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# Sarsasapogenin induces apoptosis via the reactive oxygen species-mediated mitochondrial pathway and ER stress pathway in HeLa cells

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ABSTRACT

Sarsasapogenin is a sapogenin from the Chinese medical herb *Anemarrhena asphodeloides Bunge*. In the present study, we revealed that sarsasapogenin exhibited antitumor activity by inducing apoptosis in vitro as determined by Hoechst staining analysis and double staining of Annexin V-FITC/PI. In addition, cell cycle arrest in G2/M phase was observed in sarsasapogenin-treated HeLa cells. Moreover, the results revealed that perturbations in the mitochondrial membrane were associated with the deregulation of the Bax/Bcl-2 ratio which led to the upregulation of cytochrome c, followed by activation of caspases. Meanwhile, treatment of sarsasapogenin also activated Unfolded Protein Response (UPR) signaling pathways and these changes were accompanied by increased expression of CHOP. Salubrinal (Sal), a selective inhibitor of endoplasmic reticulum (ER) stress, partially abrogated the sarsasapogenin-related cell death. Furthermore, sarsasapogenin provoked the generation of reactive oxygen species, while the antioxidant N-acetyl cysteine (NAC) effectively blocked the activation of ER stress and apoptosis, suggesting that sarsasapogenin-induced reactive oxygen species is an early event that triggers ER stress mitochondrial apoptotic pathways. Taken together, the results demonstrate that sarsasapogenin exerts its antitumor activity through both reactive oxygen species (ROS)-mediate mitochondrial dysfunction and ER stress cell death.


## 1. Introduction

Cervical cancer is the second most common cancer among women worldwide and remains a critical public health problem, although mortality rates have declined over time due to early detection and screening programs. Cervical cancer is a leading cause of death from cancer among women in developing countries, killing approximately a quarter of a million women per year [1]. Platinum-based chemotherapy drugs have been proven to be effective among the chemotherapy drugs available; however, the serious side effects of such treatments cannot be ignored. To reduce the mortality rate of cervical cancer, the development of effective drugs to treat cervical cancer with minimal side effects is of great importance. Natural products are considered to be a suitable replacement or supplement for platinum drugs to cure cervical cancer.

To date, attention has been focused on finding safe agents with biological activity and isolating active compounds from natural sources. Chemopreventive phytochemicals including berberine [2], resveratrol [3], curcumin [4] and trichosanthin [5] have been shown to have great potential as cervical cancer drugs. These compounds exert their effects through various mechanisms, such as promoting apoptosis and inhibiting DNA methyltransferase.

Apoptosis, also known as type I programmed cell death, is the most common mechanism exploited by targeted chemotherapies (cytotoxic agents and radiation therapy) that induce cancer cell death or sensitize cells [6,7]. This process can be triggered either at the plasma membrane (extrinsic pathways) and/or within cells (intrinsic pathways) [8]. Recent studies have suggested that the intrinsic pathways are initiated by the biochemical events, which affect the organelles inside the cells causing intracellular stresses. Among these organelles, mitochondria and endoplasmic reticulum (ER) play important roles in the intrinsic pathways to execute apoptosis [9].

Sarsasapogenin (Fig. 1A), a sapogenin from the Chinese medical herb *Anemarrhena asphodeloides Bunge*, has been widely used in Chinese and Japanese folk medicine. It exhibits antidiabetic activity [10], antiplatelet aggregation activity [11] and diuretic activity [12]. Despite evidence indicating the benefits of sarsasapogenin treatment for cancer resistance, there is a lack of data describing the anti-tumor activity of sarsasapogenin on cervical cancer. We therefore investigate the roles and explore the underlying mechanisms of sarsasapogenin in HeLa cells in the present study.

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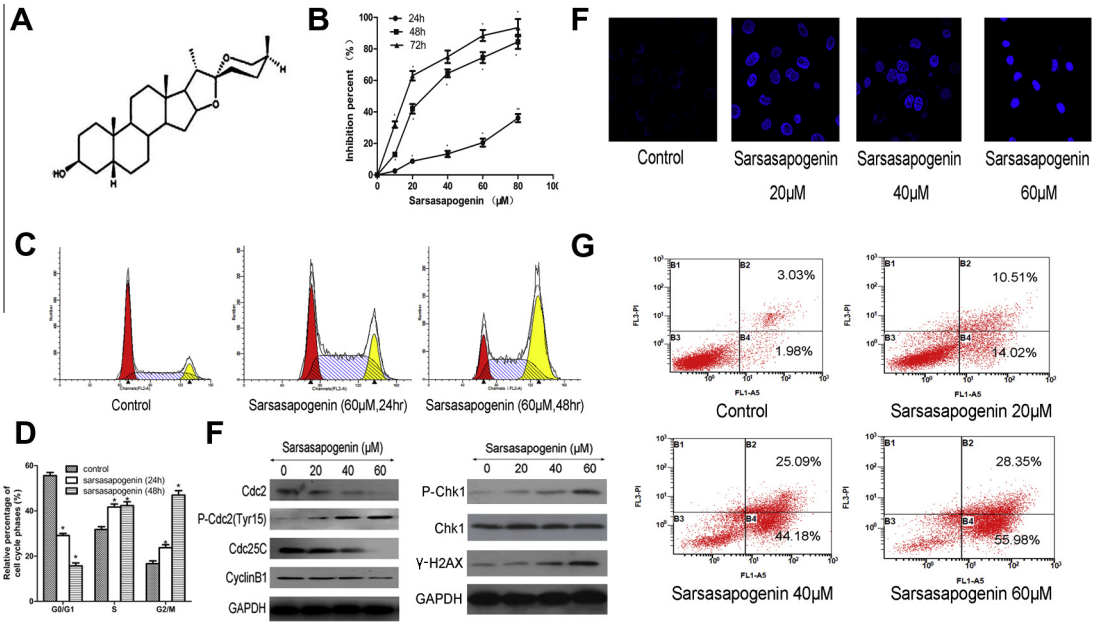


**Fig. 1.** Effects of sarsasapogenin on the cell cycle and apoptosis induction in HeLa cells. (A) Chemical structure of sarsasapogenin. (B) HeLa cells were treated with increasing doses of sarsasapogenin for various times. Results are expressed as means ± SD of data obtained in three independent experiments ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control). (C) The HeLa cells were treated with 60 μM sarsasapogenin for 24 or 48 h and then cell cycle analyzed by flow cytometry. (D) The proportions of cells in G1, S and G2 phases were calculated using Cell Quest software. Bar graphs are shown by mean ± SD from three independent experiments ($^{*}p < 0.05$ vs. control). (E) HeLa cells were treated with sarsasapogenin (0, 20, 40, 60 μM) for 48 h. Proteins (30 μg per lane) were probed with antibodies against Cyclin B1, Cdc2, Cdc25C, p-Cdc2 (Tyr15), p-Chk1 (Ser345), Chk1 and γ-H2AX as indicated. Each blot is representative of three similar experiments. (F) HeLa cells were incubated with sarsasapogenin (0–60 μM) for 48 h. Hoechst 33342-stained nuclei were visualized using confocal laser scanning microscopy (63×). The data are representative of three parallel experiments. (G) HeLa cells were treated with sarsasapogenin (0–60 μM) for 48 h, stained with Annexin V/PI and then analyzed by flow cytometry. The data represent similar results from three independent experiments.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Primary antibodies were purchased from these companies: Santa Cruz (ATF4, ATF6, XBP-1s, CHOP, GAPDH, GRP78, GRP94, PERK, eIF2S1), Cell Signaling (cleaved caspase-3, cleaved caspase-9, phospho-PERK, phospho-eIF2S1, Cyclin B1, Bcl-2, Bax, Bak, Cdc2, Cdc25C, p-Cdc2 (Tyr15), cytochrome c (cyt c), p-Chk1 (Ser345) and Chk1), Upstate Biotechnology (γ-H2AX). The fluorescent probes dihydrorhodamine 123 (DHR123), Rh123 and naphthalene-2,3-dicarboxaldehyde (NDA) were from Molecular Probes (Eugene, OR). The synthesized sarsasapogenin was obtained from The National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals, except otherwise noted, were purchased from Sigma–Aldrich chemical.

Sarsasapogenin was dissolved in ethanol at 10 mM and diluted with fresh medium to achieve the desired concentration. The final ethanol concentration in cultures was <1% (v/v), which did not influence cell growth when compared with the vehicle-free controls. HeLa cells were cultured in DMEM containing 10% fetal bovine serum in humidified air at 37 °C with 5% $CO_2$.

### 2.2. Analysis of cell viability

The effect of sarsasapogenin on cell viability was measured by the MTT assay method. After exposure to sarsasapogenin in the absence or presence of Z-VAD-FMK pretreatment for the indicated time, cells in 96-well plates were incubated with MTT. The formazan precipitate was dissolved in 200 μl of dimethyl sulfoxide, and the absorbance at 570 nm was measured using a Benchmark microplate reader (Bio-Rad, CA, USA) [13].

### 2.3. Measurement of the cell cycle

HeLa cells were cultured in the presence or absence of 60 μM sarsasapogenin for 24 or 48 h. The cells were then treated with 20 μg/ml RNaseA, followed by 25 μg/ml propidium iodide (PI). The population of cells at each stage of the cell cycle was determined by examining the intensity of PI fluorescence with a flow cytometer using an argon laser and a 570 nm bandpass filter (FACSort, Becton Dickinson).

### 2.4. Morphological analysis after Hoechst 33342 staining

HeLa cells ($1 \times 10^5$) were seeded in 6-well plates for 24 h. After sarsasapogenin treatment for another 48 h, the cells were stained with Hoechst 33342 solutions containing 5 μg/ml Hoechst 33342 in 0.1% sodium citrate. Then, the nuclear morphology of the cells was examined using confocal laser scanning microscopy (Zeiss, LSM 510, Germany).

### 2.5. Annexin V/propidium iodide flow cytometric analysis

Different stages of apoptosis were distinguished using an Annexin V-FITC/propidium iodide apoptosis kit (BioSource). Briefly, cells were cultured with sarsasapogenin at various concentrations for 48 h, and then $1 \times 10^6$ cells were harvested, washed twice with ice-cold PBS. Apoptotic (Annexin $V^+/PI^-$) or necrotic cells (Annexin $V^+/PI^+$) were evaluated by double staining with Annexin V-FITC and PI in binding buffer using by flow cytometry.

### 2.6. Measurement of mitochondrial transmembrane potential (MMP)

MMP was measured by flow cytometer using the cationic lipophilic green fluorochrome Rh123. Cells were harvested, washed twice with PBS, incubated with 1 μM Rh123 at 37 °C for 30 min,

and washed twice with PBS. Fluorescence was determined by flow cytometer with an excitation wavelength of 488 nm at FL-1 Filter.

### 2.7. Determination of the cellular redox state by ROS and GSH analyses

The oxidative stress of cells was determined by detection of cellular ROS with DHR123, and cellular reduced power was measured according to the levels of cellular reduced glutathione (GSH) with NDA. Briefly, after incubation with various concentrations of sarsasapogenin for 48 h, cells in a 6-well plate were washed and incubated at 37 °C for 30 min with 10 mM DHR123 or 500 mM NDA [14]. Cells were then washed three times with PBS, and the fluorescence intensities were measured by flow cytometry.

### 2.8. Western blot analysis

Western blot analysis was performed by standard methods. Approximately 30 μg of lysed protein were separated by SDS–PAGE gel and transferred to a nitrocellulose blotting membrane, blocked for 2 h in blocking buffer (5% bovine serum albumin solution and 0.1% Tween 20 in Tris-buffered saline (TBST)). The membrane was incubated overnight at 4 °C with the primary antibodies and the corresponding horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualized using a commercially available enhanced chemiluminescence kit (Amersham Biosciences Corp, Piscataway, NJ).

### 2.9. Statistical analysis

Unless otherwise stated, data are expressed as means ± SD and analyzed statistically by one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Effects of sarsasapogenin on the cell cycle and molecular mechanisms

To examine how sarsasapogenin inhibited the growth of HeLa cells, cell cycle analysis was performed. Sarsasapogenin induced an increase in the population of cells in S phase and an obvious accumulation of cells in G2/M phase in a time-dependent manner (Fig. 1C). In control cultures, 31% of cells were in S phase and 17% were in G2/M phase. However, the number of cells in S and G2/M phases were increased to 42% and 47% at 48 h, respectively (Fig. 1D). These results indicated that sarsasapogenin-induced cell growth inhibition could be associated with the delay at S and G2/M phase.

To further elucidate the molecular mechanisms of sarsasapogenin-induced cell cycle arrest, HeLa cells were treated with different concentrations of sarsasapogenin, and the levels of regulatory proteins were assessed. S and G2 arrest following DNA damage is associated with accumulation of hyperphosphorylated and relatively inactive Cdc2/cyclin B1 complexes, which, in turn, are regulated by Cdc25C. The phosphatase activity of Cdc25C is tightly regulated by the activation of the ATR-Chk1 kinase pathway [15]. Western blot analysis showed that p-Cdc2 and p-Chk1 were upregulated and that Cyclin B1, Cdc2 and Cdc25C were downregulated (Fig. 1E). DNA damage is one of the molecular events associated with cell cycle arrest and apoptosis, and several anti-cancer reagents induce DNA damage [16]. In the present study, sarsasapogenin caused DNA damage was evidenced by an increase in γ-H2AX protein level (Fig. 1E). These observations indicate that sarsasapogenin may trigger DNA damage and modulation of the expression or activation of cell-cycle regulatory proteins, resulting in a change in the distribution of cells in S and G2/M phase.

### 3.2. Sarsasapogenin induces apoptosis in HeLa cells

Morphological changes associated with apoptotic cell death, such as cell shrinkage and condensed and fragmented chromatin, were observed in sarsasapogenin-treated HeLa cells. Cells stained with Hoechst 33258 after exposed to 20–60 μM sarsasapogenin for 48 h showed condensed nuclei and apoptotic bodies (Fig. 1F). These results are hallmarks of apoptotic cell death, and demonstrated the ability of sarsasapogenin to induce apoptosis in HeLa cells.

Annexin V/PI binding was used to evaluate the effect of sarsasapogenin on the type of cell death. As shown in (Fig. 1G), the proportion of apoptotic cells increased in a concentration-dependent manner, and the proportion of Annexin V/PI double-positive cells also increased after treatment, indicating that sarsasapogenin might induce both apoptosis and necrosis.

### 3.3. Sarsasapogenin induces apoptosis via the caspase-dependent mitochondrial apoptotic pathway

Mitochondria plays a critical role in apoptosis induced by anticancer agents, which induce the loss of mitochondrial membrane potential (MMP) and the release of cyt c, followed by caspase-9-dependent activation of caspase-3 and the cleavage of DNA repair protein PARP [17]. As shown in (Fig. 2A), a decrease in Rh123 accumulation in HeLa cells was detected after sarsasapogenin treatment compared with the control, indicating that sarsasapogenin induced MMP disruption, which is regulated by the Bcl-2 family. Particularly, the ratio of Bax (pro-apoptotic member) to Bcl-2 (anti-apoptotic member) is critical for cyt c release and the following caspase activation [18]. Western blot analysis revealed that Bax expression increased with the suppression of Bcl-2 in a time-dependent manner which was accompanied by the accumulation of cyt c in the cytoplasmic fraction (Fig. 2B).

In this investigation, to determine whether caspase activity was essential for the observed apoptosis, the effect of Z-VAD-FMK, a general caspase inhibitor, was analyzed. As shown in (Fig. 2C), treatment with Z-VAD-FMK before the administration of sarsasapogenin decreased the level of cell death induced by treatment with 60 μM sarsasapogenin for 24 h. In addition, Z-VAD-FMK greatly decreased the proportion of apoptotic cells from 38% to 18% (Fig. 2D). We also detected a time-dependent up-regulation of cleaved caspase-9 and caspase-3 (Fig. 2E). To further identify the activation of the caspase cascade, PARP-1 (116 kDa), one of caspase downstream effectors, was examined by Western blot analysis. (Fig. 2E) showed that the cleaved fragment of PARP-1 (89 kDa) was observed after 12 h treatment of sarsasapogenin.

### 3.4. Sarsasapogenin induces the activation of ER stress pathway

It is well-established that three ER resident transmembrane proteins ATF6 (activating transcription factor 6), ERN1 (endoplasmic reticulum to nucleus signaling 1) and PERK (PKR-like ER kinase) are the stress sensors of ER [19]. Phospho-PERK can phosphorylate eIF2S1 to decrease the gene expression. In the current detection, the ER chaperones GRP78 and GRP94 were evaluated by Western blot analysis (Fig. 3A). In response to sarsasapogenin treatment, the levels of GRP78 and GRP94 increased all the way up to 24 h. P-PERK, p-eIF2S1, ATF4 and ATF6 fragments increased in the early period (after 6 h treatment), whereas their normal forms did not change (Fig. 3B), suggesting that ER stress was triggered in the sarsasapogenin-treated HeLa cells. At the same time, CHOP, a hallmark of the ER stress-mediated

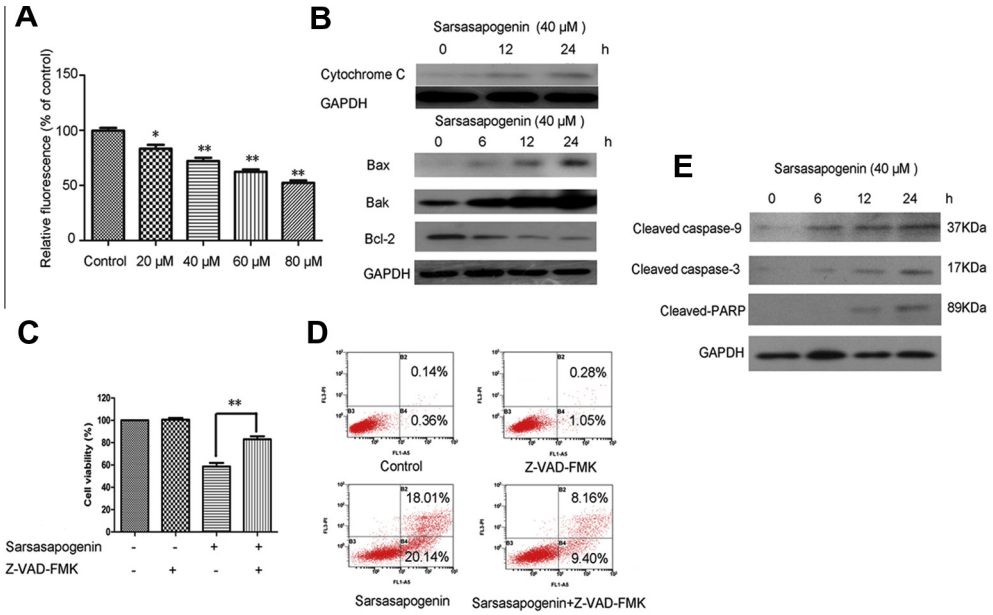


**Fig. 2.** Sarsasapogenin induced apoptosis via caspase-dependent mitochondrial pathway. (A) HeLa cells were treated with sarsasapogenin (20–80 μM) for 48 h and then stained with Rh123 to detect changes in membrane potential. Data are presented as means ± SD of three separate experiments ($^{*}p < 0.05$ and $^{**}p < 0.01$ versus control). (B) Immunoblot analysis of the impact of sarsasapogenin treatment of HeLa cells for various times on the levels of indicated proteins. (C) HeLa cells were pretreated with or without Z-VAD-FMK at 5 μM for 2 h and then treated with 60 μM sarsasapogenin for 24 h. Cell viability was determined by a MTT assay as described in the text. Each bar represents the means ± SD obtained from three experiments ($^{**}p < 0.01$ vs. sarsasapogenin treatment). (D) HeLa cells were pretreated with or without Z-VAD-FMK at 5 μM for 2 h and then treated with 60 μM sarsasapogenin for 24 h. Apoptotic cells were double-labeled with Annexin V/PI and analyzed by flow cytometry. The data represent similar results from three independent experiments. (E) Activation of caspases and their downstream effecter PARP-1 was detected by Western blotting. Each blot is representative of three similar experiments.

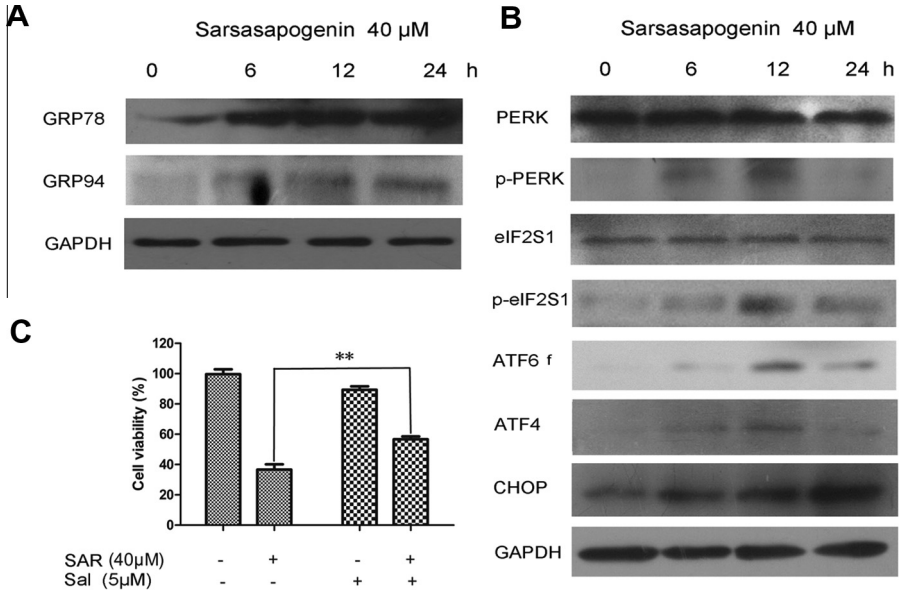


**Fig. 3.** Sarsasapogenin stimulated ER stress-mediated apoptotic pathway. (A) Western blot analysis of ER stress related chaperones GRP78 and GRP94. (B) Western blot analysis of PERK, p-PERK, eIF2S1, p-eIF2S1, ATF6 fragment, ATF4 and CHOP. Each blot is representative of three similar experiments. (C) Effect of ER stress inhibitor, salubrinal (Sal), on sarsasapogenin-induced cell death by MTT assay (Values represent means ± SD, $n = 3$ for each group $^{**}p < 0.01$ vs. sarsasapogenin treatment).

apoptosis, was also activated (Fig. 3B). We then investigated whether the inhibitor of ER stress could rescue sarsasapogenin-treated cells from apoptosis. Salubrinal (Sal), an established ER stress inhibitor, can inhibit eIF2S1 dephosphorylation and protect cells from ER stress mediated apoptosis [20]. As shown in (Fig. 3C) by MTT assay, the cell viability of sarsasapogenin-treated cells increased from 37% to 57% after pretreatment with 5 μM Sal. These results demonstrated that ER stress was partially involved in sarsasapogenin-induced apoptosis.

### *3.5. Sarsasapogenin-induced ER stress and mitochondrial dysfunction required ROS generation*

ROS and GSH levels are indexes of intracellular oxidative stress and reduced power, respectively. To determine whether sarsasapogenin induced a change in the intracellular redox state, ROS and GSH levels in the sarsasapogenin-treated cells were assessed. As shown in (Fig. 4A), after sarsasapogenin treatment, the ROS level in HeLa cells increased to 237%, and the GSH level decreased to

48%. To clearly substantiate the role of ROS in sarsasapogenin induced cancer cell death, we examined the viability of HeLa cells in the presence of NAC. As shown in (Fig. 4B), on removal of ROS by NAC, a significant increase in HeLa cell viability was observed, providing a broad indication of the role of ROS in HeLa cell viability. Tempted by the remarkable ability of ROS to induce apoptosis, we further moved on to investigate whether ROS was necessary for ER stress or mitochondrial dysfunction. We incubated HeLa cells with NAC, prior to sarsasapogenin treatment. The results showed that NAC significantly attenuated sarsasapogenin-induced activations of ATF-4, XBP-1s and CHOP (Fig. 4C). Next, we also investigated the effect of scavenging ROS by NAC on sarsasapogenin-induced mitochondrial dysfunction. Pretreatment with NAC prevented sarsasapogenin-induced mitochondrial membrane permeabilization (Fig. 4D) and inhibited the activation of caspase-9 and -3 (Fig. 4E). These results indicate that ROS play a major role in sarsasapogenin-induced ER stress and mitochondrial dysfunction in HeLa apoptosis process.

## 4. Discussion

The use of sarsasapogenin has been recommended because of its broad range of activities. However, the molecular events involved in in vitro models of various carcinomas are still unknown. In this study, we firstly elucidated that sarsasapogenin induced cytotoxic effects in human cervical cancer cells via cell cycle arrest, ROS-mediated mitochondrial pathway and ER stress pathway.

Endoplasmic reticulum (ER) serves a multiple of functions, including lipid synthesis, transport of glycoproteins destined for secretion and regulation of intracellular $Ca^{2+}$ [21]. Evidence is emerging that ER stress plays a crucial role in the regulation of apoptosis. It has been reported that ER stress triggers several specific signaling pathways, such as ER-associated protein degradation and the unfolded protein response (UPR) [22]. After sarsasapogenin exposure, PERK and eIF2S1 were phosphorylated at the early stage with the total protein level of PERK and eIF2S1 remaining unchanged. Moreover, ATF6 was cleaved to the activated form ATF6 fragmentation (Fig. 3B). The expression of ER-resident chaperones, such as GRP78 and GRP94, were also up-regulated (Fig. 3A). These signaling transduction pathways are the adaptation responses to relieve the burden of ER by interrupting the protein synthesis. If ER stress prolongs and becomes severe, these responses can trigger apoptosis. Sarsasapogenin also activated CHOP, a key transcription factor induced by ER stress, the overexpression of which could lead to growth arrest and apoptosis [23]. Besides, a significant increase in viability was noticed when we treated HeLa cells with sarsasapogenin and Sal, a selective phosphatase inhibitor of eIF2S1. All these above results suggested that ER stress was related to sarsasapogenin-induced apoptosis.

It is well known that ROS are mediators of intracellular signaling cascades, which induce the disintegration of mitochondrial membrane, following a whole train of mitochondria-associated events including apoptosis [24]. An excessive accumulation of ROS nevertheless leads to oxidative stress, cellular dysfunction and ultimately apoptosis or necrosis [25]. Here, we found that sarsasapogenin-induced apoptosis was associated with ROS generation, the activation of ER stress and the dysfunction of mitochondria. However, what are the relationships between these three signals? Sarsasapogenin induced the activation of UPR, the ER specific stress response at early stage and then activated CHOP, which may contribute to the initiation and augment of mitochondrial membrane permeabilization by dephosphorylation of Akt so as to mediate the apoptotic signals from ER to mitochondria [26]. The findings observed in previously studies confirmed that excessive production of ROS, which is known as an effective inducer of cell apoptosis, might be involved in the activation of ER stress-mediated apoptotic pathway [27]. Scavenging ROS by antioxidant NAC could attenuate sarsasapogenin-induced activation of UPR and cause a decrease of the level of CHOP. NAC also effectively prevented the disruption of mitochondrial transmembrane potential and the activation of caspase-9 and -3. Overall, these results indicated that the generation of ROS is an early event that initiates mitochondria and ER stress-mediated apoptotic pathways in HeLa cells.

In summary, our results demonstrate that sarsasapogenin induce cell cycle arrest in G2/M phase and apoptosis via the

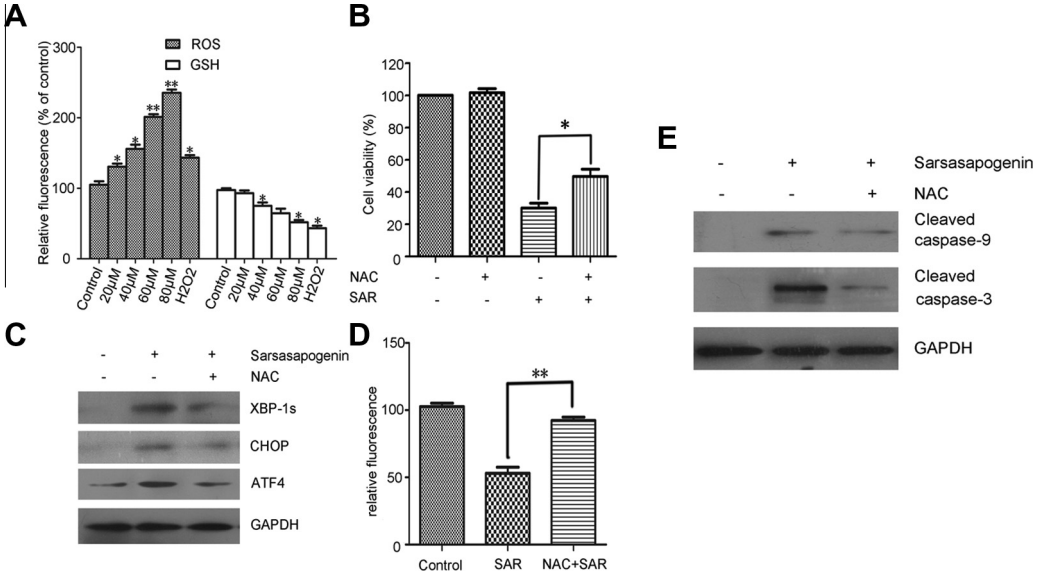


**Fig. 4.** Sarsasapogenin-induced ROS generation triggered ER stress and mitochondrial dysfunction. (A) The ROS and GSH levels were measured using DHR123 and NDA, respectively. PBS was used as a bank control, and 10 μM $H_2O_2$ (2 h incubation) was used as a positive control. Each bar represents the mean ± SD from six experiments ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs. PBS control). (B) The viability of HeLa cells was measured by MTT analysis following 48 h of culture with sarsasapogenin (60 μM) with and without ROS scavenger NAC (5 mM). Each bar represents the mean ± SD from six experiments ($^{*}p < 0.05$ vs. sarsasapogenin treatment). The cells were exposed to 60 μM UA with and without NAC (5 mM) for 48 h. (C) Immunoblot analysis for ER stress markers was performed. (D) FACS analysis of mitochondrial membrane potential after treatment. Each bar represents the mean ± SD from six experiments ($^{**}p < 0.01$ vs. sarsasapogenin treatment). (E) Immunoblot analysis for cleaved caspase-9 and -3 was performed.

caspase-dependent mitochondrial pathway and ER stress pathway. The generation of ROS is necessary for the activation of ER stress and the dysfunction of mitochondria. However, the relationship between oxidative stress and expression of cell-cycle regulatory proteins require further investigations.

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