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# Antagonism of cortistatin against cyclosporine-induced apoptosis in rat myocardial cells and its effect on myocardial apoptosis gene expression

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**Abstract. – OBJECTIVE: To investigate the role of cortistatin (CST) on cyclosporine A (CsA)-induced myocardial apoptosis in rats and determine its effect on the expressions of myocardial apoptosis genes.**

**MATERIALS AND METHODS: H9C2 cells were treated with different concentrations of CsA solution (0.04, 0.2, 1 and 5 μM) for 24, 48 and 72 h, respectively. The cell viability was detected via methyl thiazolyl tetrazolium (MTT) assay, and the appropriate dose and time were compared and determined. At the same time, CST in different concentrations (0.08, 0.04, 0.2, 1, 5 and 25 μM) was added into cell culture, and the appropriate dose was identified using MTT assay. The cellular morphology in each group was observed, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed for the detection of cell apoptosis. Moreover, in molecular mechanism research, the apoptosis-associated factors, factor associated suicide (Fas), Fas ligand (FasL) and B-cell lymphoma-2-associated X protein (Bax), were detected via quantitive Real-time polymerase chain reaction (qPCR). Finally, the levels of a protein related to myocardial apoptosis in rats were investigated via Western blotting.**

**RESULTS: The treatment with 1 μM CsA for 48 h caused significant apoptosis. The results of TUNEL staining showed the inhibitory role of CST on the myocardial apoptosis in rats induced by CsA. The detection of apoptosis factors via Real-time PCR revealed that after the induction of CsA, the expressions of Fas, FasL and Bax mRNA in cells were significantly higher than those in control group, but were significantly decreased after administration of CST. Western blotting showed that the protein expressions of Caspase 3 and Caspase 9 were remarkably elevated in cells after the use of CsA, but were significantly reduced after administration of CST ($p < 0.01$).**

**CONCLUSIONS: CST contributes to antagonistic function against the CsA-induced apoptosis of rat myocardial cells, and its effect is related to the down-regulation of expressions of apoptotic factors, Fas, FasL, Bax, Caspase 3, and Caspase 9.**

*Key Words:*
Cortistatin, Cyclosporine, Myocardial cells, Apoptosis.

## Introduction

Cyclosporine A (CsA) is a group of cyclic peptides produced by fungi, which is often used for the therapy of autoimmune diseases and organ transplantation. However, its long-term application can give rise to a wide range of biochemical effects on the body, especially severe cytotoxic effect, which mainly includes cardiac toxicity, hepatic and renal toxicity, bone marrow suppression, gastrointestinal reaction and other toxic and side effects[1,2]. The toxic effect of CsA towards the heart is more serious than other toxic and/or side effects, which is primarily due to the accumulative residue in the body. It has been demonstrated that the risk of cardiac toxicity relied on the accumulation of CsA[3]. Cortistatin (CST) shares a similar structure to somatostatin and is widely distributed in the central nervous system, peripheral endocrine organs and immune system. The emerging evidence indicated that CST exerted an anti-apoptosis effect[4,5]. However, the antagonism of CST against the CsA-induced myocardial apoptosis has not been reported yet. In this study, we establish an acute cardiotoxicity model via the induction of CsA,

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and aim to investigate the protective effect of CST on cardiotoxicity as well as the related mechanism.

## Materials and Methods

### Cell Lines

Rat H9C2 cell lines were purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

### Main Reagents

Bicinchoninic acid (BCA) protein quantification kit (Beyotime, Shanghai, China); TRIzol total RNA extraction kit (Tiangen, Beijing, China); Real-time PCR reverse transcription kit (Tiangen, Beijing, China); TUNEL kit (Roche, Basel, Switzerland) anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Caspase 3 and Caspase 9 monoclonal antibodies, secondary antibodies and fluorescent secondary antibodies (Abcam, Cambridge, MA, USA), Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) (Gibco, Waltham, MA, USA), 100 U/ml penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

## Experimental Methods

### Cell Culture

H9C2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) [containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin] in an incubator with 5% $CO_2$ under a constant temperature at 37°C. The culture solution was replaced every 2 d.

### Detection of Cell Viability via MTT Assay

H9C2 cells in the logarithmic growth phase were collected, centrifuged at 1200 rpm for 5 min and counted. Cells at a concentration of $1\times10^5$/mL were seeded into the 96-well plate (100 μL per well). After culturing for 24 h, cells were treated with different concentrations of CsA solution (0.04, 0.2, 1, and 5 μM) for 24, 48 and 72 h, and then added with 10 μL MTT solution at a concentration of 5 mg/mL. After 4 h, 100 μL triple solution were added, and after incubation for 12 h, the optical density (OD) was measured at 570 nm using a microplate reader. The cell survival rate was calculated.

### Detection of Cell Proliferation via MTT Assay

H9C2 cells were seeded into the 96-well plate at a density of $1\times10^5$/mL (100 μL per well). After incubation for 24 h, different concentrations of CST (0.08, 0.04, 0.2, 1, 5 and 25 μM) were added, respectively, and the cell proliferation was detected via MTT assay.

### Cell Status and TUNEL Staining

Cells were photographed and stained with TUNEL kit to detect the cell apoptosis according to the instruction of the manufacturer.

### Real-Time PCR

H9C2 cells were extracted according to the procedures of RNAiso Plus kit. The purity and content of the RNA samples extracted were calculated and stored at -80°C for standby application. The reverse transcription reaction solution was prepared according to the ratio in the instructions of PrimeScript® RT reagent Kit with guide DNA (gDNA) Eraser Kit, and the corresponding RNA samples were added for reverse transcription. Next, the mRNA level was measured according to the instructions of SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) kit. The corresponding RNA primer sequences are shown in Table I.

### Western Blotting

The treated H9C2 cell suspension was collected and centrifuged at 2500 rpm for 5 min; the supernatant was discarded and the cells were washed with phosphate-buffered saline (PBS) once. After centrifugation, an appropriate amount of immunoprecipitation (IP) lysis buffer was added with phenylmethanesulfonyl fluoride (PMSF) 3 min in advance according to the instructions of kit, blown and beaten evenly, followed by full lysis at 4°C for 30 min. During the lysis, Eppendorf (EP) tube was vibrated for several times to fully split cells. The lysis buffer was centrifuged at 12000 rpm at 4°C for 5 min, and the supernatant was collected as the total protein of samples. The protein concentration was measured using the BCA protein concentration assay kit and the protein was stored at -80°C for standby application.

The total protein extracted was mixed with 2 × loading buffer at a volume ratio of 1:1, boiled for 5 min, cooled naturally and stored at -80°C for standby application. 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was prepared according to the molecular

**Table I.** Primer sequences of relevant genes in Real-time PCR.

| Gene name | Primer sequence |
|---|---|
| Factor associated suicide (Fas) | 5'-3' ACATGGACAAGAACCATTATGCTGA<br>3'-5' CTGGTTTGCACTTGCACTTGGTA |
| Fas ligand (FasL) | 5'-3' CATGCAGCAGCCCATGAATTAC<br>3'-5' CTCTAGGCCCACAAGATGGACAG |
| B-cell lymphoma-2-associated X protein (Bax) | 5'-3' CAGGATGCGTCCACCAAGAA<br>3'-5' CGTGTCCACGTCAGCAATCA |
| β-actin | 5'-3' GAGCCGGGAAATCGTGCGT<br>3'-5' GGAAGGAAGGCTGGAAGATG |

weight of the target protein. The denatured protein samples were added into the loading wells of SDS-PAGE gel, and the total amount of protein per well was made equally according to the protein concentration. The protein on the gel was transferred onto the membrane under the constant current of 300 mA for an appropriate time. The protein-attached polyvinylidene difluoride (PVDF) membrane was sealed in 5% skimmed milk at room temperature for 2.5 h. The sealed PVDF membrane was washed with Tris-buffered saline and Tween 20 (TBST) for 5 min × 3 times, and then placed into the primary antibodies (Table II) for incubation at 4°C overnight. On the next day, the membrane was washed with TBST for 10 min × 3 times and placed into the corresponding secondary antibody for incubation on the shaking table at room temperature for 2 h. The membrane was washed again with TBST for 10 min × 3 times. Equal volume of reagent A and B in enhanced chemiluminescence (ECL) kit was mixed and dropped onto the PVDF membrane, followed by development in a dark place for 1 min. The image was photographed using the dynamic integral mode and analyzed by using the professional image analysis software, Gel-Pro analyzer.

### Statistical Analysis

The experimental data were presented as mean ± standard deviation (Mean ± SD). Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA). *t*-test was used for the comparisons of means between the two groups, while one-way analysis of variance (ANOVA) was used for the comparisons of means among groups. $p < 0.05$ suggested that the difference was statistically significant.

## Results

### Effect of CsA on the Viability of H9C2 Cells

The cell viability was significantly decreased in a dose-dependent manner after the treatment with different concentrations of CsA (0.04, 0.2, 1 and 5 μM) for 24, 48 and 72 h, compared with that in control group (Figure 1). After the treatment with 1 μM CsA for 48 h, the cell survival rate was significantly decreased, which indicated that the cell model was successfully established under this concentration. Therefore, 1 μM CsA for 48 h was selected as the modeling concentration for subsequent experiments.

### Effect of CST on the Viability of H9C2 Cells

We determined the effect of CST on the propagation of H9C2 cells. Of note, among diverse concentrations of CST (0.08, 0.04, 0.2, 1, 5 and 25 μM), 0.04, 0.2 and 1 μM CST effectively increased the viability of H9C2 cells (Figure 2). Therefore, 0.04, 0.2 and 1 μM CST were selected for subsequent experiments.

**Table II.** Antibodies and dilution ratios.

| Antibody | Source | Dilution ratio | Manufacturer |
|---|---|---|---|
| Caspase 3 | Rabbit | 1: 1000 | Abcam, Cambridge, MA, USA |
| Caspase 9 | Rabbit | 1: 1000 | Abcam, Cambridge, MA, USA |
| GAPDH | Rabbit | 1: 1000 | Proteintech Group, Chicago, IL, USA |

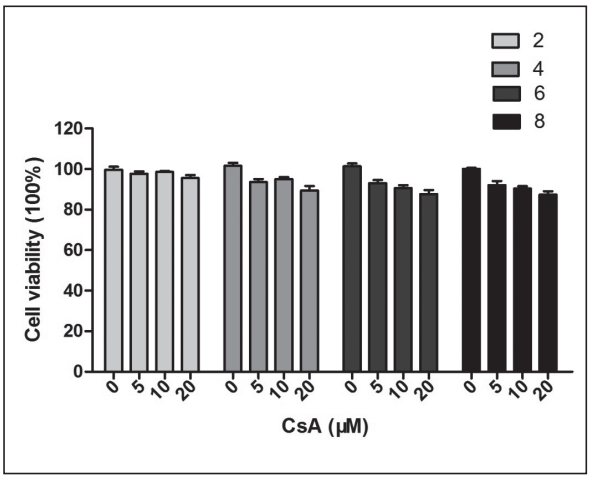


**Figure 1.** Detection of the effect of CsA on viability of H9C2 cells via MTT assay. The cell survival rate is significantly decreased after treatment with different concentrations of CsA (0.04, 0.2, 1 and 5 μM) for 24, 48 and 72 h.

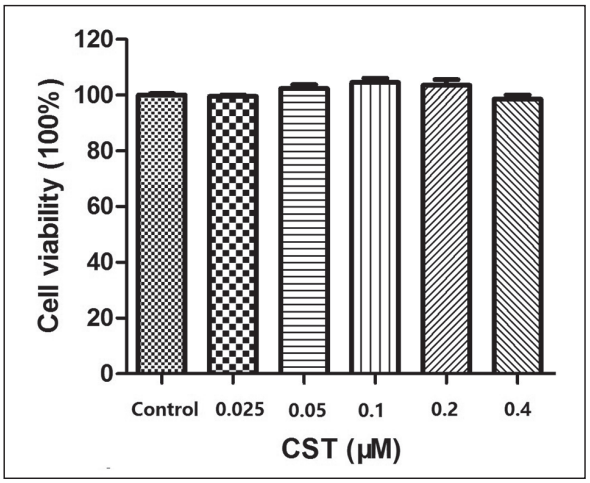


**Figure 2.** Detection of the effect of CST on viability of H9C2 cells via MTT assay. The viability of H9C2 cells is effectively increased after the treatment with 0.04, 0.2 and 1 μM CST.

### *Antagonism of CST Against CsA-Induced Apoptosis of H9C2 Cells*

After the treatment with CsA, the amount of cells was remarkably reduced, and the shape of cells was changed from elongated to oblate profile with wiredrawing (Figure 3). CST at the concentrations of 0.04, 0.2 and 1 μM could notably induce the propagation of survival cells, and to some extent alleviated cellular morphology damage caused by CsA.

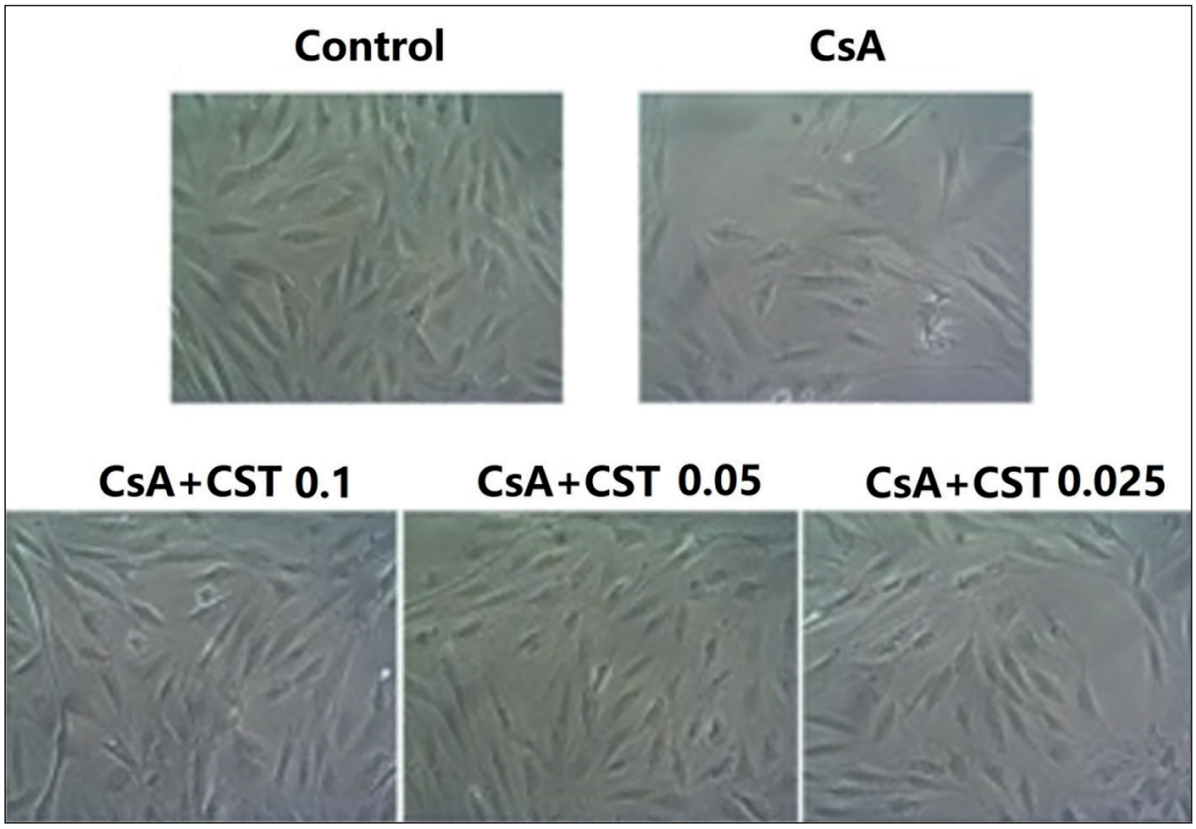


**Figure 3.** Antagonism of CST against CsA-induced apoptosis of H9C2 cells (200×).

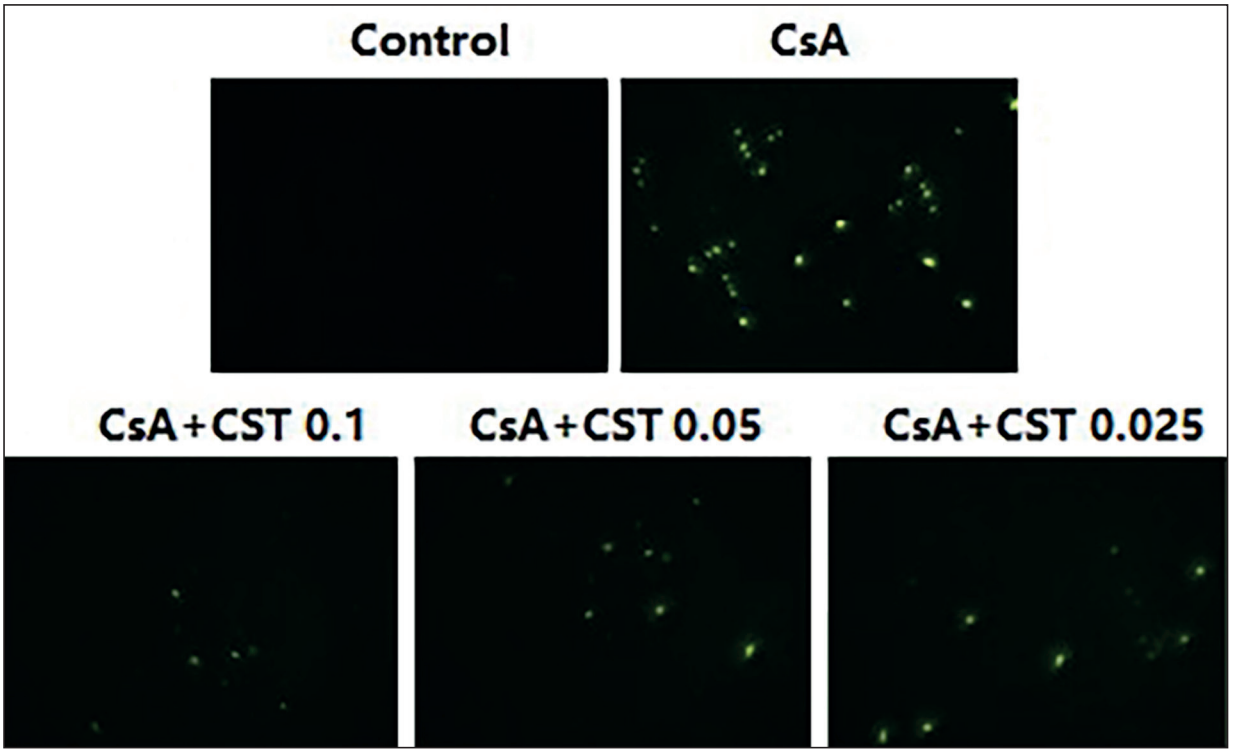


**Figure 4.** TUNEL staining results of antagonism of CST against CsA-induced apoptosis of H9C2 cells (200×).

### *TUNEL Staining Results*

There were almost no apoptotic cells in control group, but significant apoptosis occurred after the CsA induction. Notably, after the administration of CST, the cell apoptosis was appreciably inhibited in a dose-dependent manner (Figure 4).

### *Real-time PCR Results of Apoptosis-Associated Factors*

Real-time PCR was utilized to measure the expressions of Fas, FasL and Bax. Notably, our data indicated that the levels of Fas, FasL and Bax in CST administration group were significantly lower than those in model group (CsA induction group) ($p < 0.05$) (Figure 5).

### *Expressions of Caspase 3 and Caspase 9 Proteins in Each Group*

The result of Western blotting showed that the levels of Caspase 3 and Caspase 9 proteins were significantly highly expressed in the cells after the induction of CsA, compared to those in control group ($p < 0.05$). After the administration of CST, the expressions of Caspase 3 and Caspase 9 proteins were statistically decreased in a dose-dependent manner compared to that in CsA group ($p < 0.05$) (Figure 6).

## Discussion

CsA marks various effective roles and significant efficacies, but the severe cardiac toxicity limits its clinical application. Multitudes of efforts have been taken to reduce the toxicity of CsA, but the satisfactory measure has not yet been found due to its complex pathological

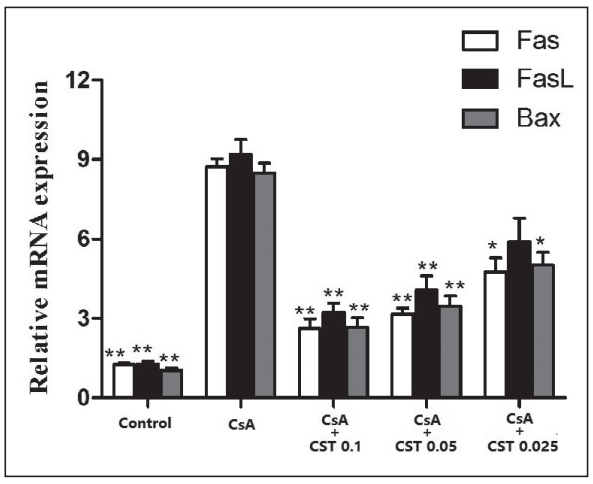


**Figure 5.** Expressions of Fas, FasL and Bax in each group. Compared with CsA1 group, *$p < 0.05$, **$p < 0.01$ (n=3).

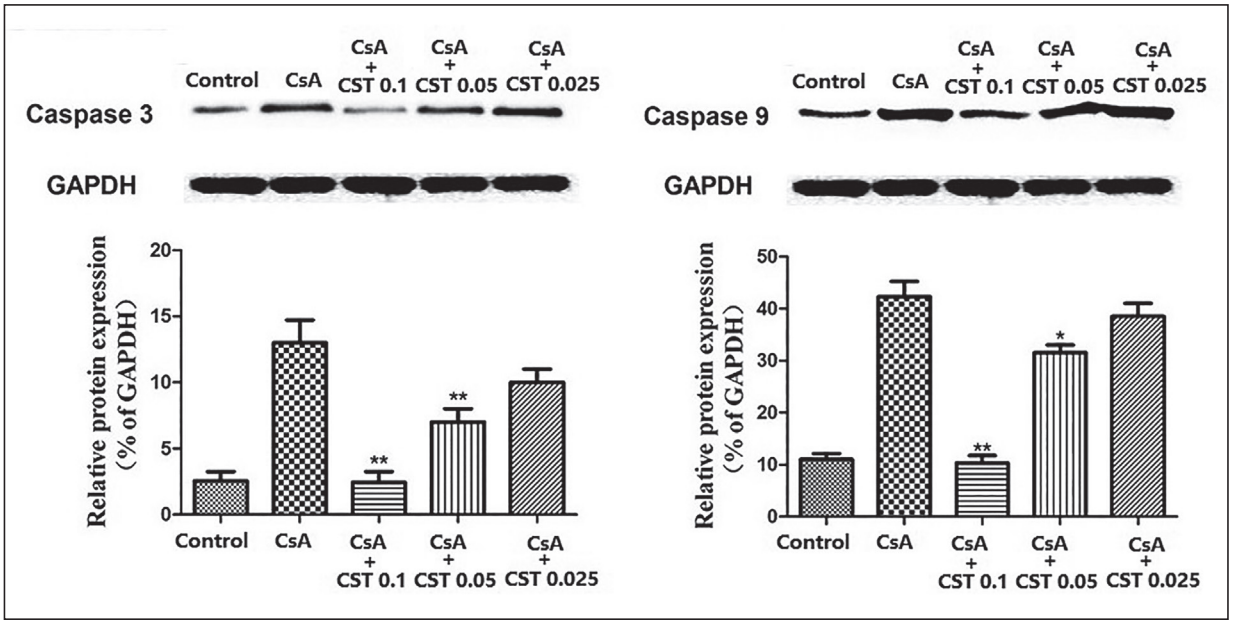


**Figure 6.** Expressions of Caspase 3 and Caspase 9 proteins in each group. Compared with CsA group, *$p < 0.05$, **$p < 0.01$ (n=3).

mechanism[6-8]. Apoptosis plays an important role in the pathological mechanism of CsA-induced cardio toxicity. Of note, accumulative evidence revealed that CST served as an anti-apoptotic factor, and performed protective effect in a variety of cardiovascular disease[9,10], which implies a possible way to alleviate cardiac toxicity caused by CsA.

Apoptosis includes a considerably complex biological process, which is associated with the activation, expression, and regulation of a range of different genes[11]. As a type of basic biological phenomenon that exists in cells, it plays a necessary role in the removal of unnecessary or abnormal cells in multicellular organisms[12,13]. On the other hand, apoptosis is strictly controlled by multiple genes, such as Bcl-2 family, Fas/FasL, Bax, Caspase family, oncogene and tumor suppressor genes[14,15]. Apoptosis is involved in the evolution of organisms, the stability of the environment and the development of multiple systems, while the disordered apoptotic process may be directly or indirectly related to the occurrence of many diseases[16], such as tumors and autoimmune diseases. Modern research[18-20] suggests that the occurrence and development of myocardial apoptosis are related to not only the cell proliferation and differentiation, but also the abnormal regulation of apoptosis, which offers novel hints to the treatment of myocardial disease.

In this study, the *in vitro* model of rat myocardial cells, H9C2 cells, was established based on the data of CsA with different concentrations (0.04, 0.2, 1, and 5 μM). The appropriate doses of CST were determined according to the result of cell viability by MTT detection. The observation of cellular morphology in each group showed that CST could resist the CsA-induced apoptosis. Moreover, the levels of apoptosis-associated factors, Fas, FasL and Bax, along with Caspase 3 and Caspase 9, were significantly higher than those in control group, but significantly decreased after administration of CST, which provides academic basis for the therapy of myocardial disease, which was in line with previous finding that myocardial cell apoptosis is related to the induction of Fas/FasL in rat model[21]. However, in-depth research is still required a pre-clinical trial of CST with the animal model as well as the safety of its clinical application needs a careful evaluation.

## Conclusions

We demonstrated that CST inhibits the apoptosis of rat myocardial cells, down regulates the expressions of apoptotic factors, Fas, FasL, Bax, Caspase 3 and Caspase 9 and alleviates the cellular morphology damage caused by CsA.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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