, washed twice with cold PBS, resuspended in PBS containing fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) for 10 min, and measured using a FACScan flow cytometer<sup>[12,13]</sup> (Becton Dickinson, Franklin Lakes, NJ, USA).

### Measurement of ROS generation

Intracellular ROS was quantified with a fluorescence plate reader using 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma)<sup>[14]</sup>. The cells on black 96-well plates were treated with tectorigenin at with tectorigenin at 20, 40, 60 and 100 µg/mL for 1, 3, 6 and 24 h, respectively, and incubated with DCFH-DA at 37°C for 30 min. After DCFH-DA was removed, the cells were washed with phosphate buffered saline (PBS). DCFH-DA-loaded cells were read on a Safire fluorescence plate reader (Tecan, Crailsheim, Germany).

### Measurement of intracellular [Ca<sup>2+</sup>]<sub>i</sub>

[Ca<sup>2+</sup>]<sub>i</sub> was monitored using fluorescent Ca<sup>2+</sup>-sensitive dye, a Fura 2-acetoxymethyl ester (Fura 2-AM)<sup>[15]</sup>. Cells were cultured and treated with tectorigenin for 1, 3, 6 and 24 h, respectively, and preloaded with 1 µmol/L Fura2-AM for 30 min in dark at 37°C in a humidified incubator. After loading with Fura2-AM, cells were collected, gently rinsed three times with D-Hanks' solution, and resuspended in D-Hanks' solution containing 0.2% BSA at 10<sup>6</sup> cells/mL. Intracellular [Ca<sup>2+</sup>]<sub>i</sub> was measured at an emission wavelength of 510 nm and an excitation wavelength of 340 and 380 nm on a Safire fluorescence



**Figure 1** Effects of tectorigenin on the viability of hepatic stellate cell -T6 (A) and -L02 cells (B). Cells were treated for 12, 24 and 48 h with tectorigenin at 10, 20, 40, 60, 80 and 100 μg/mL, respectively, followed by assessing the cell viability relative to that of untreated cells (= control). Bars represent mean  $\pm$  SD.

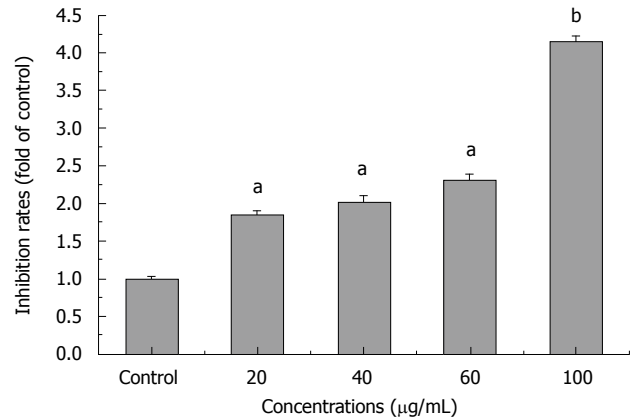
plate reader (Tecan, Crailsheim, Germany). The ratio of fluorescence intensity at 340 to 380 nm (F340/F380) was used to estimate intracellular free calcium.

#### Measurement of mitochondrial membrane potential

Change in mitochondrial membrane potential (MMP) was monitored using Rhodamine 123 (Rh-123)<sup>[16]</sup>. In brief, Rh-123 was added to cells to attain a final concentration of 3 μg/mL. After incubated at 37°C for 30 min, cells were collected, washed twice with PBS, and analyzed with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

#### Western blotting analysis

HSC-T6 cells were seeded into 60-mm dishes ( $1 \times 10^6$  cells/dish). After treated on the next day for 48 h with tectorigenin at 0, 20, 40, 60 and 100 μg/mL, respectively, HSC-T6 cells were harvested, resuspended in an ice-cold lysis buffer consisting of 50 mmol/L Tris-HCl, pH 8.0, 50 mmol/L KCl, 5 mmol/L DTT, 1 mmol/L EDTA, 0.1% SDS, 0.5% Triton X-100, and protease inhibitor cocktail tablets (Roche, IN), incubated for 10 min on ice, disrupted in a micro ultrasonic cell disrupter for 10 s and centrifuged at 750 *g* for 15 min at 4°C. The supernatant (cytosolic fraction) was removed and maintained at



**Figure 2** Proliferative inhibition of tectorigenin on hepatic stellate cells-T6 cells. Cells were treated for 48 h with tectorigenin at 0, 20, 40, 60 and 100 μg/mL, respectively. Data are expressed as fold increase over that of untreated cells. Bars represent mean  $\pm$  SD (<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01).

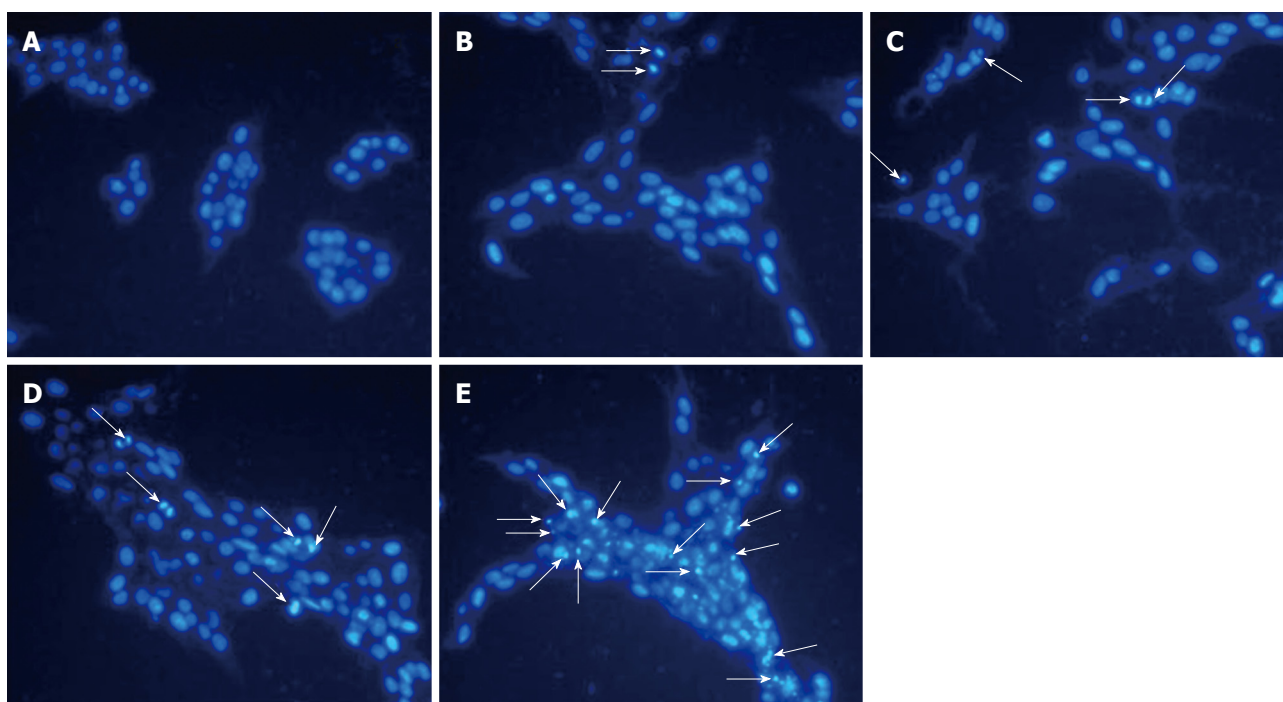
-80°C. The pellet containing mitochondria was resolved in a lysis buffer. Protein level was measured using a standard colorimetric assay kit (BCA kit). Proteins were separated by polyacrylamide/SDS gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Roche, IN). The membranes were probed with antibodies (cytochrome c and caspase-9 diluted at 1:1000, Cell Signaling Technology, MA, USA) overnight at 4°C, and incubated with a HRP coupled secondary antibody (HRP; 1:5000, Cell Signaling Technology, MA, USA). Detection was performed using a LumiGLO chemiluminescent subtract system (KPL, Guildford, UK).  $\beta$ -actin (1:200, Boster, Wuhan, China) as a loading control. Results were quantified with a scanning densitometer (Bio-Rad, USA).

#### Measurement of caspase-3 activity

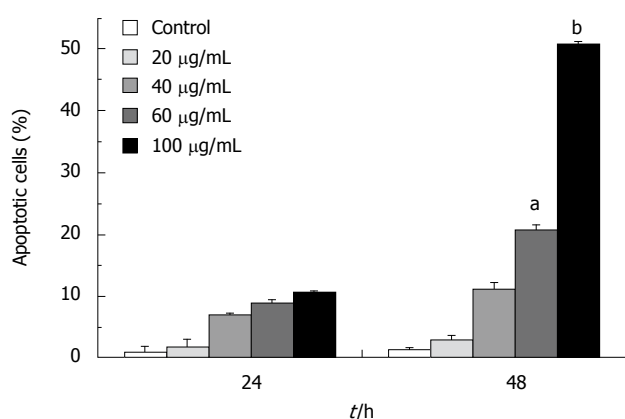
Activity of caspase-3, the main execution caspase, was detected with a caspase-3 colorimetric assay kit (KenGen Biotech, Nanjing, China) according to its manufacturer's instructions. Cultured HSC-T6 cells were washed twice with cold PBS, resuspended in a lysis buffer and left on ice for 20 min. Lysate was centrifuged at 10000 r/min for 3 min at 4°C. Supernatants were collected and protein concentrations were measured with a BCA kit. Proteins (100 μg) were incubated for 4 h at 37°C with a reaction buffer in a total volume of 105 μL containing 5 μL caspase-3 substrate, and detected with a fluorescence microplate reader (Tecan, Crailsheim, Germany) at 405 nm.

#### Statistical analysis

All data were expressed as mean  $\pm$  SD. Origin Pro 7.0 statistical package was used to determine statistical significance. Difference between two groups was analyzed by two-tailed Student's *t*-test, and difference among three or more groups was analyzed by one-way ANOVA multiple comparisons. *P* < 0.05 or *P* < 0.01 was considered statistically significant.



**Figure 3** Fluorescent staining of nuclei in hepatic stellate cells-T6 cells (200 ×). Cells were treated with tectorigenin for 48 h at 0 µg/mL (A), 20 µg/mL (B), 40 µg/mL (C), 60 µg/mL (D) and 100 µg/mL (E), respectively. The arrows in B-E indicate the apoptotic cells.



**Figure 4** Flow cytometry-evidenced apoptosis of hepatic stellate cells-T6 cells upon exposure to tectorigenin. Cells were incubated for 24 and 48 h with tectorigenin at 20, 40, 60 and 100 µg/mL, respectively, followed by being stained with fluorescein isothiocyanate-conjugated annexin V and propidium iodide. Bars represent mean  $\pm$  SD (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ ).

## RESULTS

### Effect of tectorigenin on viability of HSC-T6 and L02 cells

The percentage of cell growth was significantly different between tectorigenin-treated and untreated groups. Tectorigenin inhibited the growth of HSC-T6 cells in a dose-dependent manner (Figure 1A). Increased incubation time of HSC-T6 cells decreased the percentage of cell growth, indicating that tectorigenin also inhibits the growth of HSC-T6 cells in a time-dependent manner. Treatment with tectorigenin at 100 µg/mL resulted in a moderate cytotoxicity to L02 cells after incubated for 48 h (Figure 1B). To further investigate the inhibitory effect

of tectorigenin on proliferation of HSC-T6 cells, BrdU incorporation, another indicator of cell proliferation, was detected. Tectorigenin at 20-100 µg/mL could significantly inhibit the proliferation of HSC-T6 cells (Figure 2).

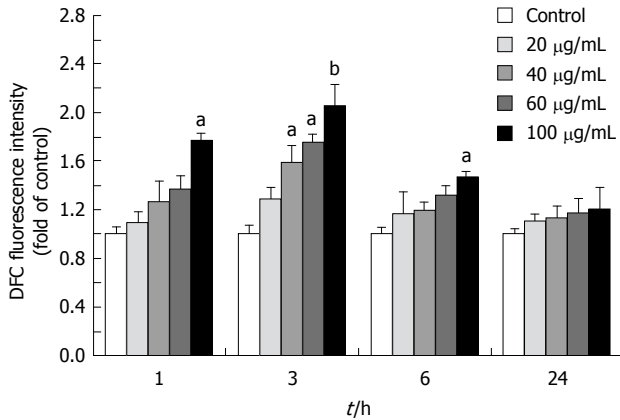
### Tectorigenin induced apoptosis of HSC-T6 cells

We studied if tectorigenin can induce apoptosis of HSC-T6 cells. In brief, apoptotic cells were visualized using DNA-binding Hoechst 33342. Regular and round-shaped nuclei were observed in control HSC-T6 cells (Figure 3). After treated for 48 h with tectorigenin at 40 and 60 µg/mL, condensed nuclei were found in HSC-T6 cells, which became smaller in size, and eventually fragmented into apoptotic bodies. Moreover, after treated with 100 µg/mL tectorigenin, the nuclei of HSC-T6 cells were further condensed with the number of apoptotic bodies sharply increased. These data suggest that tectorigenin greatly induces the condensation of chromatin and fragmentation of nuclei. The apoptosis rate of HSC-T6 cells was determined by flow cytometry analysis with annexin V-FITC and PI staining. In the control group, most cells were viable. When HSC-T6 cells were exposed for 48 h to tectorigenin at 60 and 100 µg/mL, the percentage of apoptotic cells increased to  $20.69\% \pm 2.57\%$  and  $50.67\% \pm 3.24\%$  ( $P = 0.003$ ), respectively (Figure 4), which was significantly higher than that of those not exposed to tectorigenin. In addition, tectorigenin induced apoptosis of HSC-T6 cells in a dose- and time-dependent manner.

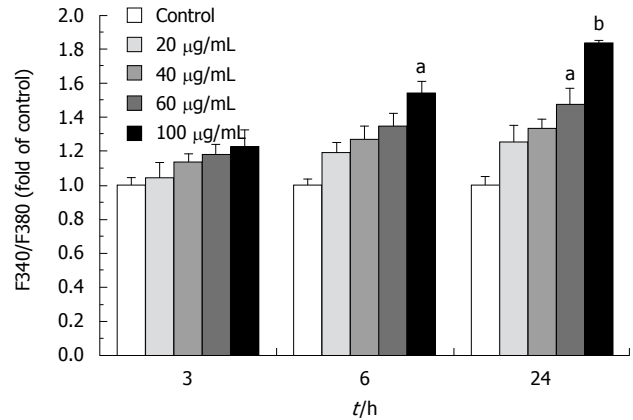
### Tectorigenin induced ROS generation in HSC-T6 cells

To determine whether tectorigenin is able to induce ROS generation in HSC-T6 cells, the level of ROS, measured using the fluorescence probe DCFH-DA, was significantly

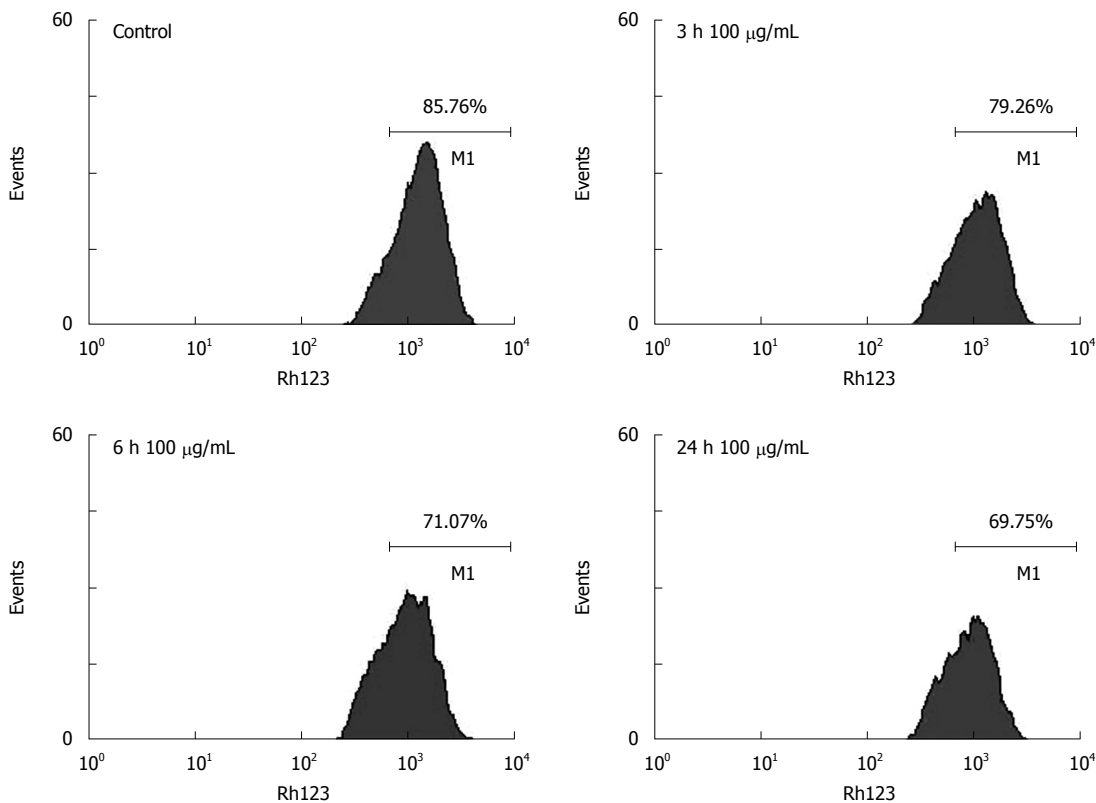




**Figure 5** Tectorigenin produces reactive oxygen species in hepatic stellate cells-T6 cells. Cells were treated with tectorigenin for 1, 3, 6 and 24 h, followed by a 30-min incubation at 37°C with reactive oxygen species detected by dichlorodihydrofluorescein diacetate. Data are expressed as fold increase over that of untreated cells. Bars represent mean  $\pm$  SD (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ ).



**Figure 6** Tectorigenin increases  $[Ca^{2+}]_i$  in hepatic stellate cells-T6 cells. Cells were treated with tectorigenin for 3, 6 and 24 h.  $[Ca^{2+}]_i$  was detected by Fura-2/AM 380nm/340nm fluorescence ratio (F340/F380). Data are expressed as fold increase over that of untreated cells. Bars represent mean  $\pm$  SD (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ ).



**Figure 7** Tectorigenin reduces mitochondrial membrane potential in hepatic stellate cells-T6 cells. After treated with tectorigenin at 100  $\mu\text{g/mL}$  for 3, 6 and 24 h, respectively, cells were harvested and stained with Rhodamine 123 to determine mitochondrial membrane potential by flow cytometry.

higher in HSC-T6 cells than in control cells after treated with 100  $\mu\text{g/mL}$  tectorigenin for 1, 3 (peaked) and 6 h (Figure 5).

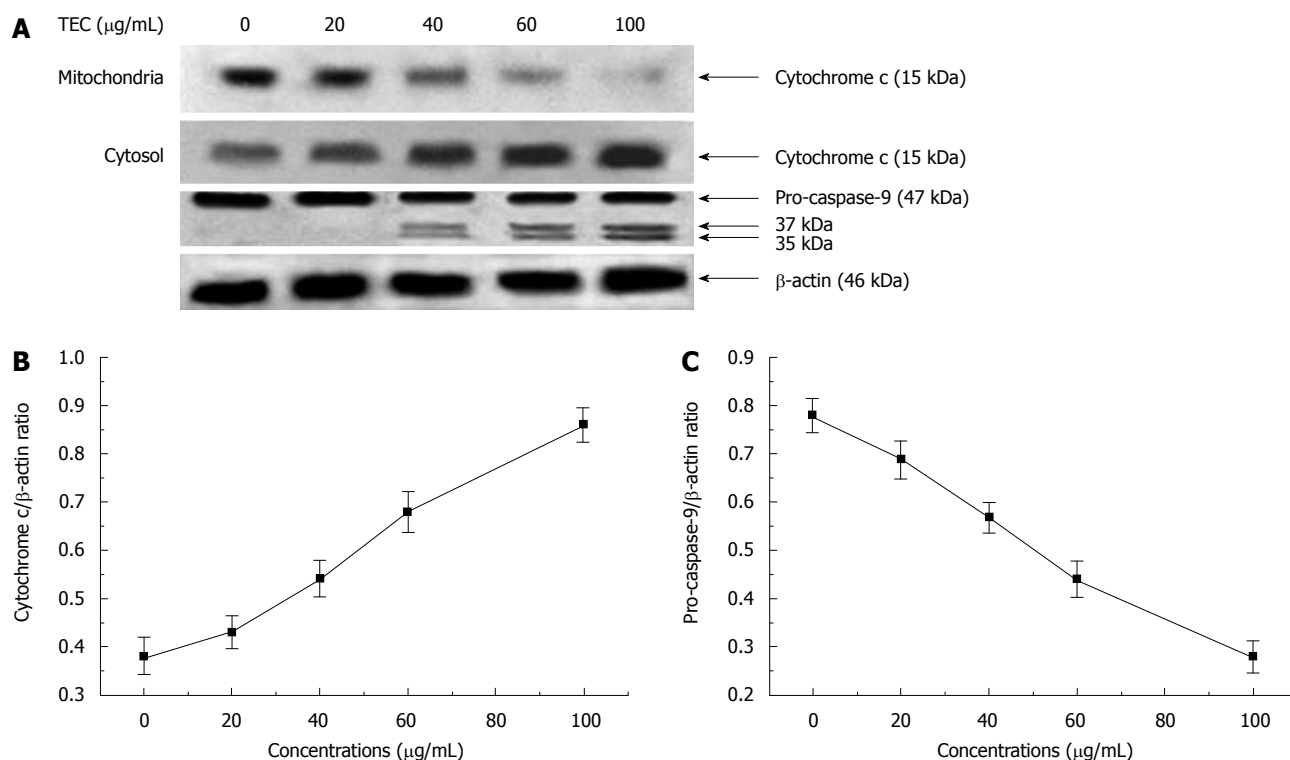
#### Tectorigenin increased $[Ca^{2+}]_i$ in HSC-T6 cells

To determine whether tectorigenin influences the level of intracellular  $Ca^{2+}$ , the level of intracellular  $Ca^{2+}$  was measured with Fura 2-AM staining. After treated with 100  $\mu\text{g/mL}$  tectorigenin for 3 h, the fluorescence ratio (F340/F380) increased to  $123.1\% \pm 7.18\%$  compared to

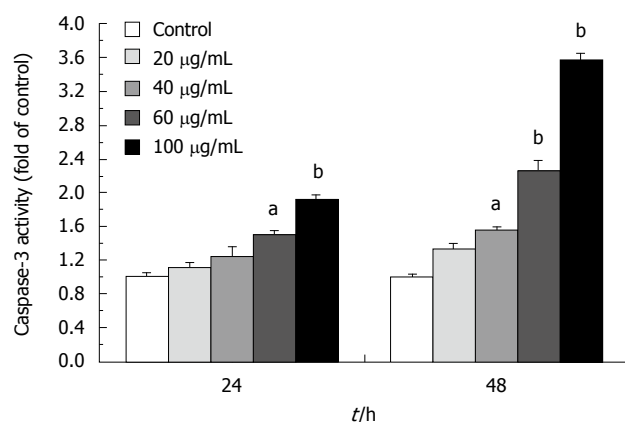
that not treated with tectorigenin (Figure 6). In addition, tectorigenin increased cytoplasmic  $Ca^{2+}$  in a time-dependent manner. When the incubation time was prolonged to 24 h, the F340/F380 value increased from  $123.1\% \pm 7.18\%$  to  $183.3\% \pm 8.64\%$  ( $P = 0.002$ ).

#### Tectorigenin decreased mitochondrial membrane potential in HSC-T6 cells

To determine whether tectorigenin decreases mitochondrial membrane potential in HSC-T6 cells, flow cytometry



**Figure 8** Western blotting analysis. After 48-h exposure to tectorigenin at 0, 20, 40, 60 and 100 µg/mL, respectively, levels of cytochrome c and caspase-9 in hepatic stellate cells-T6 cells (A), along with those of cytochrome c (B) and pro-caspase-9 (C) in cytosol, were evaluated. Protein (30 µg) from each sample was resolved on 12% SDS-PAGE and β-actin was used as a loading control.



**Figure 9** Tectorigenin activates pro-caspase-3 in hepatic stellate cells-T6 cells. Cells were treated for 24 and 48 h with tectorigenin at 0, 20, 40, 60 and 100 µg/mL, respectively. Data are expressed as fold increase over that of untreated cells. Bars represent mean  $\pm$  SD ( $^aP < 0.05$ ,  $^bP < 0.01$ ).

analysis was carried out using Rhodamine 123. Compared to control cells not treated with tectorigenin, HSC-T6 cells treated with 100 µg/mL tectorigenin for 24 h decreased the mitochondrial membrane potential (MMP) from  $85.76\% \pm 6.39\%$  to  $69.75\% \pm 5.28\%$  ( $P = 0.03$ ) (Figure 7).

### Effect of tectorigenin on release of cytochrome c and activities of caspase-9 and -3

To assess whether tectorigenin-treated cells accompany increased cytosolic translocation of cytochrome c, the activated caspases-9 and -3 were detected, and the cytosolic

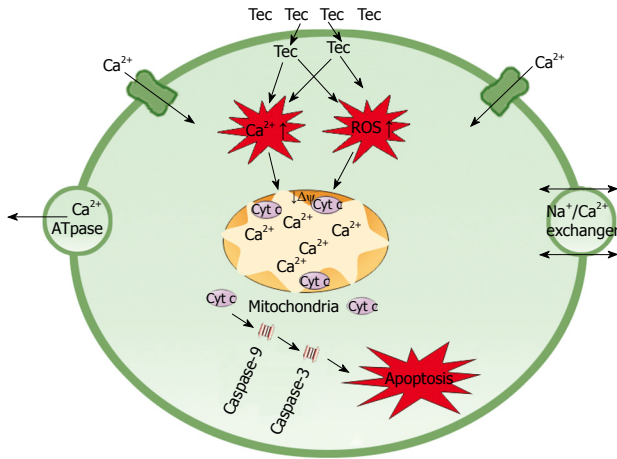
and mitochondrial levels in cytochrome c and intracellular caspases-9 and -3 were measured. The results reveal that tectorigenin releases mitochondrial cytochrome c into cytosol in a dose-dependent manner (Figure 8B). Western blotting also showed that tectorigenin induced proteolytic cleavage of pro-caspase-9 into the active form of HSC-T6 cells and tectorigenin activated the caspase-9 in a dose-dependent manner (Figure 8C). Tectorigenin (100 µg/mL) significantly increased the activity of caspase-3 in HSC-T6 cells (Figure 9).

## DISCUSSION

In the present study, tectorigenin, a bioactive compound isolated from *I. tectorum* used traditionally for severe liver disorder, suppressed the proliferation of HSC and induced apoptosis of HSC in a time- and dose-dependent manner.

As an immortalized rat liver stellate cell line, HSC-T6 cell line exhibits an activated phenotype of HSC and a fibroblast-like morphology, which presents as a useful tool in exploring hepatic fibrosis<sup>[17]</sup>. MTT and BrdU incorporation assay demonstrated that tectorigenin could inhibit the proliferation of HSC-T6 cells, with a lower cytotoxicity to human hepatocytes (L02). Furthermore, flow cytometry analysis indicated that tectorigenin could induce apoptosis of HSC-T6 cells in a dose- and time-dependent manner.

Clarification of the molecular mechanism of tectorigenin underlying the discerned apoptosis is of great importance. It has been shown that ROS generation



**Figure 10** Tectorigenin produces reactive oxygen species and increases  $\text{Ca}^{2+}$  concentration in cytoplasm of hepatic stellate cells-T6 cells, leading to the depletion of mitochondrial membrane potential and release of cytochrome c from mitochondria. Cytochrome c facilitates apoptosis of hepatic stellate cells-T6 cells by activating caspase cascade. ROS: Reactive oxygen species.

increases membrane permeability of mitochondria, triggers abrupt mitochondrial depolarization and release of cytochrome c from inner mitochondrial membrane<sup>[18]</sup>. Calcium homeostasis is an essential mechanism underlying physiological process. Since increased cytosolic calcium may induce mitochondria to take up intracellular overloaded  $\text{Ca}^{2+}$ , a much larger amount of  $\text{Ca}^{2+}$  will accumulate in mitochondria, which can potentially damage the electron transfer chain, leading to failure in maintaining the mitochondrial membrane potential<sup>[19-21]</sup>. Damages to mitochondrion result in loss of its function. For example, release of cytochrome c from mitochondria leads to cell apoptosis<sup>[22]</sup>. In this study, tectorigenin increased the ROS production and  $\text{Ca}^{2+}$  concentration in cytoplasm of HSC-T6 cells, the intracellular accumulation of ROS and  $\text{Ca}^{2+}$  further induced the loss of MMP. The disruption of MMP caused release of cytochrome c from mitochondria to cytosol. Cytosolic cytochrome c activated the pro-caspase-9 and subsequently, caspase-9 activated the downstream effector caspases-3, eventually triggered apoptosis of HSC-T6 cells (Figure 10).

In conclusion, tectorigenin suppresses the proliferation of HSC-T6 cells in a dose- and time-dependent manner, and produces a slight cytotoxicity to L02 cells. More importantly, tectorigenin induces apoptosis of HSC-T6 cells and may have anti-fibrosis potentials.

## COMMENTS

### Background

Liver fibrosis represents a significant health problem worldwide without any currently available and effective therapeutic approach. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension, and often requires liver transplantation. The most characteristic feature of liver fibrosis is excess deposition of type I collagen. A great number of researches have been performed to understand the molecular mechanism underlying liver fibrosis. Activated hepatic stellate cells (HSC) are the primary cell type responsible for the excess production of collagen.

### Research frontiers

HSC are the major cells responsible for the development of liver fibrosis and

cirrhosis. Activated HSC are proliferative and fibrogenic, with accumulation of extra cellular matrix, including  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and type I collagen. Suppression of activation and proliferation, and induction of apoptosis in HSC have been reported as the therapeutic strategies against liver fibrosis. Tectorigenin, a bioactive compound of *Iris tectorum* (*I. tectorum*), shows antioxidant, anti-inflammatory, anticancer, and hepatoprotective activities, and has been used in therapy for liver injury. However, how tectorigenin works in live fibrosis has not been unequivocally addressed. In this study, tectorigenin suppressed proliferation of HSC and induced apoptosis of HSC.

### Innovations and breakthroughs

Currently used drugs such as corticosteroids and colchicine usually show various side effects such as immunosuppression or cytotoxicity. Tectorigenin, an antioxidant, anti-inflammatory, and hepatoprotective compound isolated from *I. tectorum*, shows its inhibitory effect on proliferation of HSC and produces a low toxicity. Furthermore, *in vitro* studies suggest that tectorigenin produces its pro-apoptotic effect on HSC is probably via the mitochondrial pathway to some extent.

### Applications

By showing how tectorigenin works in HSC, this study may represent a new and future strategy for managing hepatic fibrosis.

### Peer review

This study showed that tectorigenin inhibited the growth and viability of rat hepatic HSC-T6 cells in a dose-dependent manner. The authors conclude that the anti-proliferative and pro-apoptotic effects of this component on HSC might explain its anti-fibrotic properties observed *in vivo*. This is an interesting study. However, the mechanism of tectorigenin underlying the growth and viability of HSC-T6 cells needs to be further studied.

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