—Regular Articles—

The Effect of δ-Elemene on Hela Cell Lines by Apoptosis Induction

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This study was designed to investigate the apoptosis-inducing activity of δ-elemene on Hela cells *in vitro*. MTT assay and Hoechst 33258/PI fluorescence microscopy were used for this investigation. Apoptosis was further confirmed and quantified by DNA fragmentation ELISA, Annexin V (AnV) binding of externalized phosphatidylserine and the mitochondrial probe JC-1 using flow cytometry. Generation of reactive oxygen species (ROS) was detected using CM-H2DCFDA. Western blots analysis was performed using antibodies against the pro-caspase-3, or PRAP (Poly (ADP-ribose) polymerase).The results showed that δ-elemene exhibited a marked antiproliferative effect on Hela cells in dose- and time-dependent manners, and had little inhibition to normal human liver cell line WRL-68. It was demonstrated that δ-elemene was capable of inducing DNA fragmentation in a dose- and time-dependent manner. AnV positivity and the disturbance of the polarized mitochondrial transmembrane potential ($\Delta\psi_m$) suggested that δ-elemene induced apoptotic death of Hela cells. Western blot analysis demonstrated that δ-elemene activated the caspase-signaling pathway, leading to the proteolysis conversion of pro-caspase-3 to activate caspase-3, and the subsequent cleavage of the caspase substrate PARP. Further, it was noted that the apoptotic effect of δ-elemene could be attenuated by L-Glutathione (GSH) or z-DEVD-fmk. It suggested that the increase in ROS generation might be involved in the mechanism of δ-elemene induced cell apoptosis.

Key words——δ-elemene; apoptosis; mitochondrial trans-membrane potential; ROS; caspase-3; PRAP

INTRODUCTION

Elemene is a naturally occurring compound that can be isolated from the traditional Chinese medicinal herb *Curcuma* Wenyujin, which was used to treat tumors in Chinese folk medicine.[1,2] Elemene exists as an essential oil mixture of β-, γ- and δ-elemene (Fig. 1). The major antitumor active component, β-elemene, possesses the broad-spectrum clinical activity in treatment of various tumors and is known to induce apoptosis.[3] δ-elemene is another isomeric compound of β-elemene with a different site of double bond. Our previous study was shown that δ-elemene possess no signs of bone marrow suppression and normal liver cell lines WRL-68.[4]

In this communication, the antitumor activity and the potential mechanisms of δ-elemene were investigated. There is a growing consensus that oxidative stress and the redox state of a cell play a pivotal role in regulating apoptosis.[5,6] Oxygen free radical formation's is associated with the generation and development of certain tumors. Elemene has an inhibitory effect on oxygen free radical formation in tumor cells.[7] Mitochondria are the richest source of reactive oxygen species (ROS) in the cell, converting 1—2% of reduced oxygen into superoxide.[8] Inhibition of the mitochondrial electron transport chain, resulting in subsequent release of ROS, is an early event in many forms of apoptosis.[9,10] Oxidative stress induces a number of downstream events in apoptosis, including aspartate-specific cysteine protease (caspase) activation and DNA fragmentation. The caspase cascade appears to be the main pathway by which cellular death is orchestrated. Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme selectively activated by DNA strand damage to participate in DNA repair.[11]

In the present study, we examined 1) the effect of δ-elemene induced Hela cell line death by apoptosis, 2) the effect of δ-elemene on activation of the caspase signaling pathway and PARP, 3) the role of ROS generation on δ-elemene induced cell apoptosis in Human cervical carcinoma cell lines.

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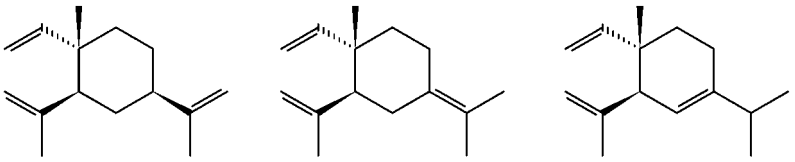


β- elemene γ-elemene δ-elemene

MW: 204.35

Fig. 1. Chemical Structure of β-Elemene, γ-Elemene and δ-Elemene

MATERIALS AND METHODS

Cell Lines Human cervical carcinoma cell line Hela and normal human liver embryonic WRL-68 line were purchased from American Type Culture Collection (ATCC, #CRL1872, #CL48, MD, USA). Hela cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; GIBCO, Invitrogen), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO, Grand Island, NY, USA). WRL-68 cells were cultured in α-Minimum Essential Medium (GIBCO BRL, Rockville, MD, USA) with 10% FBS. Cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

Reagent and Cell Culture δ-elemene and β-elemene were isolated from the essential oil of *Curcuma* Wenyujin Chen Y. H. et C. Ling using the method reported in the Ref.[1] (Assay>97%, GC).

Determination of Anti-proliferation Activity Using MTT on Hela Cell Lines Survival of cells was assessed using MTT (thiazolyl blue, Sigma, MO, USA) as previously described.[12] Briefly, Hela cells were cultured to 80% confluence and then exposed to the indicated concentrations of δ-elemene or β-elemene at 200 μM for 12, 24, 48 h. Twenty microliters of 5 mg/ml stock solution of MTT were added to each well, and incubated at 37°C for 4 h. Afterwards, 150 μl of DMSO was added to each well. The absorbance was measured by using a 96-well micro-titer plate reader at 570 nm. The percentage of cell growth inhibition was calculated as follows:

$$\text{Inhibition}(\%) = [A570(\text{control}) - A570(\text{drug})] / A570(\text{control}) \times 100$$

Nuclear Damage Observed by Hoechst 33258 Staining Apoptotic nuclear morphology was assessed using Hoechst 33258 (Sigma, USA) as previously described.[13] Hela cells after trypsin digestion (0.25%, GIBCO, Invitrogen), containing adherent and floating, were collected by centrifugation at 1000 g for 5 min, washed two times with PBS. The cells were fixed with 3.7% paraformaldehyde at room temperature for 2 h, then washed and stained with Hoechst 33258 167 μM at 37°C for 30 min. At the end of incubation, the cells were washed and re-suspended in PBS for observation of nuclear morphology using fluorescence microscopy (Nikon, Osaka, Japan).

Flow Cytometric Detection of Annexin-V (AnV) Apoptosis was measured using flow cytometry to quantify the levels of detectable phosphatidylserine on the outer membrane of apoptotic cells.[14] Briefly, Hela cells were plated at 5×10^4/ml in RPMI1640. Flasks were incubated with 200 μM δ-elemene. GSH was added 1 h prior to the treatment with δ-elemene. Cells were harvested, suspended at 1×10^6/ml, and washed two times with ice-cold PBS after 12, 24, 48 h. The cells were re-suspended in 300 μl of dilute binding buffer from the Annexin-V FITC kit (Alexis, Lausen, Switzerland). Then 5 μl propidium iodide (PI) and 5 μl of Annexin-V-FITC were added. The tubes were gently mixed and kept in the dark on ice for 10 min before analysis by flow cytometry (FACScan, Beckin Dickinson, SanJose, CA, USA). Data were analyzed using LYSIS II software. $(AnV^+)PI^-$ cells were considered early apoptotic, and $(AnV^+)PI^+$ cells were considered late apoptotic and necrotic.

Assessment of Cell DNA Fragmentation DNA fragmentation was determined by a DNA fragmentation ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany). Hela cells were cultured to 80% confluence and then exposed to different concentrations of δ-elemene for the periods of time indicated. GSH and z-DEVD-fmk were added 1 or 2 h prior to the treatment with δ-elemene.

Measurement of Generation of Reactive Oxygen Species Hydroperoxide and superoxide production was determined using 2′,7′-dichlorofluorescein

and dihydroethidium (CM-H2DCFDA; Molecular Probes Inc., Eugene, OR, USA) as described by Curtin et al.,[15] with some modifications. Briefly, after the treatment of cells with 200 μM of δ-elemene for 6, 12 and 24 h, Hela cells were collected by centrifugation and re-suspended in 200 μl of RPMI medium, after which Hela cells were loaded with CM-H2DCFDA (10 μM) for 45 min at 37℃ and then washed with PBS to remove the CM-H2DCFDA. The levels of ROS were measured by flow cytometry by determining the fluorescence intensity relative to that of the control group. GSH was added 1 h prior to the treatment with δ-elemene for 3 h.

Mitochondrial Transmembrane Potential Measurement The mitochondrial transmembrane potential ($\Delta\psi_m$) was evaluated by JC-1 assay. Treatment of cells with 200 μM of δ-elemene for 6, 12 and 24 h, Hela cells were re-suspended in 200 μl of RPMI medium, after which JC-1 (300 nM, Molecular Probes) was added for a 15 min incubation at 37℃ in the dark. The cells were then harvested by trypsin (0.25 %) and washed once with PBS. Following re-suspension in PBS, the cells were subjected to flow cytometry analysis. GSH was added 1 h prior to the treatment with δ-elemene for 3 h.

Preparation of Protein Cell Lysate After treatment with 200 μM δ-elemene for 6, 12, 24 h, Hela cells were lysed for 1 h on ice in lysis buffer [50 mM HEPES (pH 7.4), 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF)], supplemented with proteinase inhibitors: 100 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg /ml pepstatin A. Protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). The lysate was centrifuged at 16000 g at 4℃ for 10 min. The protein concentration of the resulting supernatant was measured and stored at −70℃. GSH and z-DEVD-fmk were added 1 or 2 h prior to the treatment with δ-elemene for 24 h.

Western Blotting Analysis of Caspase-3 and PARP Western blot analysis was performed as previously described[16] with some modification. Briefly, equal loading (30 μg) of protein lysates were mixed in 2× loading buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue], heated at 100℃ for 5 min, and then loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels, and the separated proteins were transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with Tween20-Tris-buffer saline [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.02% Tween 20] containing 5% nonfat milk with the primary antibodies at 1 : 500 dilution in blotting buffer overnight at 4℃ with gentle rotation. Primary incubation of the membranes was carried out using 1 : 500 dilu-

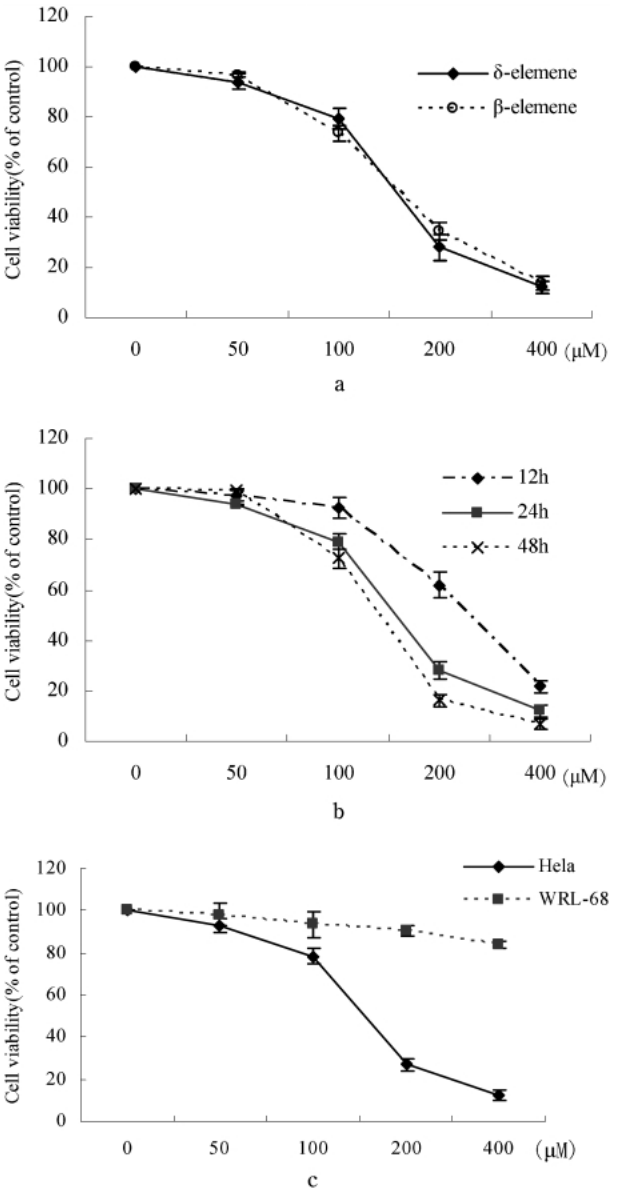


Fig. 2. Dose- and Time-dependent Inhibition of Proliferation of Hela Cells by δ-Elemene Treatment

(a) Hela cells were treated with various doses of δ-elemene compared with β-elemene for 24 h. (b) The cells were treated with 200 μM δ-elemene for various time periods. (c) The effect of δ-elemene in Hela cells and WRL-68 cells for 24 h. The results shown are representative of 3 independent experiments.

tions of a goat polyclonal caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal PARP antibody (Promega, Madison, WI), and a 1 : 1000 dilution of a goat polyclonal β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as an inner control protein. After washed 6 times for 10 min each in Tris-buffered saline (TBS), the membrane was incubated with a diluted horseradish peroxidase-labeled secondary antibody (1 : 500) in blotting buffer at room temperature for 2 h with gentle rotation. After incubation, the membrane was washed with TBS-Tween 20 at ambient temperature for 10 min six times, and rinsed once with TBS for 5 min. The membranes were visualized with an enhanced chemiluminescence system (Amersham Co., UK), according to the manufacturer's directions (Bio-Rad Laboratories, Hercules, CA). The densities of the protein bands corresponding to the appropriate size were determined and the relative amount of the target protein was expressed as the Expression index, which was calculated by the formula: the density of the target band/density of the control (β-actin) band

Statistical Analysis All results were obtained in at least three independent experiments. Data were expressed as means$\pm$S.D. Data were analyzed for statistical significance by the paired t-test. The p value of <0.05 was considered statistically significant.

RESULTS

Inhibition of Growth of Hela Cells by MTT Assay When Hela cells in culture were treated with various concentration of δ-elemene (0—400 μM) for 24 h (Fig. 2(a)) or with 200 μM δ-elemene at indicated periods (Fig. 2(b)), the growth of Hela cells was significantly inhibited in time and dose-dependent manners. The inhibitory was almost similar to that of β-elemene, and there was no significant difference between β-elemene and δ-elemene treatment (Fig 2(a),

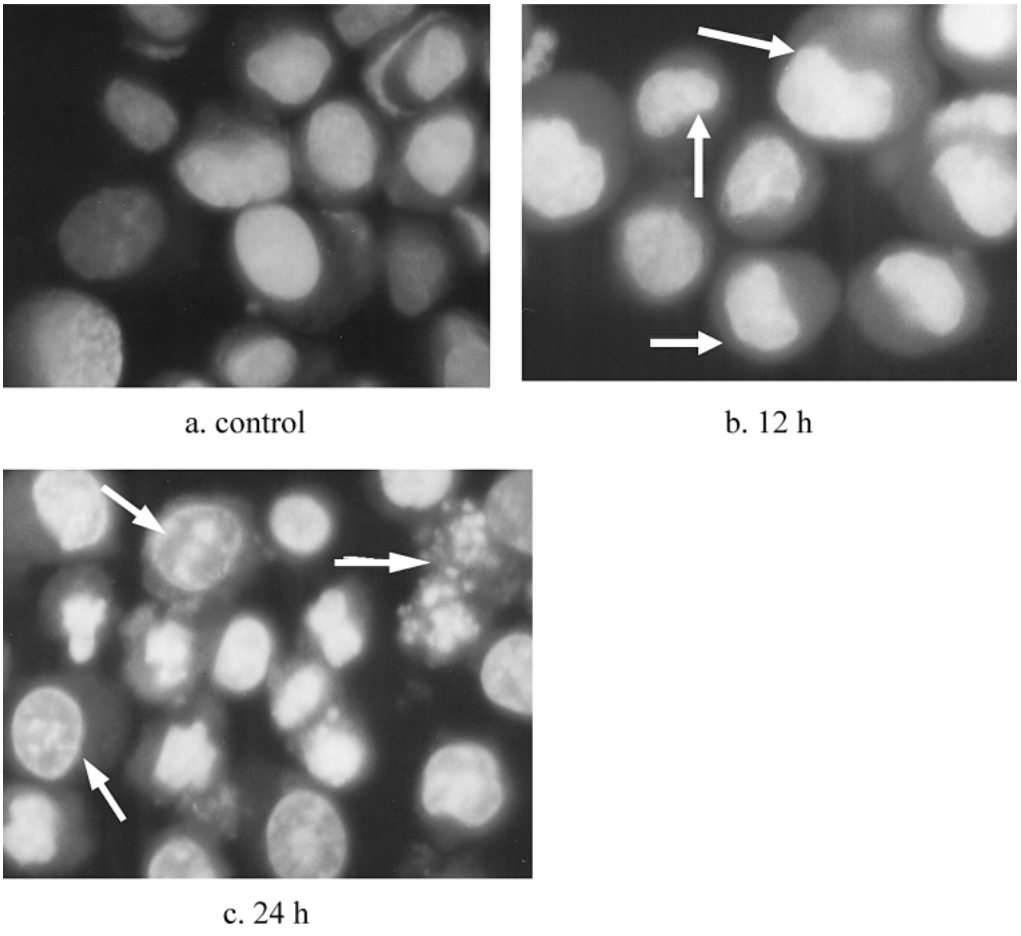


Fig. 3. Cellular Morphology of δ-Elemene Treated Hela Cells

Cells were cultured without δ-elemene (a. control) or with 200 μM δ-elemene for 12 (b) and 24 h (c). Morphological changes were observed by fluorescence microscopy ($\times$200). Red arrows indicate condensed nuclei at 12 h, yellow one indicate blebbing nuclei and granular apoptotic bodies appeared at 24 h.

$p>0.05$). The IC_{50} value of δ-elemene and β-elemene for Hela cells were 157.9 μM and 181.9 μM, respectively, while treated for 24 h. The IC_{50} of Hela cells at 12, 24, and 48 h were 243.8, 157.9, and 165.6 μM, respectively (Fig. 2(b)). However, the viability of normal human cell line WRL-68 was nearly no effecting at 200 μM after treatment with δ-elemene (Fig. 2(c), $p>0.05$ vs at 0 μM δ-elemene for 24 h).

Nuclear Damage Observed by Hoechst 33258 Staining in Hela Cells A morphological change was commonly observed in Hela cells. Originally, Hela cells appeared enlarged with prominent nuclei. In the control group (Fig. 3(a)), the cells were round in shape and stained homogeneously. After 12 h treatment with δ-elemene (Fig. 3(b)), condensation of chromatin occurred, and blebbing nuclei and granular apoptotic bodies appeared at 24 h (Fig. 3(c), arrows).

DNA Fragmentation in Hela Cells Treated with δ-Elemene δ-elemene significantly induced DNA fragmentation. The amount of DNA fragmentation peaked when the concentration of δ-elemene reached 200 μM after treatment for 24 h (Fig. 4(a), $^*p<0.001$ vs control). The time course study showed that

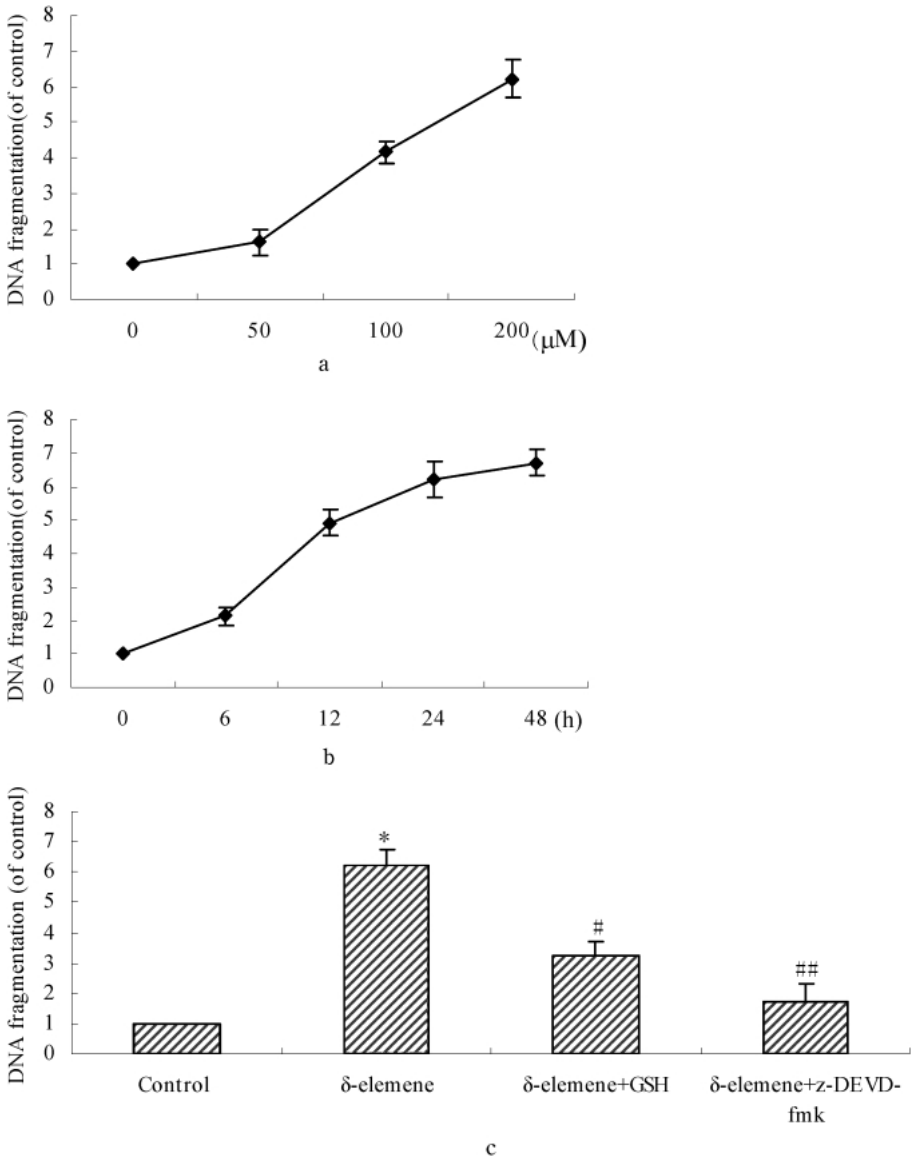


Fig. 4. Effect of δ-Elemene on DNA Fragmentation in Hela Cells

The DNA fragmentation was determined by cellular DNA fragmentation ELISA assay. Hela cells were treated with various concentrations of δ-elemene (0—200 μM) for 24 h (a) or with 200 μM δ-elemene at indicated periods (b). In the experiment using GSH and z-DEVE-fmk, Hela cell lines were pretreated with 1 mM GSH or with 2 μM z-DEVD-fmk 1 or 2 h prior to the treatment by 200 μM of δ-elemene for 24 h (c). $n=3$, mean±S.D. $^*p<0.01$ vs control, $\#p<0.05$, $\#\#p<0.01$ vs 200 μM of δ-elemene for 24 h.

the amount of DNA fragmentation was elevated after treatment for 12 h, and it peaked for 48 h after treatment with 200 μM δ-elemene (Fig. 4(b)). Furthermore, the DNA fragmentation in Hela cells treated with δ-elemene was inhibited by GSH or z-DEVD-fmk (Fig. 4(c), #$p<0.01$, ##$p<0.001$ vs 200 μM δ-elemene) for 24 h.

Apoptosis in Hela Cells Treated with δ-Elemene Results showed that apoptosis was triggered after treatment for 12 h (Fig. 5(a)). The percentage of early apoptosis represented by $(AnV)^+PI^-$ cells was significantly increased by δ-elemene treatment in a

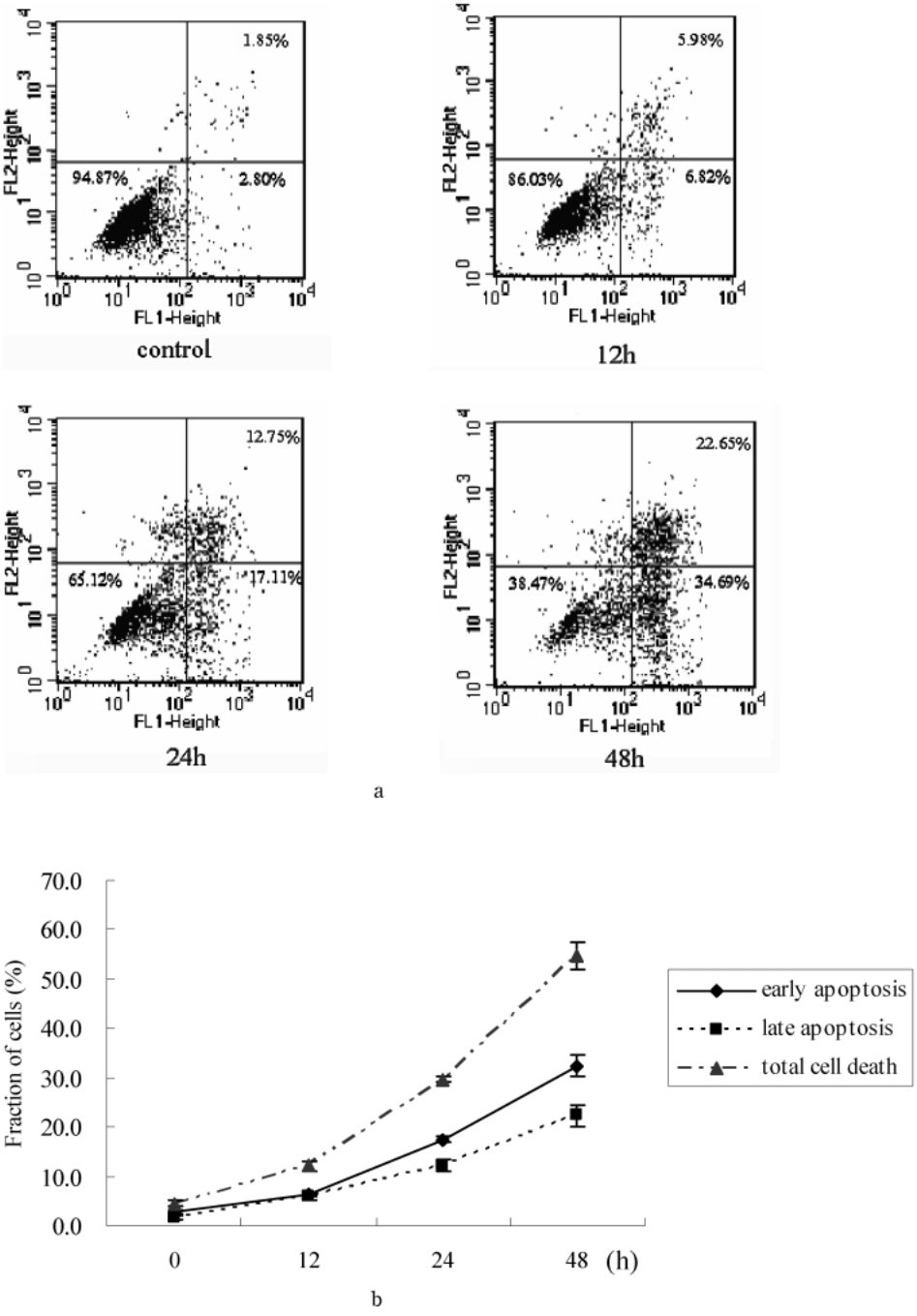


Fig. 5. The Effect of δ-Elemene on the Phosphatidylserine Distribution in Hela Cells

Hela cells were treated with 200 μM δ-elemene at indicated time (0 h, 12 h, 24 h, 48 h), and stained with FITC-conjugated Annexin V (AnV) and PI, followed by the flow cytometric analysis. (a) The light scattering and staining profiles of Annexin V-FITC and PI of each group are shown. The numerals in the quadrants indicate the percentages of cells in that particular quadrant with respect to total. Early apoptotic populations are found in the lower-right quadrants, while necrotic or late apoptotic cells are localized in the upper-right quadrant. (b) The percentage of early apoptosis, late apoptosis and total cell death in Hela cells with treatment of 200 μM δ-elemene for indicated time. Values represent mean$\pm$S.D. of 3 independent experiments performed.

time-dependent manner with 34.7% at 48 h (Fig. 5 (b)). Total cell death, which comprised early apoptotic and late apoptotic cells, calculated by the summation of the $(AnV)^+PI^-$ and $(AnV)^+PI^+$ cell populations, was also increased at 24 h. Comparing with the untreated control, more death events were observed in δ-elemene treated cells for 48 h.

Generation of ROS in Hela Cells Treated with δ-Elemene ROS generation was analyzed by flow cytometry. The treatment of Hela cells with 200 μM δ-elemene resulted in a time-dependent increase in ROS generation (Fig. 6(a)). As shown in Fig. 6(b), there was approximately 3.3 or 4.5 fold increase in generation of DCF-sensitive ROS in 200 μM δ-elemene for 3 or 6 h compared with vector control cells, a statistically significant increase ($^{**}p<0.001$). The change of ROS induced by δ-elemene was inhibited by GSH to a level (3.55%) that was almost as low as the untreated control (4.10%).

Disturbance of Mitochondria Transmembrane Potential in Hela Cells Treated with δ-Elemene The exposure of Hela cells to δ-elemene resulted in a dramatic reduction of cell population in live cells, accompanied by a concomitant increase of events in ear-

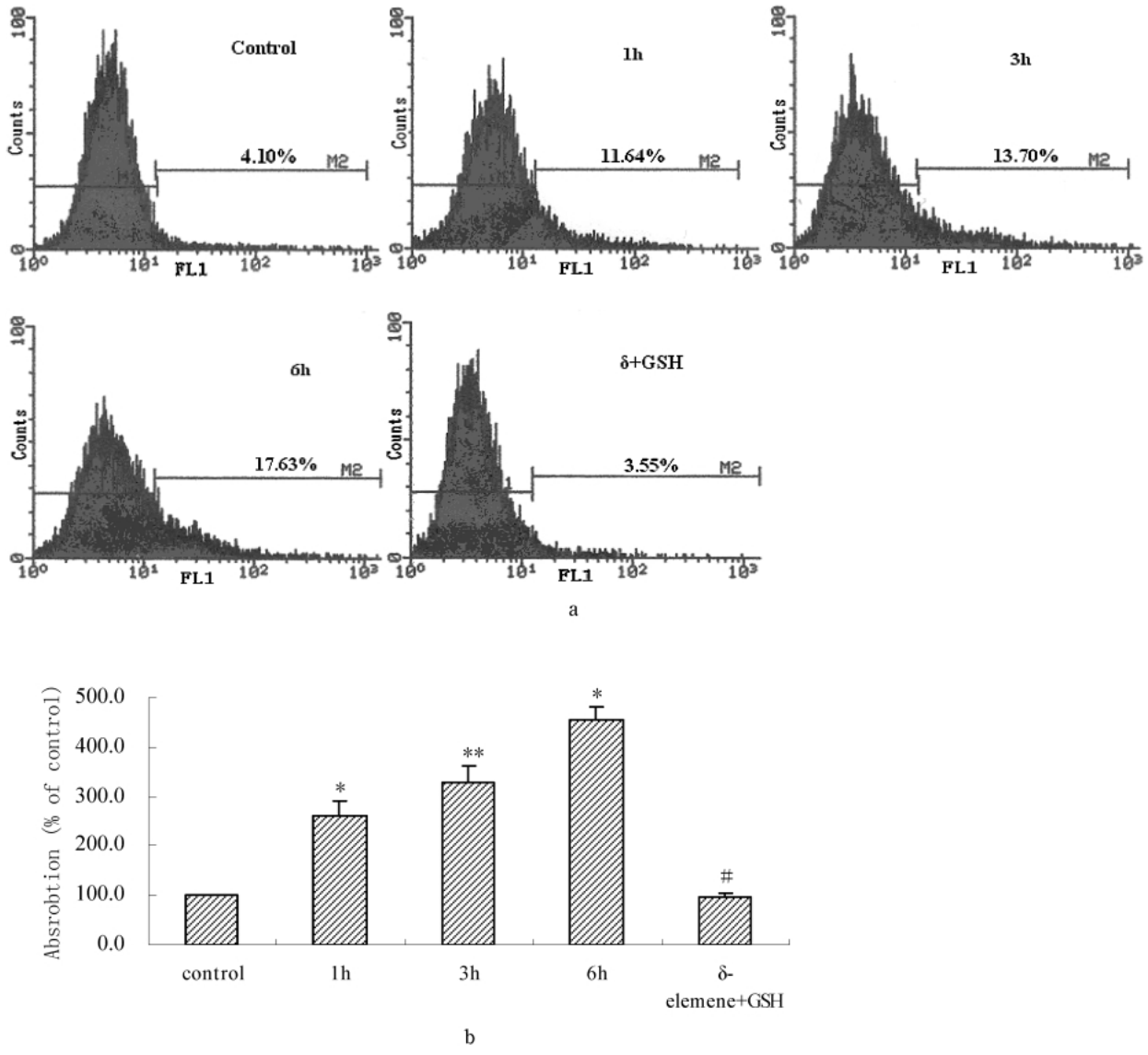


Fig. 6. Generation of ROS in Hela Cells Treated with δ-Elemene

Hela cells were treated with δ-elemene for 1, 3 or 6 h, and cells were stained with 10 μM H2DCFDA for 45 min at 37℃ and analyzed by flow cytometry (a, b). In the experiment of inhibition of GSH on generation of ROS, Hela cell lines were pretreated with 1 mM GSH 1 h prior to the treatment with 200 μM δ-elemene for 3 h. The horizontal axis shows the relative fluorescence intensity and the vertical axis shows cells number. Values represent mean±S.D. of 3 independent experiments performed. $^{*}p<0.01$, $^{**}p<0.001$ vs control, $^{\#}p<0.001$ vs 200 μM of δ-elemene for 3 h.

ly apoptosis cells in a time-dependent manner (Fig. 7 (a)). Compared with cells maintained in control cells, Hela cells with 200 μM δ-elemene treatment for 6 h exhibited green JC-1 fluorescence, which is consistent with a loss of mitochondrial membrane polarization. As shown in Fig. 7(b), the trends of increasing incidences of compromised $\Delta\psi_m$ for 6 h was 2 to 2.5 fold with respect to the untreated control. Treatment for 12 h resulted in a shift in JC-1 fluorescence to orange-red, consistent with a restoration of normal mitochondrial membrane polarization. Long exposure time essentially gave a more remarkable increase of 2.5 to 3 and 3 to 4 fold in Hela cells at 12 and 24 h respectively (Fig. 7(b), $^{*}p<0.01$, $^{**}p<0.001$, respectively, vs untreated control, $n=3$). Further, it was noted that the disturbance of $\Delta\psi_m$ could be attenuated by GSH (Fig. 7(b), $\#p<0.05$, $\#\#p<0.01$ vs 200 μM for 24 h).

Activation of Caspase-3 and Cleavage of PARP The caspase signaling pathway has been demonstrated to play a central role in cellular apoptosis. PARP, acting intranuclearly to repair damaged DNA, is one of the substrates of activated caspase. As shown in Fig. 8(a), 200 μM δ-elemene was able to cleave intact PARP (116 kDa) into an 85 kDa fragment after treatment for 6 h in time-dependent manner. The reduction of procaspase-3 indicates the activation of caspase-3. 200 μM δ-elemene was able to cleave pro-caspase-3 (32 kDa) to activate caspase-3 after treatment for 12 h in Fig. 8(a). The reduced level of procaspase-3 and elevated level of cleaved PARP were much more significant than that of the untreated cells in Fig. 8(b) and Fig. 8(c) ($^{*}p<0.05$, $^{**}p<0.01$ vs the untreated control).

Effect of GSH and z-DEVD-fmk on Activation of Caspase-3, Cleavage of PARP in Hela Cell Line To examine the effect of ROS generation and the inhibition of caspase-3 on the observed alterations of caspase-3 and PARP in Hela cells, we treated the cells with GSH 1 h prior to addition of δ-elemene, or with z-DEVD-fmk, 2 h prior to treatment with δ-elemene. The result showed that the activation of caspase-3 and the cleavage of PARP, all of which were caused by δ-elemene treatment, were inhibited by GSH (Fig. 9(a), Fig. 9(b), $^{*}p<0.05$, $^{**}p<0.01$ vs the untreated control) suggesting that the apoptotic pathway mediated by δ-elemene was associated with the production of ROS and in Hela. And z-DEVD-fmk had also significantly inhibited the activation of caspase-3 and the cleavage of PARP (Fig. 9(a), Fig. 9(c), $^{*}p<0.05$, $^{**}p<0.01$ vs the control).

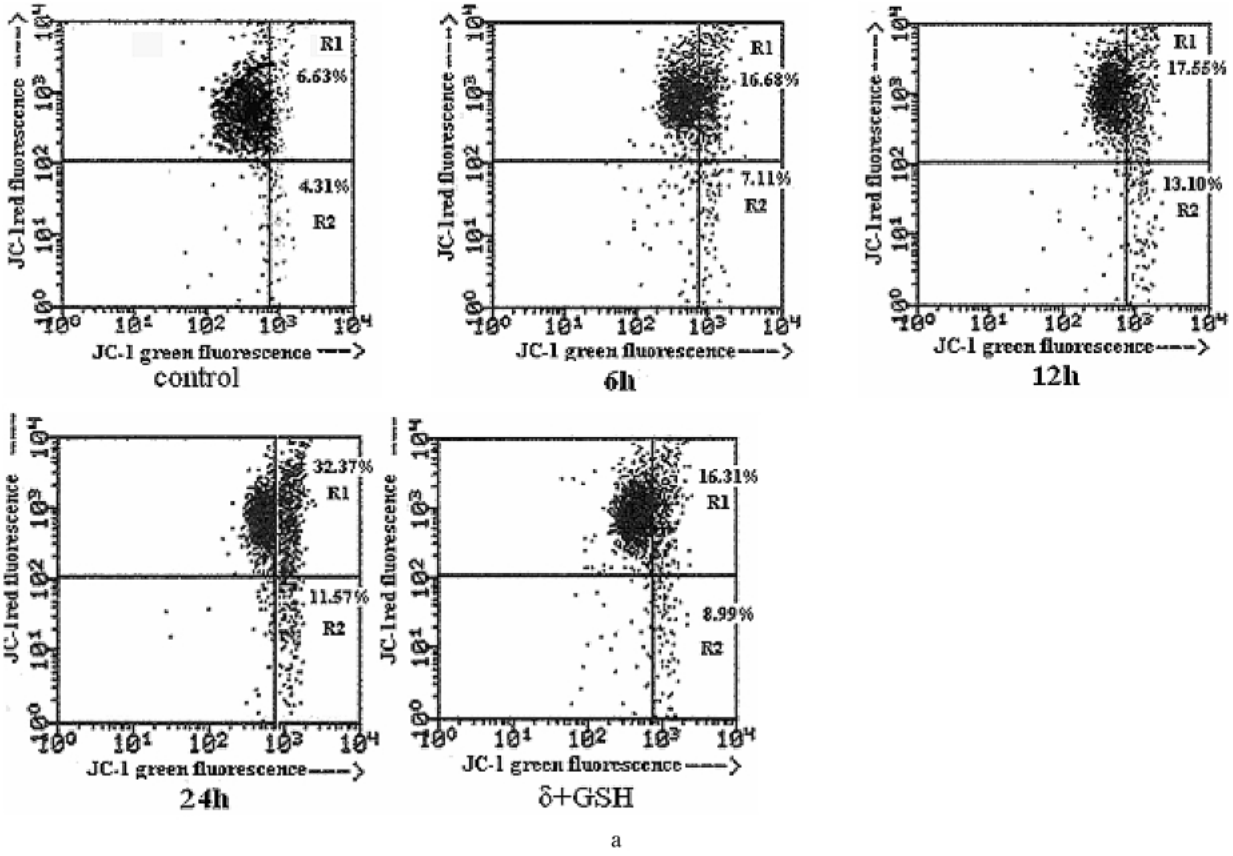
DISCUSSION

The cytotoxicity of elemene has been determined in various tumor cell lines *in vitro*.[17,18] The IC_{50} of elemene for normal peripheral blood leukocytes is 1.244 mM.[19] The present study showed that δ-elemene inhibited Hela cell growth in a time- and dose-dependent manner. The IC_{50} values of δ-elemene and β-elemene on Hela cells were 157.9 μM and 181.9 μM, respectively. They were similar to the IC_{50} (182.4 μM) of elemene for Hela cells.[20] It appears that the growth inhibitory effect of δ-elemene on Hela cells is much stronger than that on normal liver cells.[4]

In this research, induction of apoptosis by δ-elemene in a time-dependent manner was confirmed by DNA fragmentation ELISA assay, Annexin-V and JC-1 labeled flow cytometry analysis. Hoechst 33258/PI fluorescence microscopy was observed condensation of chromatin, and blebbing nuclei and granular apoptotic bodies. δ-elemene was able to significantly induce DNA fragmentation, which peaked after treatment for 48 h. The Annexin-V FITC binding assay was used to distinguish apoptotic cells from necrotic cells. The Annexin-V labeling assay showed that apoptosis and total cell death were triggered after treatment for 12 h. And the percentage of early apoptosis represented by $(AnV)^{+}PI^{-}$ cells was significantly increased by δ-elemene treatment in a time dependent manner as compared to the untreated control.

Mitochondria dysfunction has been recognized as a key step in apoptosis. JC-1 is reported to provide more accurate estimates of mitochondrial transmembrane potential ($\Delta\psi_m$) (decrease in $\Delta\psi_m$ is one of the early events of apoptosis) than Rh123 or DiOC6 (3).[21] In the presence of δ-elemene, more cells were found with brighter green fluorescence, yet lower red fluorescence intensity, which was indicative of the predominance of monomer state, implying the loss of the mitochondrial transmembrane potential ($\Delta\psi_m$).

Oxygen free radical formation is associated with the generation and development of certain tumors.[22] Elemene enhanced serum superoxide dismutase activity in patients with brain tumors.[23] It has been suggested that the agent has an inhibitory effect on ROS formation in tumors cells, which may contribute to its apoptosis effects.[20] Our results showed that ROS was



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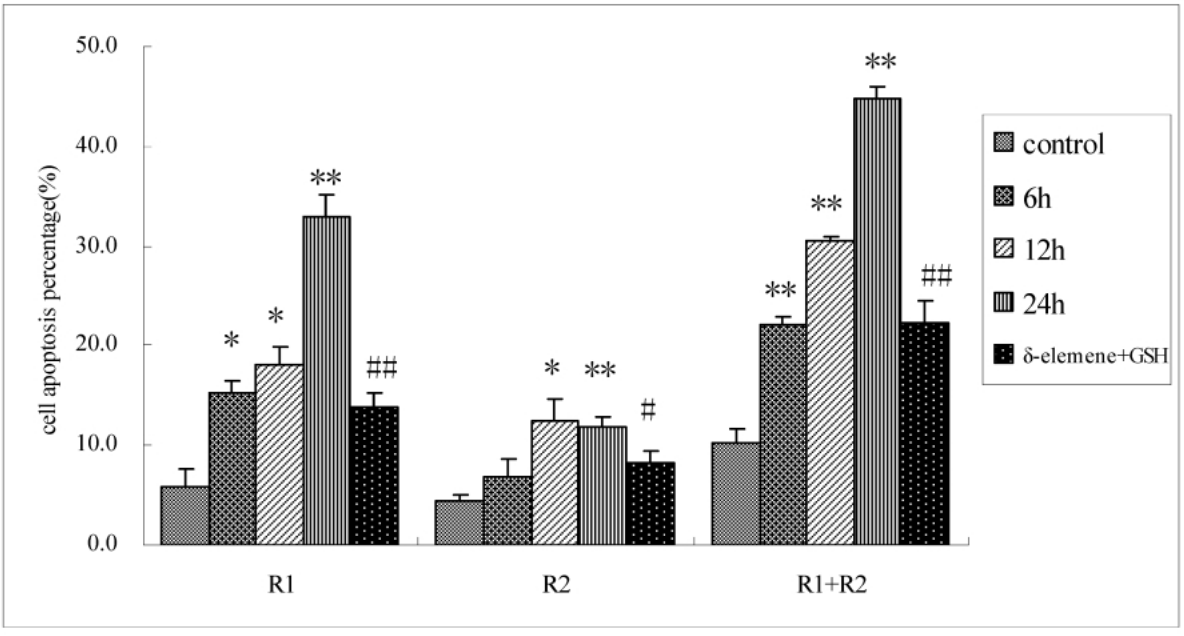

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Fig. 7. Reduction of $\Delta\psi_m$ in Hela Cells Treated with δ-Elemene

Hela cells were treated with δ-elemene for 6, 12 and 24 h, and cells were stained with JC-1 for 15 min at 37℃ and analyzed by flow cytometry. (a) After δ-elemene treatment, depolarization of $\Delta\psi_m$ became evident as indicated by increased cell population in upper right and lower right quadrant (R_2, R_3). Cells found in R_2 and R_3 had light scattering properties typical of apoptosis. In the experiment of GSH on reduction of $\Delta\psi_m$, Hela cells were pretreated with 1 mM GSH 1 h prior to the treatment with 200 μM δ-elemene for 24 h. (b) The mean and S.D. of the results were obtained from three independent experiments. Mitochondrial damage was most marked at 12 and 24 h post δ-elemene treatment. $^*p<0.05$, $^{**}p<0.01$ vs control (0 h), $^{\#}p<0.05$, $^{\#\#}p<0.01$, vs 200 μM of δ-elemene for 24 h.

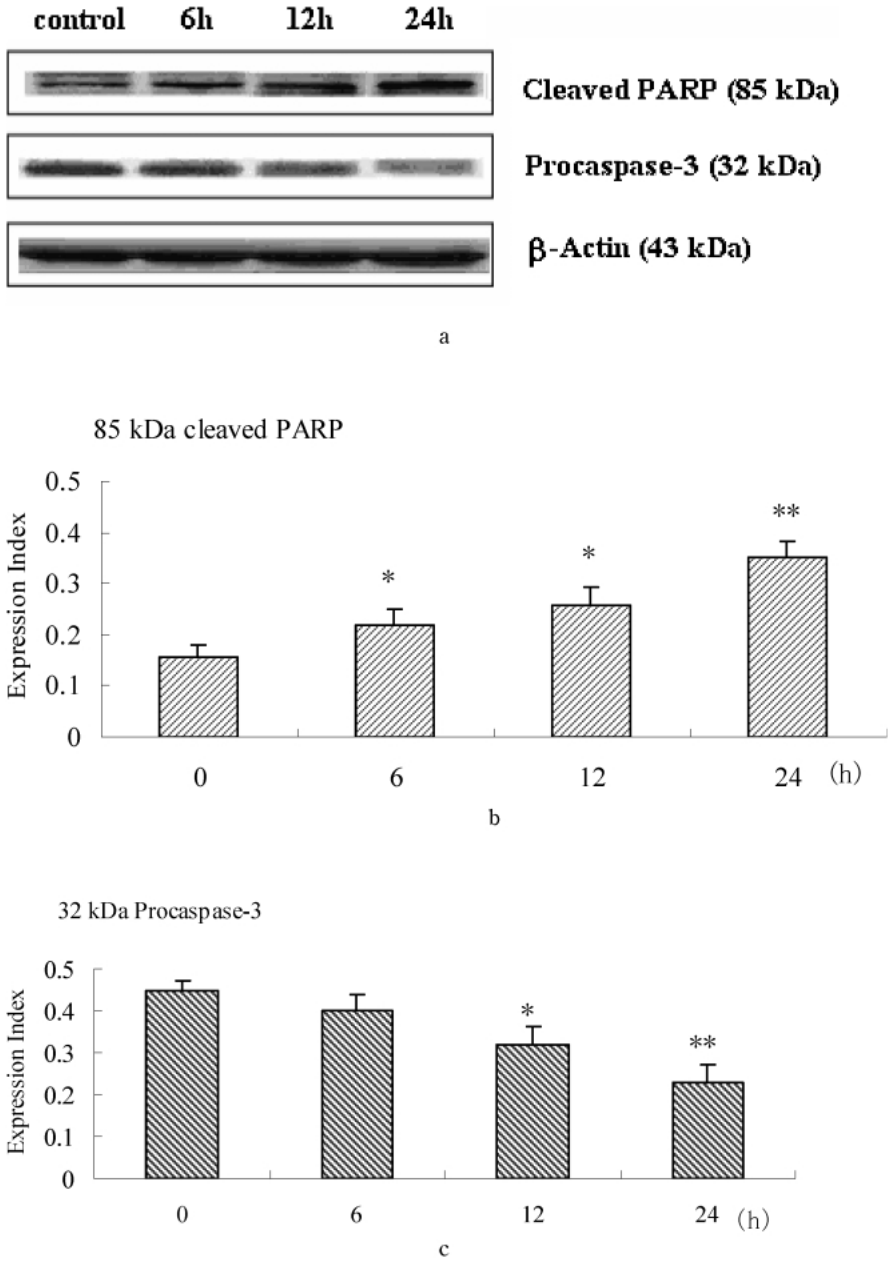


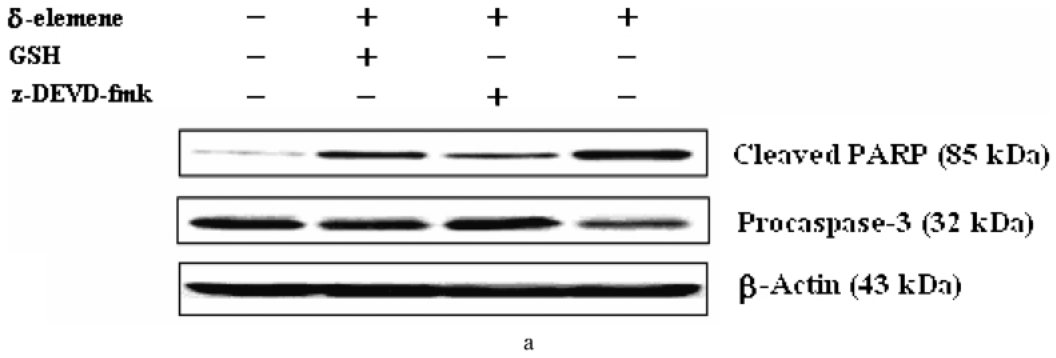
Fig. 8. Time Course of Caspase-3 Activation, PARP Cleavage in Hela Cells

Hela cells were treated with 200 μM δ-elemene at indicated periods. After treatment, total protein was extracted. The level of procaspase-3, and 85 kDa cleaved PARP fragment in total protein extract were analyzed by Western blot (a). The densities of the protein bands were determined and the relative amount of the target protein was shown as expression index. The relevant results were shown in b-c. $^{*}p<0.05$, $^{**}p<0.01$ vs control (0 h), $\sharp p<0.05$, $\sharp\sharp p<0.01$ vs 200 μM δ-elemene for 24 h.

involved in δ-elemene induced apoptosis in Hela cells. Moreover, δ-elemene induced apoptosis was reduced to a level almost as low as the control by GSH, indicating that ROS played a critical role in δ-elemene induced apoptosis in Hela cells.

Caspases involved in apoptosis are generally divided into two categories: the initiator caspases, which include caspase-2,-8,-9 and -10, and the effector caspases, which include caspase-3,-6, and -7.[24] Active caspase-3 consists of 17 and 12 kDa subunits which are derived from a 32 kDa proenzyme (procaspase-3). The caspase-3 antibody recognizes both the 32 kDa procaspase-3 and the 17 kDa subunit of active caspase-3.[25] In the present study, δ-elemene was able to activate caspase-3 in a time-dependent manner by cleaving procaspase-3 (32 kDa) to active caspase-3 (17 kDa).

DNA repair and apoptosis are two essential systems



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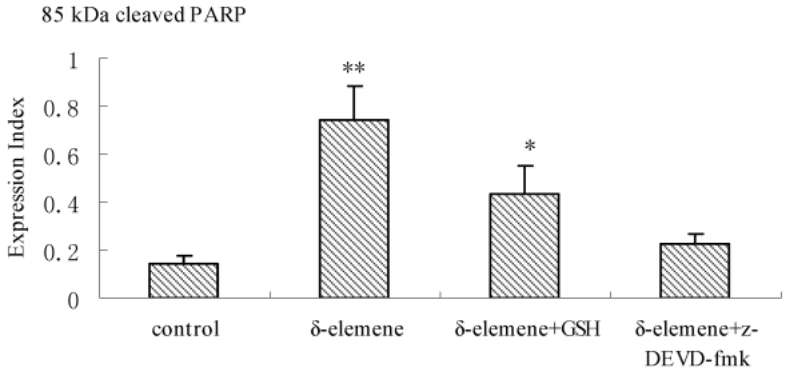
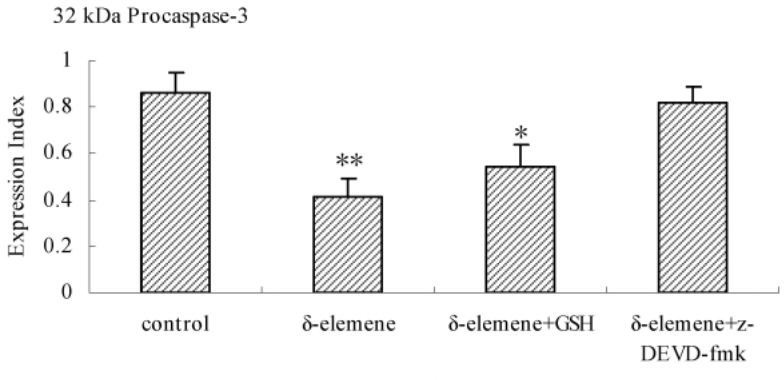


b



c

Fig. 9. Effect of GSH and z-DEVE-fmk on Activation of Caspase-3, Cleavage of PARP in Hela Cells

Hela cells were pretreated with GSH or with z-DEVD-fmk 1 or 2 h prior to the treatment of 200 μM δ-elemene for 24 h. Total protein were extracted. The levels of procaspase-3, and 85 kDa cleaved PARP fragment in total protein extract were analyzed by Western blot (a). The densities of the protein bands were determined and the relative amount of the target protein was shown as expression index. The relevant results were shown in b-c. $^{*}p<0.05$, $^{**}p<0.01$ vs control cultures (0 h), $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs 200 μM δ-elemene for 24 h.

in humans which maintain genomic integrity. Homologous recombinational repair (HRR), non-homologous end joining (NHEM), nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) are five major DNA repair pathways.[26] Poly (ADP-ribose) polymerase (PARP) is responsible for BER. One of the immediate cellular responses to DNA damage is the activation of PARP, which occurs upon binding to single- or double-strand breaks in the DNA, probably by recruiting DNA repair enzymes to the damaged DNA region.[27,28] Since PARP consumes NAD^+ and ATP, excessive DNA damage will ultimately lead to cell death through highly evoked PARP activity.[29] PARP is cleaved by caspase-3, suggesting that PARP-mediated BER is suppressed once a cell is destined to

apoptosis.[30] Our findings revealed that δ-elemene induced caspase-3 activation and a PARP breakdown, culminating in the death of Hela cells.

In summary, the results revealed that 1) δ-elemene is capable of inducing Hela cell lines death by apoptosis, 2) it is able to activate the caspase-signaling pathway, leading to the proteolytic conversion of procaspase-3 to active caspase-3, and the subsequent cleavage of the caspase substrate PARP, 3) the increase in ROS generation is involved in the mechanism of δ-elemene induced cell apoptosis. This study suggests that δ-elemene is a potent inducer of cell death of anaplastic thyroid carcinoma cells. The potential application in cancer cell apoptosis warrants further elucidation.

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