

# Parthenolide protects human lens epithelial cells from oxidative stress-induced apoptosis via inhibition of activation of caspase-3 and caspase-9

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The apoptosis of lens epithelial cells has been proposed as the common basis of cataract formation, with oxidative stress as the major cause. This study was performed to investigate the protective effect of the herbal constituent parthenolide against oxidative stress-induced apoptosis of human lens epithelial (HLE) cells and the possible molecular mechanisms involved. HLE cells (SRA01-04) were incubated with 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  in the absence or presence of different doses of parthenolide (10, 20 and 50  $\mu\text{M}$ ). To study apoptosis, the cells were assessed by morphologic examination and Annexin V-propidium iodide double staining flow cytometry; to investigate the underlying molecular mechanisms, the expression of caspase-3 and caspase-9 were assayed by Western blot and quantitative RT-PCR, and the activities of caspase-3 and caspase-9 were measured by a Chemicon caspase colorimetric activity assay kit. Stimulated with  $\text{H}_2\text{O}_2$  for 18 h, a high fraction of HLE cells underwent apoptosis, while in the presence of parthenolide of different concentrations, dose-dependent blocking of HLE cell apoptosis was observed. The expression of caspase-3 and caspase-9 induced by  $\text{H}_2\text{O}_2$  in HLE cells was significantly reduced by parthenolide both at the protein and mRNA levels, and the activation of caspase-3 and caspase-9 was also suppressed by parthenolide in a dose-dependent manner. In conclusion, parthenolide prevents HLE cells from oxidative stress-induced apoptosis through inhibition of the activation of caspase-3 and caspase-9, suggesting a potential protective effect against cataract formation.

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**Keywords:** parthenolide, human lens epithelial cells, apoptosis, caspase-3 and caspase-9

## Introduction

Notwithstanding the progress in surgical intervention techniques over the last few decades, cataracts remain by far the most common cause of human blindness worldwide [1]. It is well established that oxidative stress, which refers to the cellular damage caused by oxygen radicals, is the major contributor to cataractogenesis [2-4]. Oxidative stress plays a significant role in the degradation, oxidation, cross-

linking and aggregation of lens proteins, and also triggers the lens epithelial cell apoptosis, which is regarded as the common molecular basis of the initiation and subsequent progression of cataract [5-6].

Oxidative radicals derived from  $\text{H}_2\text{O}_2$  present in the anterior chamber and also from the mitochondrial metabolic processes in lens epithelial cells (LEC) may represent the major source of oxidative damage to the LEC [7]. Elevated levels of  $\text{H}_2\text{O}_2$  are reported in the aqueous humor of cataract patients [8], and both internal and external sources of oxidative stress induce LEC apoptosis and cause lens opacification *in vitro* [9-11]. Although the detail signaling pathways involved are still unclear, caspase-3 has been shown to play a key role in the lens epithelial cell apoptosis induced by oxidative stress [10, 12-14]. In addition, a previous study

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has suggested that  $\alpha$ B-crystallin, a molecular chaperon, can prevent LEC from apoptosis through inhibition of activation of caspase-3 [10]. However, until now there have been no effective clinical methods for preventing the LEC apoptosis induced by oxidative stress.

Recently, increasing attention has been paid to medicinal plants in an effort to find substances with potentially useful biological activities. Parthenolide, the major bioactive molecule of the Feverfew (*Tanacetum parthenium*), has a complicated role in the life and death of cells [15]. Many studies have proposed that parthenolide has proapoptotic activity either by preventing nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling or through an NF- $\kappa$ B-independent pathway, and thus it has been regarded as one of the anti-inflammatory and anti-tumoral agents [16-21]. However, other studies have argued that parthenolide might prevent cell apoptosis by suppressing apoptotic receptor expression [17]. In addition, parthenolide has also been reported to have the property of modulating the intracellular redox state by regulating the activities of GSH [22].

In the present study, we investigated the effect of parthenolide on apoptosis of human lens epithelial (HLE) cells induced by  $H_2O_2$  and the possible molecular mechanisms involved. The study demonstrates that parthenolide suppresses  $H_2O_2$ -induced HLE cell apoptosis via dose-dependent inhibition of the activities of caspase-3 and caspase-9, suggesting a potential effect against cataract formation that warrants further study.

## Materials and Methods

### Materials

All cell culture medium components were purchased from Invitrogen Life technologies unless otherwise noted. HLE cell line SRA 01-04 [23] was obtained from the Riken cell bank.  $H_2O_2$  was purchased from Sigma (St Louis, MO, USA) and prepared immediately before use in phosphate buffered (PBS) at 50  $\mu$ M. Parthenolide (Sigma, St Louis, MO, USA) was dissolved in dimethylsulfoxide at 100 mM. After storage at  $-30^\circ\text{C}$ , it was diluted to 10, 20 and 50  $\mu$ M respectively as the final concentration. Annexin V/FITC Kit was purchased from Bender MedSystems GmbH (Vienna, Austria). Antibodies used in the Western blot analysis were rabbit anti-active caspase-3 and caspase-9 polyclonal antibodies (Chemicon, CA, USA) recognizing only the cleaved large subunit (17 kDa of caspase-3 and 37 kDa of caspase-9) and rabbit polyclonal antibody against GAPDH (Santa Cruz, CA, USA). Caspase-3 and caspase-9 colorimetric activity assay kits and recombinant active caspase-3, and caspase-9 standards were from Chemicon.

### Cell culture and treatment

The HLE cells were seeded at a density of  $2 \times 10^6$ /dish in 100-mm dishes in Dulbecco's modified essential medium with 5% fetal bovine serum. When adherent cells became confluent, the cells were stimulated with 50  $\mu$ M  $H_2O_2$  in the presence of parthenolide for the indicated periods (0, 2, 4, 8, 12, 18 and 24 h). If parthenolide was

applied, the cells were incubated with different concentrations of parthenolide (0, 10, 20 and 50  $\mu$ M, respectively) for 2 h followed by the addition of 50  $\mu$ M  $H_2O_2$  and further incubation of indicated hours. In addition, morphologic observation of the HLE cells treated was performed.

### Flow cytometry analysis

Recovery of the cells was monitored by examining the levels of apoptosis at 18 h after the  $H_2O_2$  treatment. Annexin V binding and propidium iodine staining were determined by flow cytometry. The cells were treated with 50  $\mu$ M  $H_2O_2$  for 18 h, washed with ice-cold PBS and double stained with FITC-coupled annexin V protein and propidium iodine for 20 min. Flow cytometry was performed with a 488-nm laser coupled to a cell sorter (FacsCalibur; BD Biosciences, San Jose, CA, USA). Cells stained with both propidium iodide and annexin V were considered necrotic, and the cells stained only with annexin V were considered apoptotic.

### Western blot analysis

The expression of caspase-3 and caspase-9 was detected by Western blot. Cytoplasmic extracts were prepared by lysis in a lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.9), 0.5% Triton X-100, 0.6% NP-40, and protease inhibitors, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, and 2 mg/ml aprotinin). The protein contents were determined using the DC protein assay kit (Bio-Rad, Richmond, CA, USA). Protein was mixed with  $2\times$  SDS sample buffer. Forty micrograms of protein were separated in a 10% polyacrylamide gel and blotted on a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked for 2 h in blocking buffer (PBS with 7.5% non-fat dry milk, 2% BSA, 0.1% Tween), and incubated with primary antibodies (1:400 in blocking buffer) overnight at  $4^\circ\text{C}$ . Subsequently, the membranes were washed in washing buffer (PBS with 0.1% Tween-20) incubated with peroxidase-conjugated goat anti-rabbit IgG (Pierce, 1:10 000 in blocking buffer) for 1 h at room temperature, washed in PBS and developed using the ECL chemiluminescence detection system (Amersham). In all experiments Ponceau staining was carried out to control equal loading.

### Quantitative RT-PCR

Quantitative RT-PCR was performed by LightCycler technology (Roche Molecular Biochemicals) using SYBR Green I detection. In all assays, cDNA was amplified using a standardized program (10'' denaturing step; 55 cycles of 5'' at  $95^\circ\text{C}$ , 15'' at  $65^\circ\text{C}$  and 15'' at  $72^\circ\text{C}$ ; melting point analysis in  $0.1^\circ\text{C}$  steps; final cooling step). Each LightCycler capillary was loaded with 1.5  $\mu$ l DNA Master Mix, 1.8  $\mu$ l  $\text{MgCl}_2$  (25 mM), 10.1  $\mu$ l  $H_2O$  and 0.4  $\mu$ l of each primer (10  $\mu$ M). The final amount of cDNA per reaction corresponded to the 2.0 ng of RNA used for reverse transcription. Relative quantification of target gene expression was performed using a mathematical model recommended by Roche Molecular Biochemicals. The following primers were used for the experiment: CASPASE-3\_300f (5'-atggaagcgaatcaatggac-3'), CASPASE-3\_541r (5'-gagcgacggagagagactgt); CASPASE-9\_369f (5'-aacaggcaagcagcaaatgt-3'), CASPASE-9\_615r (5'-cacggcagaagtcacattg-3'). The primers used for the housekeeping gene, which was used for normalization, were as follows: GAPDH\_f (5'-accacgtccatgccatcac-3'), GAPDH\_r (5'-tccaccaccctgtgtctgta-3').

### Measurement of caspase-3 and caspase-9 activities

Cells treated were resuspended in 500  $\mu$ l of cell lysis buffer

and incubated on ice for 10 min. After centrifugation for 5 min at  $10\,000 \times g$ , supernatant was transferred to a fresh tube. Caspase-3 and caspase-9 activities in cell lysate treated were determined by a Chemicon caspase colorimetric activity assay kit. The assay is based on spectrophotometric detection of the chromophore *p*-nitroaniline (pNA) after cleavage from the labeled substrate LEHD-pNA. The free pNA can be quantified using a microtiter plate reader at 405 nm. For quantitative purposes, recombinant active caspase-3 and caspase-9 were used as the known standards of reference.

#### Statistical analysis

All the experiments were performed at least three times. The results are expressed as the mean  $\pm$  SD. Statistical significance was analyzed by one-way analysis of variance.  $p < 0.05$  was considered statistically significant.

## Results

### *Parthenolide inhibited morphologic changes of HLE cells induced by H<sub>2</sub>O<sub>2</sub>*

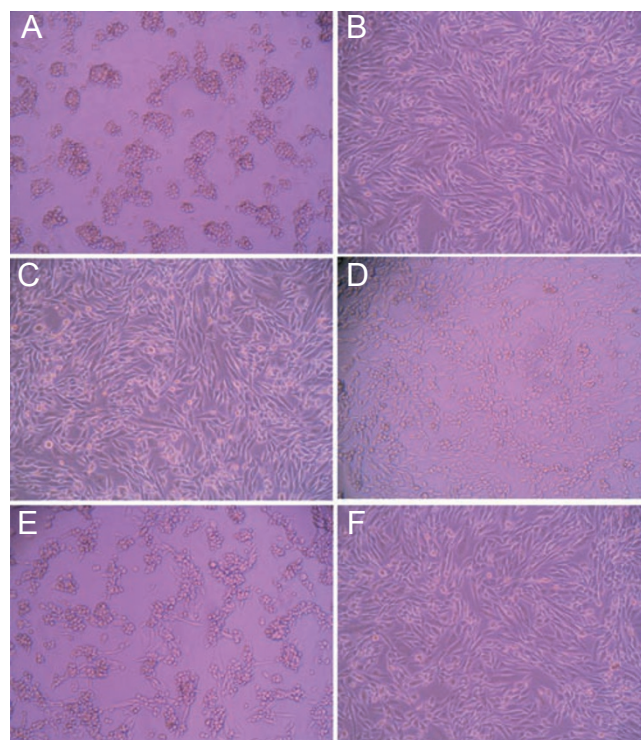
In the present study, morphologic observation of HLE cells treated with H<sub>2</sub>O<sub>2</sub> (50  $\mu$ M) in the presence or absence of different concentrations (10, 20 and 50  $\mu$ M) of parthenolide or only with 50  $\mu$ M parthenolide was performed to study the effect of this herbal extract on HLE cells. After H<sub>2</sub>O<sub>2</sub> treatment, a high fraction of cells exhibited apoptosis-like morphologic changes, such as detachment, and cytoplasmic-condensation leading to rounding. However, the proportion of cells with abnormal morphology suggestive of apoptosis decreased with the increasing parthenolide doses, indicating a dose-dependent prevention effect by parthenolide (Figure 1).

### *Parthenolide blocked HLE cells apoptosis induced by H<sub>2</sub>O<sub>2</sub>*

To obtain a definitive quantification of the effect of different concentrations of parthenolide on H<sub>2</sub>O<sub>2</sub>-induced HLE cells apoptosis, the percentage of apoptotic cells was detected by Annexin V-FITC and PI double staining methods (Figure 2). A significant increase of apoptosis was observed in HLE cells treated with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> compared with the control ( $44.32 \pm 3.10\%$  vs  $2.90 \pm 0.55\%$ ,  $p < 0.01$ ). The percentages of apoptotic cells treated together with H<sub>2</sub>O<sub>2</sub> and parthenolide of 10, 20 and 50  $\mu$ M were  $33.54 \pm 0.43\%$ ,  $12.41 \pm 2.05\%$  and  $5.71 \pm 2.64\%$ , respectively. Parthenolide significantly reduced the percentage of apoptotic cells compared with the H<sub>2</sub>O<sub>2</sub> treated cells in a dose-dependent manner ( $p < 0.01$  at all three concentrations).

### *Parthenolide reduced caspase-3 and caspase-9 expression induced by H<sub>2</sub>O<sub>2</sub>*

Once we confirmed that parthenolide can protect HLE cells from H<sub>2</sub>O<sub>2</sub>-induced apoptosis, we attempted to investigate the potential molecular mechanisms involved.

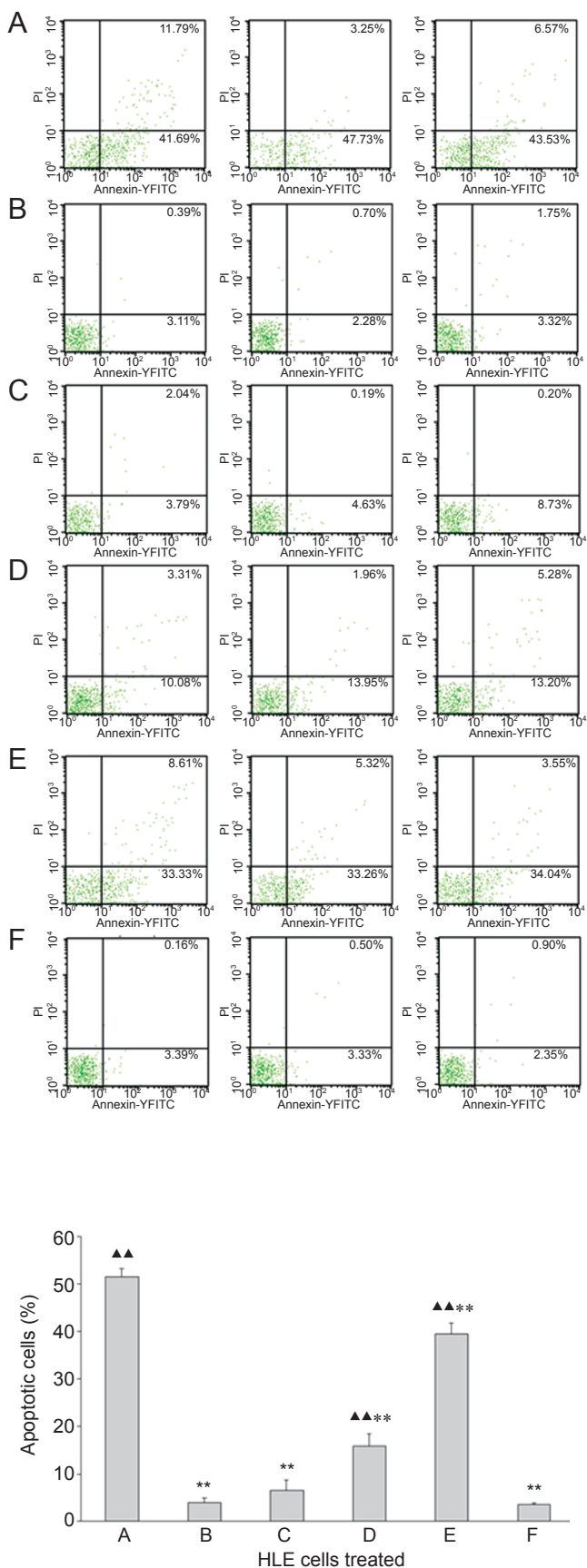


**Figure 1** Parthenolide inhibited morphological changes of HLE cells induced by H<sub>2</sub>O<sub>2</sub>. When treated by H<sub>2</sub>O<sub>2</sub> (50  $\mu$ M) for 18 h, a large fraction of cells demonstrated apoptosis signs such as detachment and cytoplasmic condensation leading to rounding. However, the proportion of cells with abnormal morphology decreased in parallel with the concentrations of parthenolide. (A) HLE cells with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> treatment; (B) HLE cells without H<sub>2</sub>O<sub>2</sub> or parthenolide treatment (Normal); (C-E) HLE cells with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> treatment and 50, 20 or 10  $\mu$ M parthenolide treatment, respectively; (F) HLE cells with only 50  $\mu$ M parthenolide treatment (Control). Magnification:  $10\times$

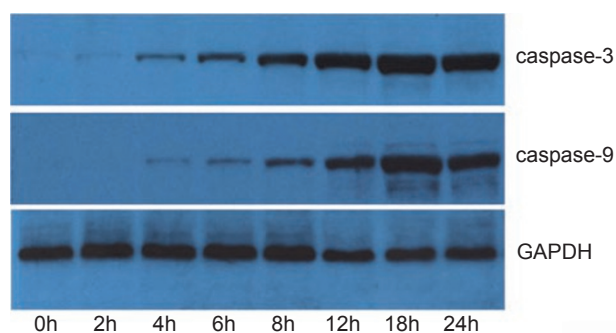
We examined the expression of caspase-3 and caspase-9 at the protein and transcriptional levels. We measured the dynamic expression of the cleaved form of caspase-3 (17 kDa) and caspase-9 (37 kDa) by Western blot at 0, 2, 4, 6, 8, 12, 18 and 24 h after treatment with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> and observed that the induction of activated caspase-3 and caspase-9 proteins increased in a time-dependent manner and both reached peak levels at 18 h after treatment (Figure 3). Parthenolide at concentrations above 20  $\mu$ M significantly inhibited the protein expression of caspase-3 and caspase-9 in HLE cells after 18 h H<sub>2</sub>O<sub>2</sub> treatment, as shown in Figure 4.

As expected, the mRNA expression of caspase-3 and caspase-9 in H<sub>2</sub>O<sub>2</sub>-induced HLE cells paralleled the protein expression with different treatments. Parthenolide significantly blocked the mRNA expression of caspase-3

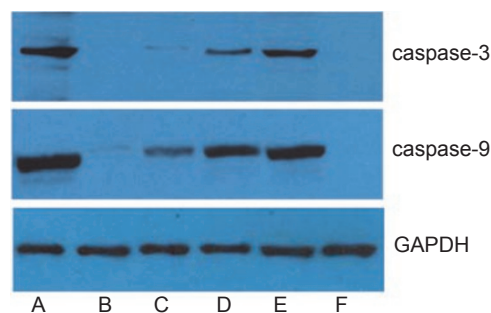




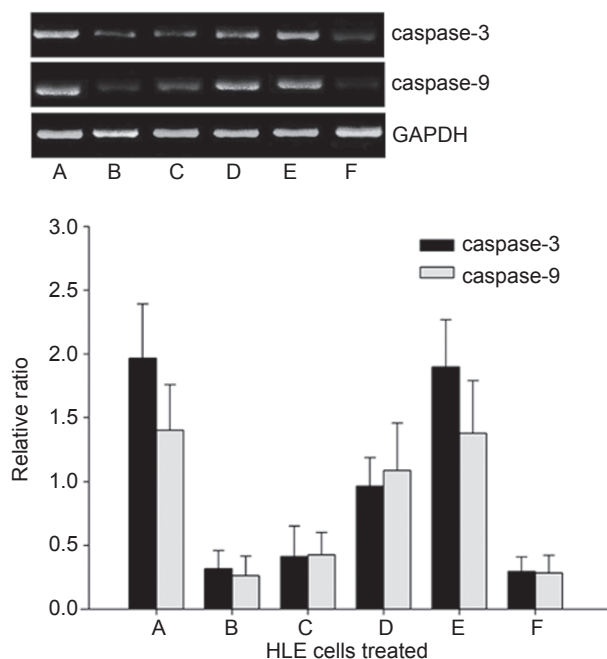
**Figure 2** Parthenolide blocked apoptosis of HLE cells induced by H<sub>2</sub>O<sub>2</sub>. The apoptotic cells were detected by Annexin V and propidium iodide double staining methods. H<sub>2</sub>O<sub>2</sub> treatment significantly increased HLE cells apoptosis, but, in the presence of different doses of parthenolide, the induction of apoptosis was significantly blocked in a dose-dependent manner. Top: (A) HLE cells with 50 μM H<sub>2</sub>O<sub>2</sub> treatment; (B) HLE cells without H<sub>2</sub>O<sub>2</sub> or parthenolide treatment (Normal); (C-E) HLE cells with 50 μM H<sub>2</sub>O<sub>2</sub> treatment and 50, 20 or 10 μM parthenolide treatment, respectively; (F) HLE cells with only 50 μM parthenolide treatment (Control); bottom: ▲▲*p*<0.01 compared with normal control; \*\**p*<0.01 compared with H<sub>2</sub>O<sub>2</sub> treatment alone.



**Figure 3** H<sub>2</sub>O<sub>2</sub> increased the expression of caspase-3 and caspase-9 in a time-dependent manner. We measured the dynamic expression of caspase-3 and caspase-9 by Western blot analysis at 0, 2, 4, 6, 8, 12, 18 and 24 h after treatment with 50 μM H<sub>2</sub>O<sub>2</sub> and observed that the induction of the cleaved form of caspase-3 (17 kDa) and caspase-9 (37 kDa) proteins increased in a time-dependent manner and reached peak levels at 18 h after treatment.



**Figure 4** Parthenolide inhibited the expression of caspase-3 and caspase-9 at the protein level in H<sub>2</sub>O<sub>2</sub>-induced HLE cells. The expression of caspase-3 and caspase-9 in treated HLE cells was detected by Western blot analysis. Parthenolide significantly decreased the protein expression of caspase-3 and -9 in H<sub>2</sub>O<sub>2</sub> (50 μM)-treated HLE cells, and the inhibition effect was positively correlated with the concentrations of parthenolide. (A) HLE cells with 50 μM H<sub>2</sub>O<sub>2</sub> treatment; (B) HLE cells without H<sub>2</sub>O<sub>2</sub> or parthenolide treatment (Normal); (C-E) HLE cells with 50 μM H<sub>2</sub>O<sub>2</sub> treatment and 50, 20 or 10 μM parthenolide treatment, respectively; (F) HLE cells with only 50 μM parthenolide treatment (Control).

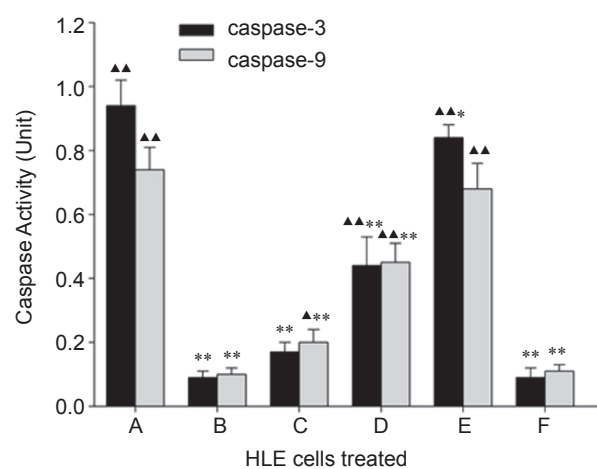


**Figure 5** Parthenolide reduced the expression of caspase-3 and caspase-9 at the transcription level in H<sub>2</sub>O<sub>2</sub> induced HLE cells. The expression of caspase-3 and caspase-9 mRNA in treated HLE cells was detected by quantitative RT-PCR. Parthenolide inhibited significantly the mRNA expression of caspase-3 and caspase-9 at a concentration above 20 μM in H<sub>2</sub>O<sub>2</sub> (50 μM) treated HLE cells. Top: PCR products were separated on 1.5% agarose gel with ethidium bromide in TBE-buffer; bottom: the expressions of caspase-3 and caspase-9 mRNA were detected by LightCycler technology using SYBR Green I. (A) HLE cells with 50 μM H<sub>2</sub>O<sub>2</sub> treatment; (B) HLE cells without H<sub>2</sub>O<sub>2</sub> or parthenolide treatment (Normal); (C-E) HLE cells with 50 μM H<sub>2</sub>O<sub>2</sub> treatment and 50, 20 or 10 μM parthenolide treatment, respectively; (F) HLE cells with only 50 μM parthenolide treatment (Control).

and caspase-9 in H<sub>2</sub>O<sub>2</sub>-treated HLE cells, and the inhibition effect was positively correlated with the concentrations of parthenolide. In addition, comparing with caspase-9, the caspase-3 mRNA was more profoundly suppressed at a low concentration of parthenolide (20 μM) relative to that in HLE cells treated with H<sub>2</sub>O<sub>2</sub> only (Figure 5).

*Parthenolide suppressed the activation of caspase-3 and caspase-9 induced by H<sub>2</sub>O<sub>2</sub>*

We used a Chemicon caspase colorimetric activity assay kit to quantify the effect of parthenolide of different concentrations on H<sub>2</sub>O<sub>2</sub>-induced activation of caspase-3 and caspase-9 in HLE cells (Figure 6). A significant increase of caspase-3 and caspase-9 activities was detected in HLE cells treated with 50 μM H<sub>2</sub>O<sub>2</sub> compared with the



**Figure 6** Parthenolide suppressed the activation of caspase-3 and caspase-9 in H<sub>2</sub>O<sub>2</sub> induced HLE cells. Caspase-3 and caspase-9 activities in treated cells were measured by a Chemicon caspase colorimetric activity assay kit. H<sub>2</sub>O<sub>2</sub> treatment significantly increased HLE cell apoptosis, but in the presence of different doses of parthenolide, the activation of caspase-3 and caspase-9 were significantly suppressed in a dose-dependent manner. (A) HLE cells with 50 μM H<sub>2</sub>O<sub>2</sub> treatment; (B) HLE cells without H<sub>2</sub>O<sub>2</sub> or parthenolide treatment (Normal control); (C-E) HLE cells with 50 μM H<sub>2</sub>O<sub>2</sub> treatment and 50, 20 or 10 μM parthenolide treatment, respectively; (F) HLE cells with only 50 μM parthenolide treatment. ▲*p*<0.05 compared with normal control; ▲▲*p*<0.01 compared with normal control; \**p*<0.05 compared with H<sub>2</sub>O<sub>2</sub> treatment alone; \*\**p*<0.01 compared with H<sub>2</sub>O<sub>2</sub> treatment alone.

control (0.94±0.08 vs 0.09±0.02, 0.74±0.07 vs 0.10±0.02; *p*<0.01 in both cases). The activities of caspase 3 in HLE cells treated together with H<sub>2</sub>O<sub>2</sub> and parthenolide of 10, 20 and 50 μM were 0.84±0.04, 0.44±0.09 and 0.17±0.03 respectively, and the activities of caspase 9 were 0.68±0.08, 0.45±0.06 and 0.20±0.04, respectively. Parthenolide significantly suppressed the activation of caspase-3 and caspase-9 in the H<sub>2</sub>O<sub>2</sub>-treated HLE cells in a dose-dependent manner.

**Discussion**

It is generally accepted that apoptosis of lens epithelial cells is associated with the development of cataracts, and oxidative stress is regarded as a major cause of lens opacity. This study was undertaken to examine the effect of parthenolide against H<sub>2</sub>O<sub>2</sub>-induced apoptosis of HLE cells and the possible molecular mechanisms involved. The present study demonstrates that parthenolide could suppress H<sub>2</sub>O<sub>2</sub>-induced HLE cells apoptosis in a dose-de-

pendent manner *in vitro*. The results show that after H<sub>2</sub>O<sub>2</sub> treatment, a high fraction of HLE cells exhibit apoptotic-like signs. However, in the presence of parthenolide, the proportions of apoptotic cells were significantly decreased. It has been shown that parthenolide can sensitize tumor cells to apoptosis in several previous studies [18, 21]. More recently, however, studies have shown that, at low doses, parthenolide functions as an antioxidant that can reduce oxidative stress-induced apoptosis via suppressing the activation of CD95 ligand expression in T cells [17]. In contrast, at high doses, parthenolide by itself induces oxidative stress-induced apoptosis [15]. To know if the observed effects of parthenolide on apoptosis are reproducible in HLE cells, we performed a dose-response experiment with concentrations of 10, 20 and 50 μM. Interestingly, all the concentrations of parthenolide exert a protective effect against oxidative stress, and the inhibitory effect against apoptosis, as measured by cell morphology study and flow cytometry, was dose-dependent. The present study suggests different mechanisms of action of parthenolide on cell apoptosis regulation in different cell lines.

After observing that the parthenolide actions in SRA01-04 HLE cells were different from those reported in other cell lines, we studied the potential pathway involved. As is well known, apoptotic cell death can be induced through either the death receptor or the mitochondria-mediated signaling pathways [24]. Many stimuli that cause oxidative stress are sufficient to induce apoptosis through the mitochondrial pathway. Previous studies have demonstrated that caspase-3 plays a key role in the HLE cell apoptosis induced by oxidative stress [10, 12-14], suggesting a mitochondria-mediated signaling pathway is involved. In addition, *in vitro* studies have identified caspase-9, Apaf1 and cytochrome *c* as participants in a complex important for caspase-3 activation [25], and there is reduced apoptosis in caspase-9 deficient mice [26]. Therefore, to investigate the roles of the effector caspase-3 and the initiator caspase-9 in the HLE cell apoptosis induced by oxidative stress, and the molecular mechanism by which parthenolide protects HLE cells from apoptosis, we examined the expression of caspase-3 and caspase-9 using the methods of Western blot analysis and quantitative RT-PCR. Stimulation of HLE cells by H<sub>2</sub>O<sub>2</sub> resulted in a significant increase in caspase-3 expression both at the transcription and protein levels, but in the presence of parthenolide of different concentrations (10, 20 and 50 μM), the H<sub>2</sub>O<sub>2</sub>-induced caspase-3 levels were significantly reduced in a dose-dependent manner. Interestingly, similar results were observed when caspase-9 was examined (Figures 4 and 5). In addition, as measured by a Chemicon caspase colorimetric activity assay kit, the activation of caspase-3 and caspase-9 was also suppressed. Thus, our findings point to the existence of a pathway

dependent on both caspase-9 and caspase-3 during the apoptosis of HEL cells stimulated by oxidative stress. Meanwhile, parthenolide suppresses the apoptosis of HLE cells, at least in part, through inhibition of the expression of caspase-3 and caspase-9, both at the transcription and protein levels.

The present study suggests that the HLE cells stimulated by H<sub>2</sub>O<sub>2</sub> undergo mitochondria-mediated apoptosis dependent on both caspase-3 and caspase-9. Furthermore, the herbal constituent, parthenolide, can protect HLE cells from apoptosis by inhibiting the activation of caspase-3 and caspase-9. Since the association of HLE cells apoptosis and cataract formation has been well documented, our study suggests parthenolide has potential in cataract prevention.

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