

Original Article

ROS attenuates the antitumor effect of Raddeanin on ovarian cancer cells Skov3

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Abstract: Epithelial ovarian cancer is the fourth commonest cause of female cancer death, but no proper evidence had proved that surgery could prolong the survival time. Hence, new effective chemotherapy is necessary to improve the survival. Raddeanin A (RA), anoleanane-type triterpenoid saponin, is isolated from *Anemone raddeana*. Previous study had proved that RA exerted antitumor activity through inhibiting proliferation and promoting apoptosis of some kinds of cancer cell. ROS is a double-edged sword for tumors and might contribute to therapy resistant. In this study, we discuss at the first time whether ROS was involved in the antitumor effect of RA on Skov3 cells, and analysis the mechanism. The results showed that after be treated by RA, the proliferation of Skov3 was inhibited, and this effect can be enhanced by ROS inhibitor NAC. Pretreated with NAC can enhance the cell cycle block but not apoptosis induced by RA. Moreover, as a by-product of RA, ROS induced autophagy can attenuate RA's antitumor activity, and autophagy inhibitor 3-MA could recover RA's antitumor effect. These results demonstrated that ROS and autophagy could be considered as two pro-tumor factors in some conditions. The combination of RA and ROS inhibitor or autophagy inhibitor or both of them may be the novel strategies at least in ovarian cancer therapy.

Keywords: ROS, Raddeanin A (RA), epithelial ovarian cancer, Skov3 cells, autophagy, cell cycle

Introduction

Epithelial ovarian cancer is the fourth commonest cause of female cancer death in the developed country. One of the reasons for the high death rate is the recurrence, especially in the patients with platinum-resistant [1]. The median survival time of those recurrent ovarian cancer (ROC) patients was only 12 months. Unfortunately, there is no proper evidence to prove that surgery could prolong the survival time of ROC patients [2]. Hence, it is necessary to found new effective chemotherapy for improving survival.

Anemone raddeana Regel, which has been included in Chinese Pharmacopeia, is a traditional Chinese medical herb for the treatment of cancer. Among all of the identified oleanane-typed saponins from it, Raddeanin A (RA) have been reported with the antitumor activity for

some kinds of cancer cells [3, 4]. Previous studies had reported the significant effect of RA on the growth of the tumor cells such as liver cancer, lung cancer and gastric cancer cells [4]. What's more, Ying-Yun Guan has found that RA can suppress the angiogenesis and growth of human colorectal tumor by inhibiting VEGFR signaling pathway [5]. Our previous study has proven the antitumor activity of RA on Skov3, an ovarian cancer cell line, but the factors influencing its biological activity are uncertain.

ROS, a group of molecules including superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}) and hydrogen peroxide (H_2O_2), can be involved in oxidative stress, leading to proteins, lipids and DNA damage [6]. Interestingly, research has also reported that ROS could promote tumor cells to survival [7], which indicated that ROS might induce the drug resistance.

ROS attenuates the effect of Raddeanin on Skov3

As a tumor regulator, ROS can mediate not only cell proliferation, but also autophagy [8]. Autophagy is the process by which cells recycle cytoplasm and dispose of excess or defective organelles [9]. Autophagy can act as a positive or negative role for the proliferation of tumor, which seems to be correlated to autophagy levels induced by ROS accumulation at different stages of tumor growth [10, 11]. On one hand, autophagy protects against the production of ROS that induces the cell damage and DNA mutation described to induce tumorigenesis [12, 13]. However, during the later stages of tumor process, autophagy is required for promoting cell tolerance to stress caused by chemical drugs [6]. The role of autophagy in cancer chemotherapy is complex. The relation of RA resistance and ROS-induced autophagy is unclear.

In this study, NAC and 3-MA were used representatively to test whether ROS and ROS-induced autophagy could influence the chemotherapeutic effect of RA via comparing the regulation of autophagy proteins and the effects of cell proliferation, cell apoptosis, and cell cycle of Skov3 *in vitro*.

Materials and methods

Ovarian cancer cell line skove3

Skov3 cells were obtained from the American Type Culture Collection (Bethesda, BD). The cell lines were cultured in IMDM medium supplemented with 10% fetal bovine serum (Gibicol, MD) and maintained in a humidified cell incubator with 5% CO₂ at 37°C.

Reagents and chemicals

RA was purchased from Yuanye bio-technology (Shanghai, China), NAC was purchased from Biyuntian bio-Technology (Shanghai, China), 3-MA were purchased from Selleck chemicals (Houston, TX, USA).

MTT assay

In vitro cytotoxicity of RA in ovarian cancer cell Skov3 were measured by MTT (3-[4,5-diethyl, as previously-thiazol-2-yl]-2,5-diphenyltetrazolium-bromide, Sigma-Aldrich, St Louis, MO, USA) reagent, as previously described [14]. Briefly, ovarian cancer cells were cultured in 96-well

plates with 3×10^3 per well and incubated at 37°C in the presence of variable concentrations of RA with 0 μmol/L, 2.5 μmol/L and 5 μmol/L. After 48 h, MTT was added to a final concentration of 1 mM. After 4 hours, formazan crystals were dissolved by the addition of DMSO. Optical densities were measured using a visible light microplate reader at 490 nm. The data for the cell lines are presented as mean ± standard error from 3 independent experiments.

Analysis of apoptosis and cell cycle

Ovarian cancer cells were treated with the variable concentrations of RA for 3 and 6 hours. The cells were harvested and fixed with ice-cold 70% (v/v) alcohol for 24 h. The cells were pelleted, washed with PBS, and resuspended in PBS containing 50 μg/mL propidium iodide (PI), 0.1% Triton X-100 (v/v), and 1 μg/mL DNase-free RNase. DNA content was determined by flow cytometry analysis using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) as previously described [14]. Cell cycle analysis was performed using Multicycle software (Phoenix Flow Systems, Inc., San Diego, CA, USA).

To detect apoptosis, Skov3 cells treated with RA were harvested and centrifuged at 1000 g for 10 min. The cell pellets were suspended into 500 μl binding buffer, and incubated with FITC-conjugated annexin V (Sanjian, Tianjin, China) and propidium iodide (PI) at room temperature in dark for 15 min. Then, the samples were analyzed by flow cytometry. Early apoptosis cells with PI-negative staining and annexin V-positive staining were enumerated [15].

DHE staining

Ovarian cancer cells were treated with the indicated drugs for 3 hours or 6 hours at 37°C. After were washed with PBS twice, the cells were incubated with DHE at 37°C for 30 minutes and then visualized with fluorescence microscope.

DCHF-DA staining

Skov3 cells were seeded at a density of 3×10^5 cells per well and then were treated with various indicated treatments. All the cells were harvested in full serum media, spun down for 5

ROS attenuates the effect of Raddeanin on Skov3

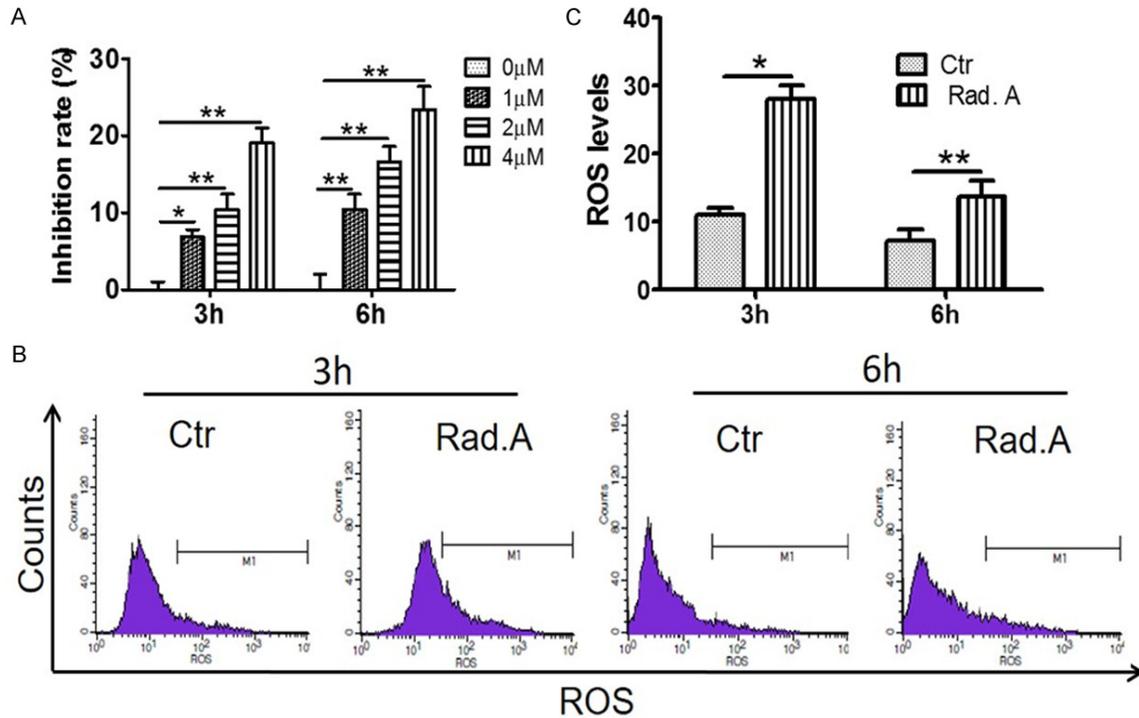


Figure 1. The inhibition rate and ROS level in Skov3 cells after exposing to RA for 3 h and 6 h. A. MTT assay of Skov3 cells treated with RA (0, 1, 2 and 4 μ M for 3 h and 6 h). Either 3 hours or 6 hours after treatment, the inhibition rates of Skov3 at distinct treatment groups (1 μ M, 2 μ M and 4 μ M) were all significantly increased compared to the control group (0 μ M). B and C. Flow cytometry data with DCFH-DA staining for ROS level in the cells treated with RA (4 μ M for 3 h and 6 h). The production of ROS in RA groups was significantly increased compared to that of control group, and the 6 h group showed significant reduction in ROS level increase as compared to 3 h group.

minutes at 1000 rpm and resuspended in PBS containing 10 μ M DCFH-DA. Cells were maintained at 37°C in dark for 30 minutes. Then cells were pelleted and resuspended in PBS and finally analyzed by flow cytometry (Becton Dickinson model FACS Calibur) [16].

AO staining

The Skov3 cells were treated with indicated different treatments for indicated time points followed by staining with 1 μ M acridine orange for 15 min. Cells were then washed with PBS and visualized with fluorescence microscope [17].

Western blot analysis

Soluble proteins were extracted (in the presence of complete protease and phosphatase inhibitors, Roche Applied Sciences, Indianapolis, IN, USA) and subjected to SDS-polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF)

membranes (Thermo Fisher Inc., Rockford, IL, USA) and immunoblotted with anti-p53, -p21, -ATR, -CDK4, -cyclin B1, -Chk1 or -beta-actin antibody (proteintech group, USA), as previously described [14]. Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA), as described by the manufacturer.

Statistical analysis

Data are presented as the mean \pm SE and represent three independent experiments. Statistical analyses were performed with SPSS17.0, difference among the groups was compared with one-factor analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant.

Results

The inhibition rate of RA may be regulated by the ROS level of Skov3 in vitro

Our previous study proved the inhibition activity of RA on Skov3, to further identify the sen-

ROS attenuates the effect of Raddeanin on Skov3

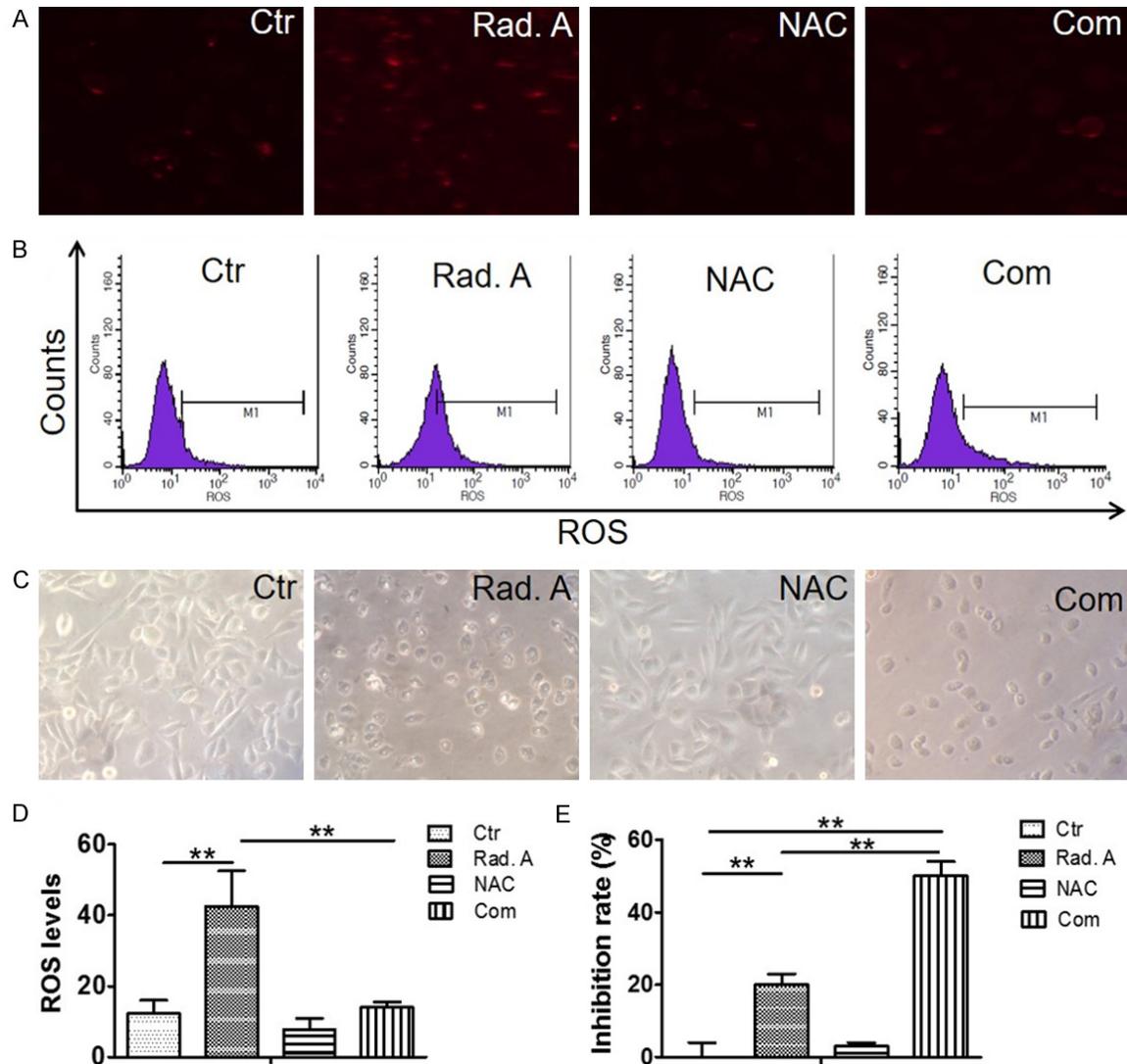


Figure 2. ROS decrease the inhibition rate induced by RA in Skov3 cells. A. DHE staining of ROS levels in four groups: control group, RA group (4 μ M for 6 h), NAC group (0.01 mM for 6 h) and combine group (0.01 mM NAC pretreatment for 1 h and 4 μ M RA treatment for 6 h). The elevated levels of superoxide were observed in RA group as compared to the control and combine groups. B and D. Flow cytometry data with DCFH-DA staining for ROS level in the cells treated by different ways. The levels of ROS in the control and combine group were lower than the RA group. C. Effect of different treatments on the morphological changes under the light microscope. The percentage of cells with rounded morphology in combination group was significantly increased compared to that of RA group. E. MTT assay of Skov3 cells treated with the four treatments. The inhibition rate of combine group was significantly increased compared to that of RA group.

sitivity of Skov3 to RA in different times, here, MTT assay was used to detect the survival cells in each groups. As shown in **Figure 1A**, either 3 hours or 6 hours after treatment, the inhibition rates of Skov3 treated with RA at distinct dose groups (1 μ M, 2 μ M and 4 μ M) were all significantly increased compared to the control group (0 μ M) respectively. Interestingly, the inhibition rates were significantly increased in 6 h groups compared to that in 3 h groups. The data sug-

gested that RA at different doses could inhibit the proliferation of Skov3 and the anti-proliferation effect would be enhanced after treatment for 6 hours.

To explore whether ROS was participate in the mechanism of the anti-proliferation effect, flow cytometry was employed to detect the ROS level. The result showed that ROS in RA groups (3 h and 6 h) was significantly increased com-

ROS attenuates the effect of Raddeanin on Skov3

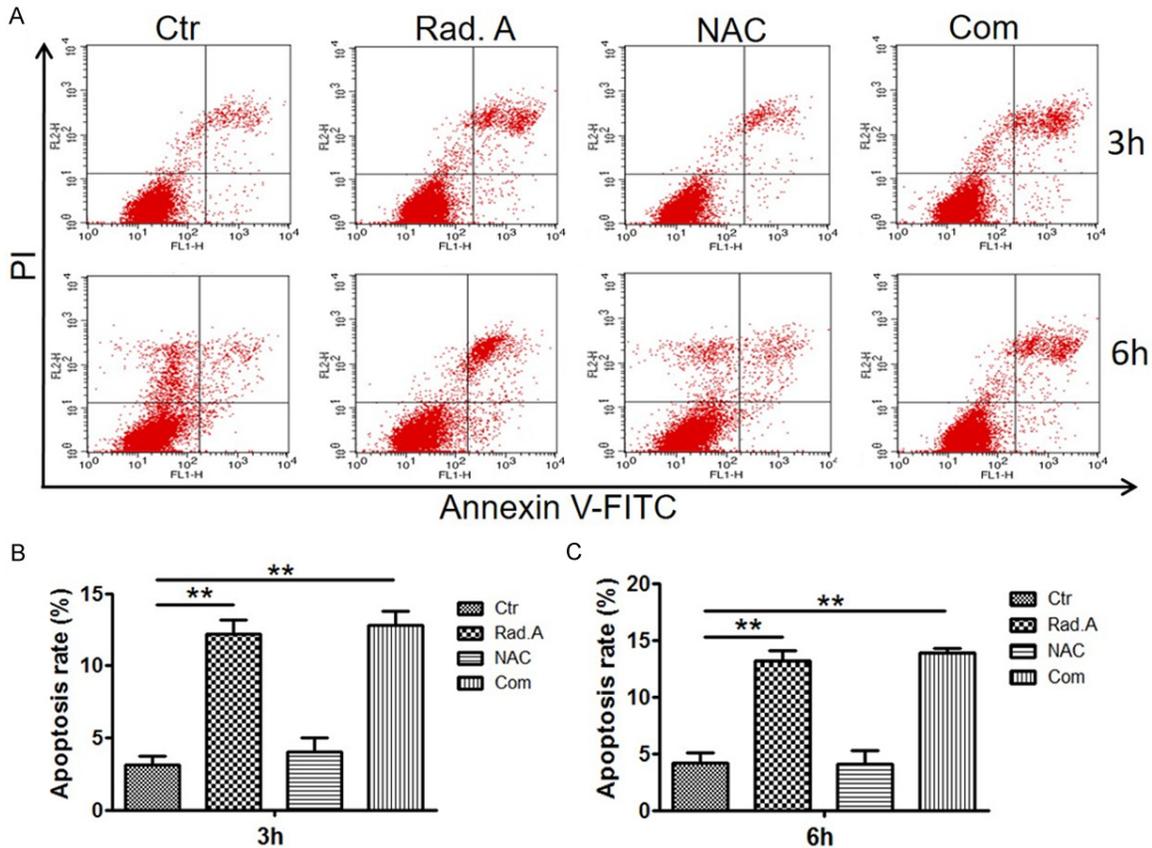


Figure 3. The decrease of inhibition rate caused by ROS was not via the apoptosis mechanism in Skov3 cells. A. FCM raw data with Annexin-V-FITC and PI staining cell cycle arrest. B and C. The apoptosis rates were not significantly increased in combine group (3 h or 6 h) as compared to RA (4 μ M) group (3 h or 6 h).

pared to that of control group (Figure 1B and 1C). However, ROS lever in 6 h group treated with RA decreased significantly compared to 3 h group. All these findings implied that the increase of the sensitivity of Skov3 to RA might be relevant to the decrease of ROS.

RA combined with NAC can enhance the sensitivity of Skov3 to RA

To further identify the role of ROS in the sensitivity of Skov3 to RA, NAC (0.01 mM for 1 h) was used to suppress ROS. Flow cytometry and DHE staining results showed that the level of ROS was significantly decreased in NAC group as compared to control group. Moreover, ROS level was significantly increased in RA group as compared to control group, whereas ROS was significantly reduced in the cells implemented with the combinational treatment as compared to the cells treated with RA alone (Figure 2A, 2B and 2D). Blow the graph, the percentage of

cells with rounded morphology in combination group was significantly increased compared to that of RA group (Figure 2C). MTT results showed that the inhibition rate of combine group was significantly increased compared to that of RA group (Figure 2E). All these results suggested that RA combined with NAC had effectively inhibited the ROS, which could increase the inhibition rates of RA in Skov3.

The inhibition of ROS enhance the sensitivity of Skov3 to RA may not via cell apoptosis mechanism but via cell cycle

To further elucidate the mechanism involved in ROS inhibition mediated enhancement of sensitivity to RA in Skov3, flow cytometry was employed to detect the apoptosis rate of the cells. As shown in Figure 3, the apoptosis rates of Skov3 were all significantly increased in RA group (4 μ M for 3 or 6 h) compared to that in control group. However, the apoptosis rates

ROS attenuates the effect of Raddeanin on Skov3

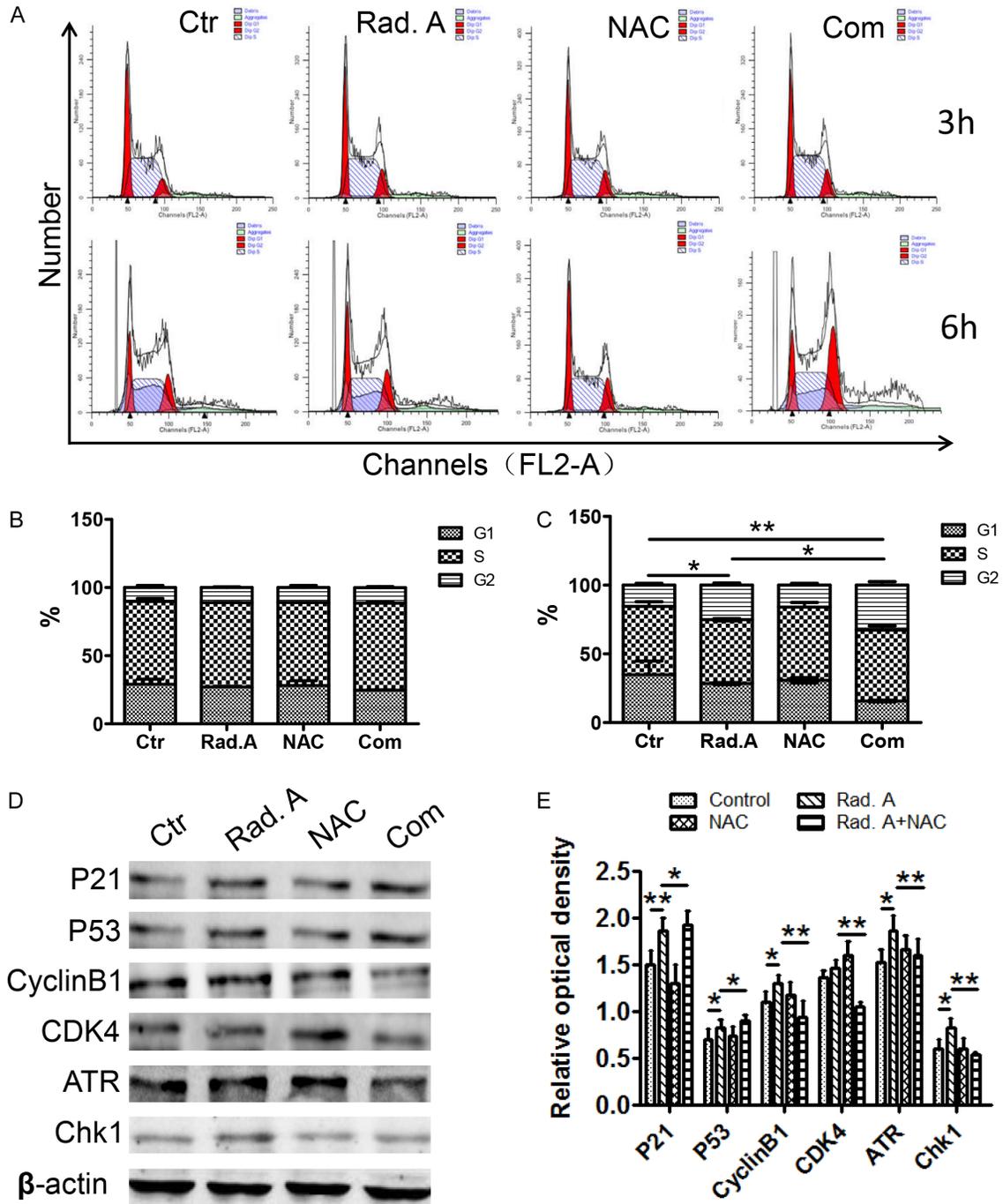


Figure 4. The decrease of inhibition rate caused by ROS was related to the cell cycle arrest mechanism in Skov3 cells. A. FCM raw data with PI staining for cell cycle arrest. B. Values represent the percentage of the cells in each cell cycle phase in 3 h group. There was no significantly difference among the groups after treated for 3 h. C. Values represent the percentage of the cells in each cell cycle phase in 6 h group. After treated by NAC (0.01 mM for 1 h) combined with RA (4 μ M for 6 h), population of the cells in G1 phase significantly decreased but that in G2 phase significantly increased as compared to the cells treated with RA alone. D. Western blot assay for protein expression of p53, p21, CDK4, ATR, Chk1 and cyclin B1. E. Relative optical density (OD) value from each group was showed as average from Western blot analysis. p53 and p21 were up-regulated but ATR, cyclin B1, CDK4 and Chk1 were down-regulated in combination group compared to that of RA group.

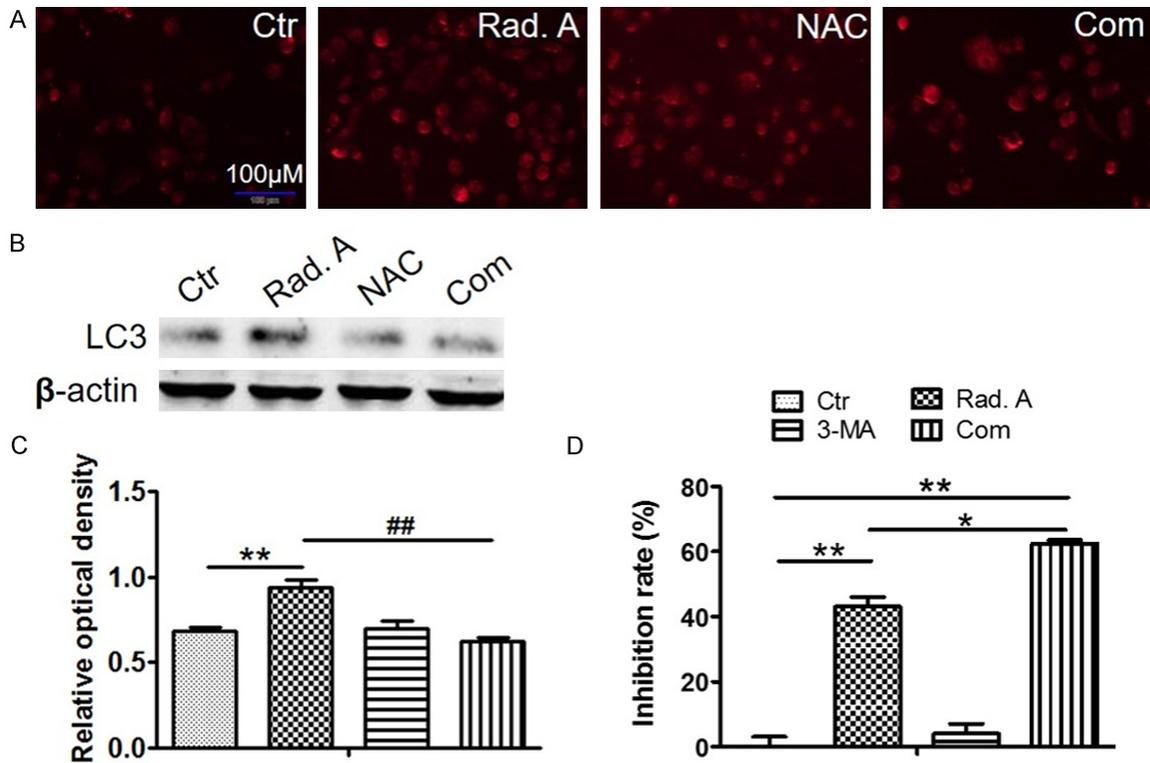


Figure 5. RA increased autophagy level through producing low level ROS. A. AO staining of autophagy levels in four treatments in 6 h. The elevated levels of autophagy were observed in RA (4 μ M for 6 h) group as compared to the NAC (0.01 mM for 1 h) and combine groups. B. Western blot assay for protein expression of LC3. C. Relative optical density (OD) value from each group was showed as average from Western blot analysis. The cells implemented with the combination showed lower expression of LC3 protein than the cells only treated with RA. D. MTT assay of Skov3 cells treated with four groups: control group, RA group (4 μ M for 6 h), 3-MA group (1 mM for 6 h) and combine group (1 mM 3-MA pretreatment for 1 h and 4 μ M treatment for 6 h). The inhibition rate was significantly increased in the cells treated by RA combined with 3-MA compared to that of the cells in RA group.

were not significantly increased in the combine group (3 h or 6 h) as compared to RA group (4 μ M for 3 or 6 h). These results indicated that the apoptosis process might not involve in the mechanism that mediated the enhancement of sensitivity to RA in Skov3 via inhibiting ROS.

To testify whether the mechanism is related to cell cycle block, flow cytometry was employed to detect the population of cells in G0/G1, S and G2/M phases. The results showed that after treating by NAC (0.01 mM for 1 h) combined with RA (4 μ M for 3 or 6 h), population of the cells in G1 phase significantly decreased but that in G2 phase significantly increased as compared to the cells treated with RA alone in 6 h group (Figure 4A and 4C). However, there was no significantly difference in 3 h group (Figure 4A and 4B). Additionally, Western blot was employed to detect the expressions of p53, p21, CDK4, ATR, Chk1 and cyclin B1, which are cell cycle regulatory proteins. The results showed that p53 and p21 were up-

regulated but ATR, cyclin B1, CDK4 and Chk1 were down-regulated in combination group compared to that of RA group, which indicated that the inhibition of ROS could down-regulate the expressions of ATR, cyclin B1, CDK4 and Chk1 but up-regulate the expressions of p53 and p21 (Figure 4D and 4E). These implied that the inhibition of ROS could inhibit Skov3 going through the G2/M checkpoint and then induce cell cycle arrest. So, we can draw a conclusion that the decreasing of ROS induced by RA could block cell cycle but has no remarkable effect of apoptosis. In other words, the inhibition of ROS could increase the sensitivity of Skov3 to RA through blocking cell cycle.

RA combined with NAC can increase the inhibition rate through inhibiting autophagy

To illuminate another ROS-related mechanism that reinforced the chemosensitivity of RA in

Skov3, AO staining, MTT and Western blot were employed to detect the level of autophagy, the inhibition rate of RA and the expression of LC3, a representative protein of autophagy. The cells implemented with the combination of NAC (0.01 mM for 1 h) and RA (4 μ M for 6 h) showed lower expression of LC3 protein than the cells only treated with RA and the elevated levels of superoxide were observed in the RA group as compared to the NAC and the combine groups. (**Figure 5A-C**), which was accordant with the changes of ROS level (**Figure 2A** and **2B**). Furthermore, the inhibition rate was significantly increased in the cells treated by RA combined with 3-MA (1 mM for 1 h pretreatment), an autophagy suppression, compared to that of the cells in RA group. Taken together, these results suggested that ROS could impact the chemosensitivity of RA in Skov3 through regulating autophagy mechanism.

Discussion

The progress of tumor is directly related to the proliferation of cells. This study found that RA could inhibit the proliferation of Skov3 *in vitro*. ROS is caused by an imbalance in the redox status of the body. The increasing of ROS can lead to tissue damage, and thus stimulate the uncontrolled growth of cells which initiate the process of carcinogenesis [18]. But ROS is a double-edged sword for tumors and might contribute to therapy resistant [19]. In many kinds of tumors, the high level ROS production had been considered as a critical factor in the mechanism of anti-tumor effect of chemotherapy or radiotherapy [20-22]. However, low level ROS mediated cellular proliferation process, including acceleration of cell cycle, induction of autophagy and metastasis, improvement of tumor cells survive. And ROS was reported to play a key role in the initiation and progression of various types of cancers [7, 23-25].

The sensitivity of tumor to chemotherapy is based on the inhibition rate of proliferation. In this study, the sensitivity of Skov3 to RA was enhanced after be treated for 6 hours compared to 3 hours, whereas the level of ROS was simultaneously decreased (**Figure 1A-C**). Moreover, the increasing of inhibition rate of RA in Skov3 was definitely related to the decreasing of ROS (**Figure 2**). Which indicate that although RA could inhibit the development of Skov3, its by-product such as low level

ROS can promote the proliferation of Skov3, which might be one of the therapy resistance mechanism of RA. Moreover, pre-treating with NAC enhanced the anti-proliferation effect of RA, indicated that the sensitivity of Skov3 to RA was potentiated when the cells lost the protection of ROS. This also highlights the advantage that a positive impact in inhibition rate of NAC-pretreatment which directly decrease the ROS level in Skov3 cells.

The concept that programmed cell death by apoptosis serves as a natural barrier to cancer development has been established by many researchers over the last two decades [26]. The induction of apoptosis has been regarded as the key mechanism of anti-tumor chemotherapy [27]. High level ROS caused by most chemotherapy lead to cell apoptosis [28]. However, no significant decrease of apoptosis rates was detected in the combination groups compared to the RA group, after be treated for either 3 h or 6 h in this study (**Figure 3**), implying that the increasing of apoptosis rate in Skov3 induced by RA was not through producing high level ROS. Moreover, low level ROS could promote the cell division [28], which was accordant to our research (**Figure 2**). Nevertheless, the apoptosis rates were not increased in NAC-pretreatment groups (**Figure 3**), which indicated that the inhibition of ROS enhanced the sensitivity of Skov3 to RA was not via cell apoptosis mechanism.

Although the regulation mechanism of ROS in tumors is not completely clear, more and more studies stressed that ROS's function as a tumor regulator seemed to occur at multiple mechanisms, such as apoptosis, cell cycle and autophagy. Thus, we supposed whether ROS inhibition enhanced the anti-tumor effect of RA in Skov3 via block cell cycle. Normal cell progression from G1-S-G2-M-G0 during the division cycle is tightly controlled by the cyclins, the cyclin-dependent kinase inhibitor (CKI) and the cyclin-dependent kinases (CDKs). This study proved that RA could induce G2/M arrest of Skov3 cells by down-regulating the level of Cyclin B1, and the protein complex of cyclin B1 and CDK1 urges cells through G2/M phase [29]. P21 and P53 are two major members of CKI family. P21 is an efficient inhibitor of CDK4, and it can arrest cell in G2 when overexpressed. The G2/M checkpoint, an important site that prevents cells from entering mitosis when DNA

is damaged, is mediated by the activation and accumulation of p53, which then up-regulates the p21 to maintain G2 arrest in response to DNA damage [30]. Recently, it had been reported that the production of low level ROS could promote the cell cycle process through inducing the transcription and translation of the cyclins and CDKs [31]. In this study, western blot results showed that the level of cyclin B1 was significantly decreased in combined group compared to RA group and the expression of p53/p21 was significantly up-regulated in combined group compared with RA group (**Figure 4D** and **4E**), which was correspond to the G2/M arrest results (**Figure 4A** and **4C**). These findings suggested that while RA was effective for holding the Skov3 cells at the G2 phase, its effect had been weakened by the low level ROS. In other words, it was well in line with our previous idea that ROS inhibition enhanced the anti-tumor effect of RA in Skov3 via cell cycle block mechanism.

The checkpoint kinase 1 (Chk1) is an essential kinase that is phosphorylated by ataxia-telangiectasia-mutated and Rad-3 related (ATR) and required for the G2/M DNA damage checkpoint, and it was regarded as a tumor suppressor for many years [32]. However, increasing evidence suggests that Chk1 may actually promote tumor growth, and the enhanced activation of Chk1 led to resistance of cancer cells, including prostate and lung cancer, to chemotherapy [33]. In this study, the increasing of Chk1/ATR expression (**Figure 4D** and **4E**) directly or indirectly caused by the ROS, by-product of RA, possessed survival advantages over Skov3 cells, because the more Chk1 protein Skov3 had, the better they were to facilitate the DNA damage in G2 phase caused by the RA chemotherapy [30, 33]. All these observations implied that low level ROS promoted Skov3 survive and proliferation through negatively impacting the sensitive of Skov3 to RA via cell cycle mechanism. This model provided a strong support to target ROS in human ovarian cancer therapy.

The another regulation mechanism of ROS in tumor is autophagy which is regulated by numerous stresses such as hypoxia, ROS level and chemotherapies [6]. Alterations in ROS levels and autophagy play a crucial role in cancer initiation and progression, and both are recog-

nized as the potential targets for cancer treatment. It had been reported that Ciclopiroxolamine (CPX) could induce autophagy in human rhabdomyosarcoma cells by ROS [8]. Hahm et al found that the use of antioxidants in prostate cancer inhibited autophagy levels [34]. In the present study, for the first time, we found that RA induced LC3 expression, which was representative for autophagy, in Skov3 cells. Interestingly, low level ROS was simultaneously increased by RA as previous describe. Thus, NAC, the ROS scavenger, was used to identified the relation between ROS and autophagy. Our results that NAC remarkably attenuated RA-induced LC3 expression suggested that RA triggered autophagy by induction of low level ROS (**Figure 5A-C**). In order to further discuss the RA resistance mechanism caused by low level ROS in Skov3, 3-MA, an autophagy inhibitor was employed to pretreat the Skov3 cells. The present observations that the disruption of autophagy in the presence of 3-MA increased the RA-induced inhibition rate of Skov3 cells clearly indicated that RA-induced autophagy was a drug resistance mechanism at least in ovarian cancer cells (**Figure 5D**). All these findings identified that the autophagy induced by low level ROS produced by RA was a potential drug resistance mechanism in the RA therapy of ovarian cancer, and we also provided a promising strategy for ovarian cancer therapy that the combination of RA and an autophagy inhibitor.

In conclusion, this study showed that low level ROS induced autophagy and then led to the decrease of inhibition rates of RA in human ovarian cancer Skov3 cells. Disruption of ROS using NAC increased RA-induced inhibition rates, indicating that RA-induced low level ROS was a pro-proliferation mechanism in Skov3 cells. Further, we found that the mechanism was not related to the apoptosis pathway, but the dysregulation of cell-cycle arrest in G2 phase. Additionally, the ROS-mediated autophagy was involved in our study. 3-MA, an inhibitor of autophagy, induced the inhibition rates of RA, suggesting that ROS-mediated autophagy was a pro-survival mechanism in the cells. Our findings suggested that the combination of RA and ROS inhibitor or autophagy inhibitor or both of them may be the promising strategies at least in ovarian cancer therapy.

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Disclosure of conflict of interest

None.

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ROS attenuates the effect of Raddeanin on Skov3

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