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Original Article

Alantolactone and ZnO nanoparticles induce apoptosis activity of cisplatin in an ovarian cancer cell line (SKOV3)

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Abstract

Background and purpose: Ovarian cancer is one of the leading causes of cancer mortality in women. Despite the increase in cases of this cancer, the current therapeutic strategy is not effective. This study aimed to investigate the effect of cisplatin (Cis) with alantolactone (ALT) and ZnO nanoparticles (ZnONPs) in inducing apoptosis in SKOV3 ovarian cancer cells line.
Experimental approach: To evaluate the viability of SKOV3 cells and determine the IC_{50} of Cis, ALT, and ZnONPs, MTT assay was used. Real-time PCR and western blotting were used to evaluate the expression levels of genes (XIAP, cyclin D1, Bcl-2, Bax, and MDM2) and proteins (XIAP, cyclin D1, Bcl-2, Bax), respectively. Also, cellular ROS levels were assessed by fluorimetry.
Findings / Results: Our results showed that ALT and ZnONPs significantly increased the response to Cis in SKOV3 cells compared to the control and this response is remarkably increased in the triple combination (ALT-Cis-ZnONPs). The expression of XIAP, cyclin D1, and Bcl-2 genes and proteins in the groups treated with ALT, Cis, and ZnONPs as a single agent, double and triple combination were significantly reduced compared to the control, while Bax was generally shown an increase. Also, the level of intracellular ROS is higher in the treatment groups than in the control group and the highest increase was observed in the triple combination.
Conclusion and implications: Taken together, our data demonstrated the potential therapeutic approach of using ALT and ZnONPs that may enhance the apoptotic effects of Cis on the SKOV3 cells.

Keywords: Alantolactone; Apoptosis; Cisplatin; Ovarian cancer; SKOV3; ZnONPs.

INTRODUCTION

Ovarian cancer is one of the deadliest gynecology-related cancers. The incidence risk of ovarian cancer in women is approximately 1.5% and the risk of death is approximately 1% (1-4). A growing body of evidence showed that changes in the pattern of gene expression in cancer cells are based on the progression of tumors. Platinum-based antitumor drugs such as cisplatin (Cis) and carboplatin are common therapeutic strategies for ovarian cancer. However, as treatment progresses, most patients lose their sensitivity to these agents. One of the main limitations in the treatment process for patients with ovarian cancer is their resistance to chemotherapy compounds (5). For this reason, it is necessary to provide new strategies to deal with resistance to chemotherapy. Current treatment protocols are associated with numerous side effects in patients with ovarian cancer, so much attention has been paid to the medicinal value of natural compounds and herbs, and these compounds have been used successfully in the treatment of cancer and other diseases.

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The cytotoxic effects of various herbal medicines such as phenolic acids, flavonoids, and sesquiterpenes on cancer cells have been demonstrated through different mechanisms (6).

Sesquiterpene lactones (SLS) include a large group of secondary metabolites that have a variety of biological activities. These molecules are the largest group of natural compounds that have different structures and are mostly identified in Asteraceae. These compounds have been considered because of their high therapeutic activities (7,8). In recent years, the anticancer properties of SLS have attracted the attention of many researchers. One of these compounds, alantolactone (ATL), is a natural compound of the SLS family, which is the main medicinal component of the plant *Inula helenium* and it has various biological activities such as anti-inflammatory, antibacterial, antifungal, antiseptic, and anticancer properties (9,10). Studies have shown that ATL has anticancer activity against various human tumor cells, such as colorectal, cervical, gastric, lung, liver, and breast cancer cells through a variety of mechanisms, including increasing the production of reactive oxygen species (ROS) (11), induction cell cycle arrest, glutathione depletion, inhibition of nuclear factor kappa B (NF-κB), Signal transducer and activator of transcription 3 (STAT3), and Bax/Bcl signaling pathways (12,13).

In recent years, the use of nanotechnology in medical processes is also growing. Therefore, a better understanding of the mechanisms of interaction between nanoparticles and living tissues is essential to assess the biological consequences associated with nanotechnologies. Physicochemical and biochemical properties of nanoparticles, such as surface properties, charge, size, or adsorption of biological components are important factors that play a role in their interaction with cells, including cell stress reactions and biological properties of cells (14). Zinc oxide nanoparticles (ZnONPs) are one of the compounds recognized by the Food and Drug Organization as safe compounds. The ZnONP is a biocompatible and nontoxic nanoparticle that has also been used as the carrier of drugs. Some of ZnONPs properties are high chemical stability, low dielectric constant, high catalytic activity, absorption of infrared and ultraviolet light, and most importantly its antibacterial features (15). Compared to other nanomaterials, ZnONPs have been considered by researchers due to their low toxicity and higher adsorption power (16,17). Various studies in recent years have shown that ZnONPs exert their antitumor effects through intracellular ROS production and reduction of reduced glutathione-glutathione disulfide (GSH-GSSG) ratio (18). Also, compared to normal cells, ZnONPs have selective cytotoxicity 28-35 times against cancer cells (19). This property identifies zinc nanoparticles as a potential antitumor agent for the treatment of various tumors.

Given that studies have been performed to investigate the antitumor mechanisms of Cis, ALT, and ZnONPs show that these compounds use common mechanisms to induce apoptosis. Therefore, we aimed to investigate the effects of concomitant use of Cis with ALT and ZnONPs on SKOV3 ovarian cancer cells and evaluate some factors involved in the apoptotic and B-cell lymphoma protein 2 (Bcl-2)-associated X (Bax)/Bcl-2 pathway. Combination therapy with the simultaneous use of two or more drugs with nanoparticles is a potential tool to overcome drug resistance which can significantly improve therapeutic efficacy by applying synergistic effects and eliminating unwanted side effects. In addition, targeting multiple signaling pathways may be a new model for cancer treatment.

MATERIALS AND METHODS

Reagents

Dulbecco's modified eagles medium (DMEM) and fetal bovine serum (FBS) were provided from Gibco (Invitrogen, UK). Penicillin G, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained by Sigma-Aldrich (St Louis, MO, USA). ALT (Cat. No: SML0415-Germany) was dissolved in DMSO and then diluted in a cell culture medium to make working solutions. ZnONPs used in this study were purchased from US Research Nanomaterials (USA; stock # us355) (Fig. 1).

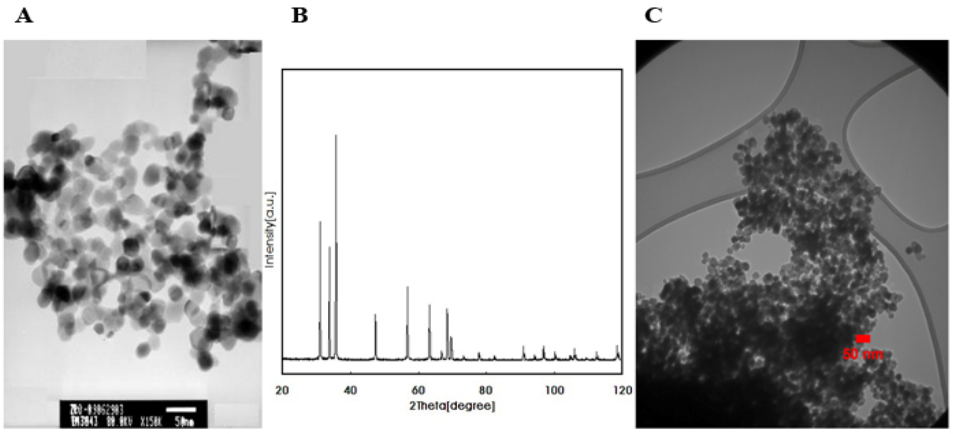

Fig. 1. (A) SEM image, (B) XRD pattern, and (C) TEM image of the ZnO nanoparticles at a scale of 50 nm.

Penicillin-streptomycin, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Biowest (France). General kit (RiboEx LS Total RNA) was obtained from South Korea. The first-strand cDNA synthesis kit and SYBR Green polymerase chain reaction (PCR) master mix were purchased from Yekta Tajhiz Azma, Iran. Primary antibodies against β-actin, Bax, Bcl-2, cyclin D1, and X-linked inhibitor of apoptosis (XIAP) were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Cell culture

The human ovarian cancer cell line, SKOV3 was obtained from Pasteur Institute cell bank of Iran (Tehran, Iran) and cultured under standard cell culture conditions in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin at a temperature of 37 °C in a humidified incubator with a 5% CO2 atmosphere. Subculture was performed when cells reached 80-90% confluence.

Cell viability assay

To measure the effect of Cis, ALT, and ZnONPs on the viability of SKOV3 cell line, MTT assay was used. For this assay, 4×10^3 cells/well were seeded in a 96-well plate and cultured. This cells were treated with ALT and Cis at different concentrations (0, 1, 5, 10, 25, 50, 75, 100, and 150 µM) and also ZnONPs at 0, 5, 10, 25, 50, 100, 200, 250, and 300 µM, for 24, 48, and 72 h. Control cells were also treated with DMSO. Then, 24 h were selected as the best time among these times, and finally to evaluate the cell survival, the cells which were categorized in the following 6 groups were treated with the calculated IC_{50} of ALT, Cis, and ZnONP for 24 h. The cells in groups 1-3 were treated with IC_{50} of Cis, ALT, and ZnONP, as single agent, respectively; double-combination treatments were including group 4, IC_{50} of Cis + IC_{50} of ALT; group 5, 0.5 IC_{50} of (Cis + ALT); group 6, 0.5 IC_{50} of (Cis + ZnONPs); and group 7, as triple-combination treatment, 0.5 IC_{50} of (Cis + ALT + ZnONPs).

Briefly, after the treatment, the plate was removed from the incubator and the culture medium was poured out and 40 µL of MTT solution was added to the wells. The plate was kept at 37 °C for 4 h. After 4 h, 100 µL of DMSO was added to each well. Finally, the optical density (OD) was recorded at 570 nm by a microplate reader (Biotech ELx800, USA) with a reference wavelength of 630 nm. All tests were set up in triplicates. Half maximal inhibitory concentration (IC_{50}) was calculated by Combosyn software (Combosyn, Inc., Paramus, USA).

Primer design and real time-PCR

The SKOV3 cells in groups 1-7 were treated with Cis, ALT, and ZnONPs for 24 h. After 24 h total RNA was extracted from SKOV3 cells in froups 1-7 which were treated with different concentrations of Cis, ALT, and ZnONPs using GeneAll reagent kit (Biotechnology CO, LTD, South Korea). Then, total RNA was qualitatively and quantitatively evaluated by gel electrophoresis and nanodrop (Thermo Fisher Scientific, USA) at 260-280 nm.

Table 1. List of primers used in the real-time polymerase chain reaction

Genes	Sequence of primer	Product size	Melting points (°C)
MDM2	CAGAGTCTTGCTCCATCACC ATGCCTGTAATCCCAGTTACTTG	125	55
B-actin	ATTTTGAATGATGAGCCTTCGT TGTGCACTTTTATTCAACTGGT	151	55
XIAP	TCGAAGTGAATCTGATGCTGT ACTGAGTATATCCATGTCCCAA	126	53
Cyclin D1	AGGGTACTCAACCTAAGTTCG TTGCCACCTCCCTTCAACA	147	53
Bcl-2	CTCTCCCCGCGACTCCTG AATGCATAAGGCAACGATCCCA	91	54
Bax	CCGTGGACACAGACTCCC AAGTAGAAAAGGGCGACAACC	103	56

Complementary DNA (cDNA) was synthesized using the first-strand cDNA synthesis kit according to the manufacturer's instructions. For cDNA synthesis, 1 µg of RNA was used as the template. Real-time PCR was performed using SYBR Green PCR master mix that reaction mixture contained 2 µL template cDNA (2000 ng), 1 µL (10 pM) of primers mixture, 7 µL of SYBR green master mix, 4 µL nuclease-free water.

The primers of the target genes and the β-actin gene as a housekeeping gene are presented in Table 1. The reaction mixture was incubated under the following conditions: 95 °C, 5 min, 1 cycle (holding step); 95 °C, 10 s, 40 cycle (denaturation); 54 °C, 30 s, 40 cycles (annealing); 72 °C, 20 s, 40 cycles (extension). To evaluate the gene expression between the control and treated groups cells were normalized to β-actin mRNA levels and then gene expression was calculated by the $2^{-\Delta\Delta ct}$ method.

Western blot method

Western blotting was used to determine the several protein levels including Bax, XIAP, cyclin D1, Bcl2 in the SKOV3 cells in groups 1-7 which were treated with Cis, ALT, and ZnONPs for 24 h. Then, the RIPA buffer was used to obtain total lysates from the treated and control cells. The proteins were electrophoretically separated and transferred into the polyvinylidene difluoride (PVDF) membrane. Skimmed dry milk 5%was used to block the membrane. The membranes were then incubated overnight at 4 °C with specific antibodies. After this step, secondary horseradish peroxidase-conjugated antibody was added and incubated for 4 h at room temperature. Protein bands were visualized by using enhanced luminol-based chemiluminescent (ECL) western blotting detection reagent.

Cellular ROS detection assay

The evaluation of ROS is commonly performed by a single measurement of fluorescence (ROS assay kit: ab113851, Abcam). The precise concentration of DCFDA depends on the cell line being used but a general starting range would be 10 - 50 µM (25 µM). The DCFDA inactively goes into the cell, where it reacts with ROS to shape the highly fluorescent compound dichlorofluorescein (DCF). In this study, the DCFDA assay kit was used to evaluate ROS values by fluorimetry. The SKOV3 cells in groups 1-7 were treated with Cis, ALT, and ZnONPs for 24 h. After 24 h, SKOV3 cells were incubated with 100 µL of DCFDA (25 µM) for 45 min and then exposed to tert-butyl hydrogen peroxide (TBHP). For this purpose, according to the protocol, initially, the SKOV3 cells were cultured. Then, the cells were stained by DCFDA at 37 °C. Subsequently, the fluorescent values are read by the fluorimeter and calculated using Ex/Em = 485/535 nm equation. Also in this method, TBHP is used as a positive control. Finally, blank readings were reduced from all measurements and the fold changes were determined compared to the control (diluent treated cells if performing toxicity studies). All test steps were performed in triplicate.

Statistical analysis

Graph Pad Prism 6 and SPSS 16 were used for Statistical analysis. The differences between control and treated cells were assessed by ANOVA and Tukey's method and P-value < 0.05 was considered a significant difference. Results were expressed as mean ± SD.

RESULTS

ALT and ZnONPs increase the cytotoxic effects of Cis on SKOV3 cells

MTT assay was performed to survey the anti-proliferative effects of ALT, ZnONPs, and Cis on SKOV3 cells. IC_{50s} for ALT and Cis were 44.75 and 30.76 µM at 24 h, 10.41 and 6.93 µM at 48 h, and 11.32 and 5.57 µM at 72 h, respectively. IC_{50} for ZnONPs was 16.56 µM at 24 h, 9.66 µM at 48 h, and 8.05 µM at 72 h. Our results showed that ALT and ZnONPs significantly reduced the effective concentration of Cis in SKOV3 cells and facilitated the effects of Cis (Fig. 2).

Effect of Cis, ALT, and ZnONPs on the expression level of MDM2, XIAP, cylinD1, Bcl-2, and Bax genes

The expression of MDM2, XIAP, cylin D1, Bcl-2, and Bax genes in the groups treated with ALT, Cis, and ZnONPs as a single agent, double combination, and triple combination was presented evaluation by RT-PCR. The expression of these genes in the groups treated with ALT, Cis, and ZnONPs compared to the control group is shown in Fig. 3. The results indicated significantly different fold changes of genes expression of treated groups compared to the control group. In general, the expression of XIAP, cyclin D1, and Bcl2 genes in the treated groups with ALT, and Cis as a single agent was significantly reduced compared to the control group, while the Bax gene has generally shown an increase. Also, the expression of XIAP, cylin D1, and Bcl-2 in the double-combination groups was significantly reduced and expression of Bax increased in these groups. In the triple-combination, 0.5 IC_{50} (ALT-Cis-ZnONPs) led to a significant down-regulation of XIAP, cyclin D1, and Bcl2 and up-regulation of Bax gene. Also, there were no significant changes in the expression level of MDM2 gene (Fig. 3).

Effect of Cis, ALT, and ZnONPs on the protein expression

As shown in Fig. 4, western blotting was performed to evaluate the levels of Bax, Bcl-2, and cyclinD1 and XIAP protein. The results of Bax protein showed the expression level of this protein increases in treated groups. . Among the treatment groups, the comparison of single-agent groups with combination groups showed that double and triple combination groups increased the level of expression compared to single agents. Also, the results related to Bcl-2 and XIAP proteins showed that there were differences between several groups. Compared to the single-agents, double and triple combination groups are effective in reducing the expression level of Bcl-2 and XIAP proteins.

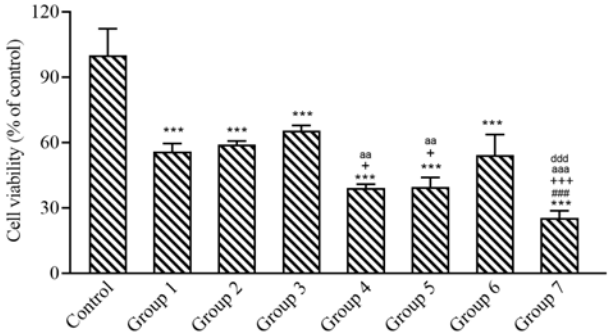

Fig. 2. ALT and ZnONPs reduce the effective dose of Cis. The SKOV3 cells in groups 1-3 were treated with IC_{50} of Cis, ALT, and ZnONP, as single agent, respectively; double-combination treatments were including group 4, IC_{50} of Cis + IC_{50} of ALT; group 5, 0.5 IC_{50} of (Cis + ALT); and group 6, 0.5 IC_{50} of (Cis + ZnONPs); and group 7, as triple-combination treatment, 0.5 IC_{50} of (Cis + ALT + ZnONPs). Data are expressed as mean ± SD of three independent experiments. $^{***}P < 0.001$ Indicates statistically significant changes compared to the control group; $^{###}P < 0.001$ versus group 1; $^{+}P < 0.05$ and $^{+++}P < 0.001$ versus group 2; $^{aa}P < 0.01$ versus group 3; $^{ddd}P < 0.001$ versus group 6. ALT, Alantolactone; Cis, cisplatin; ZnONP, zinc oxide nanoparticle.

The results of cyclin D1 protein also showed a decrease in the mentioned groups compared to the control group. Also, double and triple combination groups have better-reducing effects on cyclin D1 than single-agent groups. Our results showed that ALT and ZnONPs can facilitate and enhance the effects of Cis on proteins involved in the cell cycle and apoptosis.

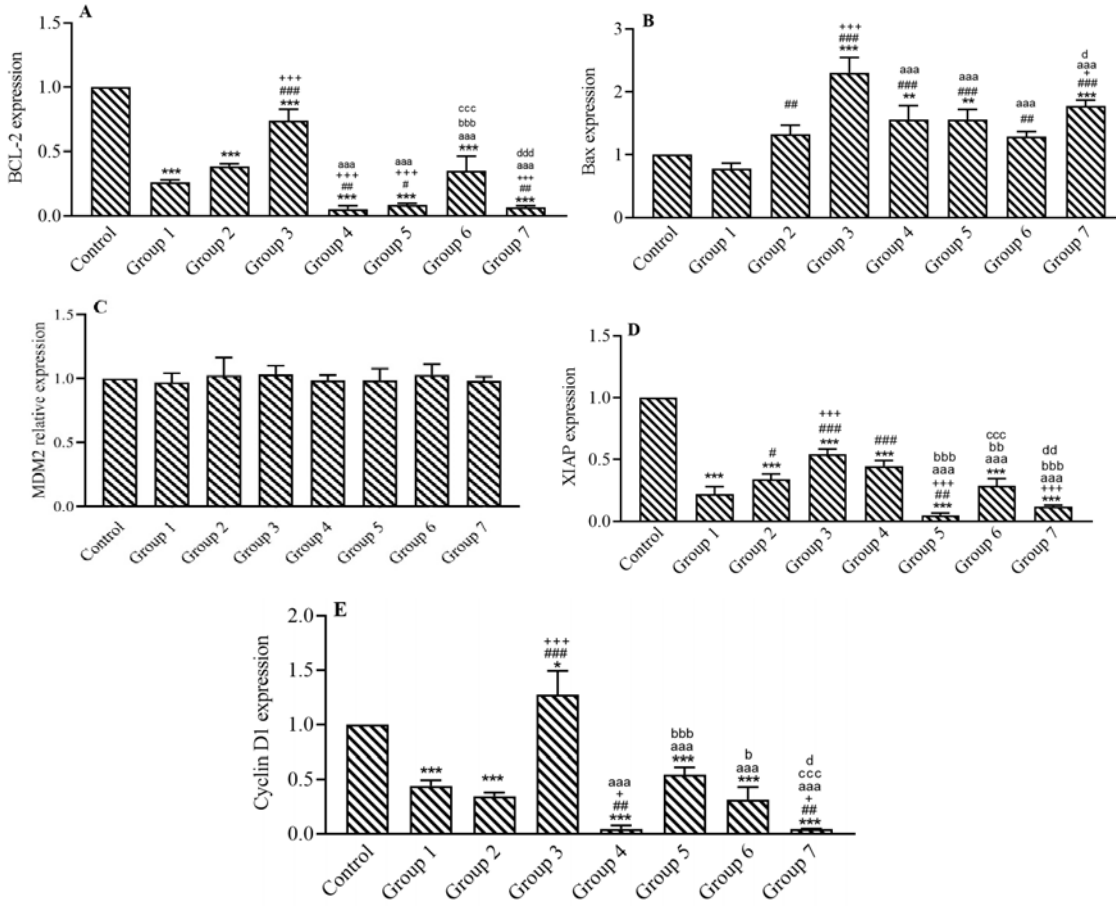

Fig. 3. Effects of Cis, ALT, and ZnONPs on mRNA relative expression of (A) Bcl-2, (B) Bax, (C) MDM-2, (D) XIAP, and (E) Cyclin D1. Expression of mRNA levels that have been normalized to β-actin mRNA expression is presented. The SKOV3 cells in groups 1-3 were treated with IC_{50} of Cis, ALT, and ZnONP, as single agent, respectively; double-combination treatments were including group 4, IC_{50} of Cis + IC_{50} of ALT; group 5, 0.5 IC_{50} of (Cis + ALT); and group 6, 0.5 IC_{50} of (Cis + ZnONPs); and group 7, as triple-combination treatment, 0.5 IC_{50} of (Cis + ALT + ZnONPs). Data are expressed as mean ± SD of three independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate statistically significant changes compared to the control group; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus group 1; $^{+}P < 0.05$ and $^{+++}P < 0.001$ versus group 2; $^{aaa}P < 0.001$ versus group 3; $^{b}P < 0.05$, $^{bb}P < 0.01$, and $^{bbb}P < 0.001$ against group 4; $^{ccc}P < 0.001$ versus group 5; $^{ddd}P < 0.001$ versus group 6. ALT, Alantolactone; Cis, cisplatin; ZnONP, zinc oxide nanoparticle; Bcl-2, B-cell lymphoma protein 2; Bax, Bcl-2-associated X; MDM-2, murine double minute 2; XIAP, X-linked inhibitor of apoptosis.

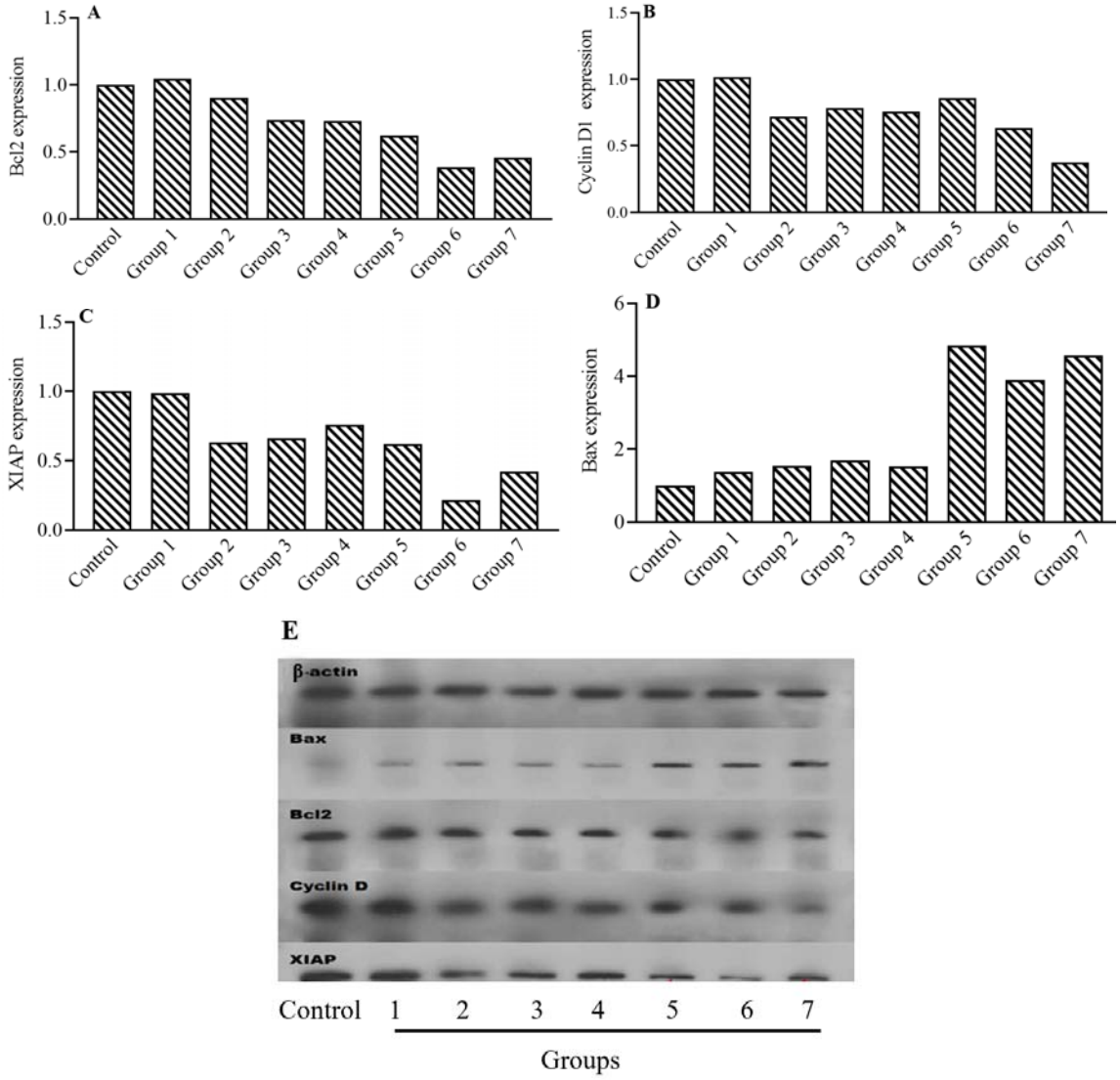

Fig. 4. Evaluation of effects of Cis, ALT, and ZnONPs on the protein expression of (A) Bcl2, (B) cyclin D1, (C) XIAP, and (D) Bax using western blot. The SKOV3 cells in groups 1-3 were treated with IC_{50} of Cis, ALT, and ZnONP, as single agent, respectively; double-combination treatments were including group 4, IC_{50} of Cis + IC_{50} of ALT; group 5, 0.5 IC_{50} of (Cis + ALT); and group 6, 0.5 IC_{50} of (Cis + ZnONPs); and group 7, as triple-combination treatment, 0.5 IC_{50} of (Cis + ALT + ZnONPs). ALT, Alantolactone; Cis, cisplatin; ZnONP, zinc oxide nanoparticle; Bcl-2, B-cell lymphoma protein 2; Bax, Bcl-2-associated X; MDM-2, murine double minute 2; XIAP, X-linked inhibitor of apoptosis.

ALT, ZnONPs, and Cis effects on the antioxidant balance of SKOV3 cells

The status of oxidants and antioxidants was examined in SKOV3 treated cells for 24 h (groups 1-7). Results showed that ROS levels were significantly higher in treated cells (except groups 1 and 2) as the highest levels were seen in the triple-combination (Fig. 5).

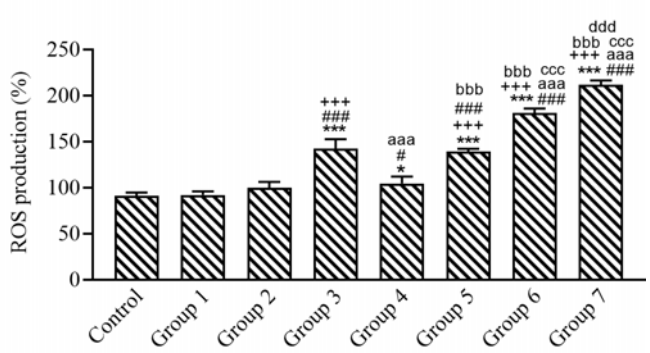

Fig. 5. Evaluation of effects of Cis, ALT, and ZnONPs on ROS production. The SKOV3 cells in groups 1-3 were treated with IC_{50} of Cis, ALT, and ZnONP, as single agent, respectively; double-combination treatments were including group 4, IC_{50} of Cis + IC_{50} of ALT; group 5, 0.5 IC_{50} of (Cis + ALT); and group 6, 0.5 IC_{50} of (Cis + ZnONPs); and group 7, as triple-combination treatment, 0.5 IC_{50} of (Cis + ALT + ZnONPs). Data are expressed as mean ± SD of three independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate statistically significant changes compared to the control group; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus group 1; $^{+++}P < 0.001$ versus group 2; $^{aaa}P < 0.001$ versus group 3; $^{bbb}P < 0.001$ against group 4; $^{ccc}P < 0.001$ versus group 5; $^{ddd}P < 0.001$ versus group 6. ALT, Alantolactone; Cis, cisplatin; ZnONP, zinc oxide nanoparticle; ROS, reactive oxygen species.

DISCUSSION

Ovarian cancer is one of the most common gynecologic cancers and is the fifth most common cancer in women in terms of mortality rate. Platinum-based chemotherapeutic agents such as Cis have been used as the first-line treatment in this type of cancer. However, most patients with ovarian cancer become easily resistant to platinum-based chemotherapeutic agents (20). Therefore, identifying a new chemotherapy agent or improving the current therapeutic agent is one of the main goals of scientists. In this regard, the use of natural products alone or in combination with common drugs due to their extensive structural and functional properties has attracted the attention of many researchers. Nanomedicine and the use of nanoparticles is an emerging field that has evidence of its effectiveness in the treatment of cancer. Given that many scientists have suggested that the use of natural products and nanoparticles with common chemotherapy agents can reduce the toxicity of high-dose chemotherapy. Also, various studies on the antitumor-molecular mechanism of ZnONPs, ALT, and Cis showed that all three compounds exert their antitumor activity through the production of ROS. Therefore, in the present study, we investigated the effects of ZnONPs and ALT on apoptotic activity and cell cycle arrest of Cis in the SKOV3 ovarian cancer cell line. The results of our study showed that the expression level of XIAP, cyclin D1, and Bcl-2 in the groups treated with ALT, Cis, and ZnONPs alone had a significant decrease compared to the control group while the Bax increased. It was also observed that if the three agents are combined (ALT-Cis- ZnONPs) further effects are seen. Also, the results related to ROS levels in our study showed that in the triple-combination group compared to single-agent and control, ROS levels increased more. Our results were consistent with the results obtained by Wahab *et al.* which also showed that ZnONPs induce apoptosis in cancer cells by increasing ROS levels (21). The results of our study also indicated that the use of ZnONPs at 16.56 µM resulted in death in 50% of cells (IC_{50}) after 24 h. In the study by Selvakumari *et al.* 31 µg of ZnONPs cause apoptotic death in 50% of cells (22).

ZnONPs have been proposed to have anticancer activity and have received increasing attention as a potential anticancer drug. Clinical and epidemiological results of some studies have shown that ZnONPs reduce the risk of several cancers, such as ovarian, lung, and breast (23,24). Pilot data from clinical trials recommend both dosage and types of ZnONP are crucial for its anticancer activity (25). In the current research, we focused on anticancer effects (half of IC_{50}) of ZnONPs against SKOV3 cells and evaluated several concentrations of ZnONPs, with particular

emphasis on the apoptotic pathway. Our results showed that ZnONPs through altering the expression of genes and proteins involved in apoptosis and cell cycle induced apoptosis, improved Cis apoptotic activity, and cell cycle arrest in ovarian cancer cells. Our results showed that ZnONPs could facilitate the effects of Cis on cell cycle inhibition and induction of apoptosis in SKOV3 cells.

Studies in recent years have shown that many compounds, especially natural compounds, are involved in inducing apoptosis in cancer cells by promoting ROS overload. ALT is one of the main active compounds derived from *Inula helenium,* which has been reported by researchers as a potential candidate for the treatment of various types of cancers (10). A growing body of evidence suggested that one of the antitumor mechanisms of ATL is increasing intracellular ROS production, which happens through depletion of GSH or inhibition of thioredoxin reductase. ROS, overloaded by ALT, induces apoptosis in cancer cells, and the use of ROS inhibitor blocks ALT apoptotic activity.. For example, Ding *et al.* reported that in colorectal cancer cells, ALT through overproduction of ROS and extensive oxidative DNA damage leads to upregulation of Bax and caspase-3, and downregulation of Bcl-2. They also reported that ALT caused cell cycle arrest at G1in colorectal cancer cells (6). Also, Jiang *et al.* reported that ALT activates the apoptotic pathway through poly(ADP-ribose) polymerase (PARP) cleavage and increased Bax / Bcl2 (26). Khan and colleagues also reported that ALT induces apoptosis in glioblastoma cells through ROS generation and GSH depletion (12).

According to the results of our study, it is clear that ALT may increase apoptosis in cancer cells through different pathways. Our results showed that ALT together with ZnONPs can significantly increase the apoptotic activity of Cis and cell cycle arrest in ovarian cancer cells. One of these pathways, which has been mentioned in the results of previous studies (27), is the overproduction of ROS in these cells as a signal of apoptosis. One of the possible effective pathway is the Bax / Bcl-2 pathway. ALT with ZnONPs increases ROS levels in these cells and may be activates the caspase pathway by stimulating BAX and inhibiting Bcl2 expression. Finally, caspases increase apoptotic signals. Normally, the protein kinase B (AKT) stimulates Bax / Bcl2 leading to increased cell proliferation. However, in the presence of ALT, with an excessive increase in ROS, the inhibition signal affects Bax / Bcl-2 and thus inhibits cell proliferation and leads to the promotion of apoptosis in ovarian cancer cells.

CONCLUSION

According to the results of our study, we can conclude that when ALT is used in combination with ZnONPs, greater amounts of ROS are generated in cancer cells and it can improve the effects of Cis on ovarian cancer. Overproduction of ROS can further affect signaling pathways and enhanced apoptosis through upregulation of pro-apoptotic proteins such as Bax, also down-regulation of anti-apoptotic proteins such as XIAP and Bcl2 in cancer cells. Our results showed that ALT in combination with ZnONPs could improve the effects of Cis and act as a potential therapeutic strategy by reducing the effective concentration of Cis.

Conflict of interest statement

All authors declared no conflict of interest in this study.

Acknowledgments

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Authors' contribution

Sh. Alipour and Sh. Gholizadeh-Ghaleh Aziz supervised the project. Sh. Alipour, Sh. Gholizadeh-Ghaleh Aziz, and Gh. Babaei performed the experiments. Sh. Alipour and S. Abolhasani analyzed and interpreted the data. Sh. Alipour wrote the manuscript in consultation with Sh. Gholizadeh-Ghaleh Aziz. The final version of the manuscript was approved by all authors.

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