SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 29: 207, 2025

Glycyrrhizin enhances the antitumor activity of cisplatin in non-small cell lung cancer cells by influencing DNA damage and apoptosis

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Received September 18, 2024; Accepted January 7, 2025

DOI: 10.3892/ol.2025.14954

Abstract. The objective of the present study was to elucidate the mechanism by which glycyrrhizin enhances the antitumor activity of cisplatin in non-small cell lung cancer. Initially, A549 cells were treated with different concentrations of glycyrrhizin (0.25-8 mM) or cisplatin (10-160 μM) for 48 h to investigate the effect of glycyrrhizin combined with cisplatin on A549 cells *in vitro*. Subsequently, A549 cells were divided into control (untreated), CP (20 μM cisplatin), GL (2 mM glycyrrhizin) and CP + GL (20 μM cisplatin + 2 mM glycyrrhizin) groups to elucidate the underlying mechanism of glycyrrhizin. After 48 h incubation, the viability and colony-forming ability of the cells were assessed using MTT and colony formation assays. Apoptosis levels and cell cycle progression were analyzed using flow cytometry and western blotting was used to evaluate apoptosis- and cell cycle-related proteins. Additionally, comet assays and western blotting were used to evaluate DNA damage and relevant proteins. The results demonstrated both glycyrrhizin and cisplatin individually reduced A549 cell viability in a concentration-dependent manner. Cisplatin demonstrated a lower half-maximal inhibitory concentration (IC_{50}) at higher glycyrrhizin concentrations, with an IC_{50} value of ~35 μM with 2 mM glycyrrhizin. Furthermore, the combined treatment of glycyrrhizin and cisplatin synergistically reduced cell colony-forming ability, induced apoptosis and arrested the cell cycle at the G_2 phase, showing greater efficacy when compared with either treatment individually. In addition, western blotting analysis demonstrated that, in comparison with treatment with cisplatin or glycyrrhizin alone, the combined treatment markedly increased the protein expression levels of B-cell lymphoma 2-associated X protein, cleaved-caspase-3/caspase-3, γH2AX, phosphorylated-checkpoint kinase 1 and phosphorylated-p53/p53, while notably reducing the protein levels of B-cell lymphoma 2, cyclin D1, cyclin-dependent kinase 2 and cyclin-dependent kinase 4. The findings of the present study indicate that glycyrrhizin enhances the antitumor efficacy of cisplatin in non-small cell lung cancer cells by modulating DNA damage and apoptosis.

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Key words: glycyrrhizin, DNA damage, apoptosis, cisplatin, non-small cell lung cancer cells

Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, with an estimated 2 million new cases diagnosed each year (1). Of patients with lung cancer, ~85% are attributed to non-small cell lung cancer (NSCLC) (2), which is further classified into lung adenocarcinoma (LUAD) and lung squamous cell carcinoma. Notably, 30% patients with NSCLC are diagnosed at advanced stages (3), reducing the likelihood of effective intervention and leading to poor treatment outcomes. With a 5-year survival rate of only 15%, the prognosis for patients with NSCLC remains poor and recurrence is common (2,4). Tobacco smoking remains the primary etiological factor for all lung cancer subtypes, with research indicating that smoking contributes to >80% of lung cancer cases in numerous nations (5). Consequently, smoking cessation is considered to be crucial in the prevention of lung cancer, necessitating ongoing enhancements in treatment strategies.

In this context, cisplatin, a chemotherapy agent used for >30 years, has shown varying efficacy across different cancer types. Particularly, cisplatin has emerged as the established treatment standard for advanced NSCLC (6). Cisplatin has the ability to undergo hydration within cancer cells, acquiring a positive charge that enables its integration into DNA to create cross-links and disrupt the DNA structure, leading to apoptosis of cancer cells (7,8). Lung cancer comprises cells or clonal populations with unique molecular profiles, resulting in intratumoral diversity and vulnerability to genetic alterations, which markedly impede the therapeutic efficacy of cisplatin (9). Therefore, developing novel treatment approaches is essential to enhance the therapeutic potential of cisplatin and improve the prognosis of patients with NSCLC.

Among various drug candidates, Chinese herbal medicine has a history of >50 years in the treatment of lung cancer (10). A number of approved cancer therapies are modified natural products or semi-synthetic derivatives, including compounds such as camptothecin, paclitaxel and vincristine (11). In addition, numerous monomers derived from Traditional Chinese

Medicine possess individual anticancer properties. For instance, flavonoids such as luteolin and baicalein (12), alkaloids such as berberine and oxymatrine (13) and terpenoids, such as triptolide and andrographolide (14), all demonstrate therapeutic potential, along with other compounds such polyphenols, anthraquinones and polysaccharides (15). Compared with conventional treatments for NSCLC, Chinese herbal medicines offer a distinct and innovative pharmacological mechanism, accompanied by lower toxicity (16,17). Notably, integrative medicine is effective in the management of NSCLC, improving the efficacy of chemotherapy and radiotherapy while reducing side effects such as bone marrow suppression, nausea and vomiting (18).

Glycyrrhizin is a triterpenoid compound that is extracted from the roots of the licorice plant, which initially gained attention for its antiviral and anti-inflammatory effects (19). In previous years, studies (20-22) have reported that glycyrrhizin also possesses notable anticancer properties, demonstrating antitumor effects across several cancer types. Its primary mechanism of action involves increasing reactive oxygen species levels, activating caspase-3 and reducing the mitochondrial membrane potential, leading to cell cycle arrest and DNA damage in cancer cells, thereby inhibiting cell proliferation. For instance, in leukemia, glycyrrhizin inhibits the AKT/mTOR/STAT3 signaling pathway, leading to a reduction in the expression of cyclin D1 and survivin, thereby promoting apoptosis (23). In gastric cancer, glycyrrhizin inhibits the phosphorylation of the PI3K/AKT pathway and reduces the expression of cyclin D1, survivin and p65. In addition, glycyrrhizin enhances the activity of B-cell lymphoma 2-associated X protein (Bax) and poly(ADP-ribose) polymerase and subsequently inhibits cancer cell proliferation and induces apoptosis (24).

Glycyrrhizin has also demonstrated potential in the treatment of NSCLC. Research has indicated that glycyrrhizin can inhibit the growth of LUAD cells (25). Notably, Deng *et al* (26) demonstrated that glycyrrhizin not only enhances the antitumor effect of cisplatin in LUAD xenograft mice but also reduces liver and kidney damage caused by cisplatin treatment. These studies suggest that glycyrrhizin may serve as an adjuvant to cisplatin, improving the therapeutic efficacy of NSCLC while minimizing the side effects of cisplatin. However, the molecular mechanisms underlying the combined use of glycyrrhizin and cisplatin remain unclear and require further investigation.

Materials and methods

Cell culture and grouping. A549 cells, a NSCLC cell line (American Type Culture Collection no. #CCL-185), were purchased from the Cell Resource Center of Peking Union Medical College (Beijing, China). Prior to experiments, the cells were subjected to polymerase chain reaction analysis to ensure that they were free from mycoplasma contamination. Subsequently, A549 cells were maintained in Roswell Park Memorial Institute 1640 medium (cat. no R8758; Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS; cat. no 12103C; Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin (cat. no P4458; Sigma-Aldrich; Merck KGaA) and 100 μg/ml streptomycin (cat. no S9137; Sigma-Aldrich; Merck KGaA). Cell culture was performed in a 37˚C cell culture incubator (cat. no COI-160-ST; Beijing Labgic Technology Co., Ltd.) with 5% CO_2. Upon reaching 80-90% confluence, the cells were subcultured and those that had undergone three consecutive passages were utilized for experimental purposes. To evaluate the effect of glycyrrhizin on the sensitivity of A549 cells to cisplatin, cells in each group were randomly assigned to different treatment conditions. The control group consisted of untreated A549 cells. The treatment groups received cisplatin (20 μM) (21), glycyrrhizin (2 mM) (19) or a combination of both drugs at the same concentration. The cells were incubated at 37˚C for 48 h before harvest for further analysis. Cisplatin and glycyrrhizin were purchased from Sigma-Aldrich (Merck KGaA).

MTT assay. The MTT assay kit (cat. no E-CK-A341; Wuhan Elabscience Biotechnology Co., Ltd.) was employed to assess the viability of the normal human lung epithelial cell line BEAS-2B (American Type Culture Collection) and A549 cells under different treatment conditions. BEAS-2B cells were used as a normal control to evaluate the cytotoxicity of glycyrrhizin and cisplatin. BEAS-2B cells and A549 cells were seeded into 96-well plates at a density of $2x10^4$ cells/ml, with a volume of 200 μl per well. The plates were randomly assigned to experimental groups and placed in an incubator until the cells were fully adhered. Experimental groups were then treated with several concentrations of glycyrrhizin (0, 0.25, 0.5, 1, 2, 4 and 8 mM) and cisplatin (0, 10, 20, 40 and 160 μM), either alone or in combination. After 48 h incubation, 20 μl MTT solution (5 mg/ml) was added to each well. Following 4 h incubation, 100 μl dimethyl sulfoxide was added to the wells and the optical density at 490 nm was measured using an iMark microplate reader (Bio-Rad Laboratories, Inc.) to determine cell viability. The viability of cells in each experimental group was calculated relative to the control group, which exhibited 100% viability for A549 cells, while BEAS-2B cells served as a comparative indicator for the cytotoxicity of treatments.

Cell colony formation assay. A cell colony formation assay was utilized to evaluate the effect of cisplatin and glycyrrhizin, both separately and in combination, on the colony-forming ability of A549 cells. Briefly, A549 cells were seeded into 12-well plates at a density of 500 cells/ml, with 1 ml per well. After 24 h incubation in a cell culture incubator, the cells were randomly assigned to different treatment groups and exposed to cisplatin (20 μM), glycyrrhizin (2 mM) or a combination of both. Following 48 h incubation, the cells were washed with sterile phosphate-buffered saline (PBS) buffer (cat no. ST447-1L; Beyotime Institute of Biotechnology), replenished with fresh complete medium and further incubated in a cell culture incubator at 37˚C for 14 days to allow colony formation. The cells were subsequently fixed with a fixative solution (1:7 mixture of 100% acetic acid and pure methanol) at room temperature for 20 min. After washing the cells three times with sterile PBS, they were stained with 0.5% crystal violet solution (cat. no C805211; Shanghai Macklin Biochemical Co., Ltd.) at room temperature for 1 h, rinsed with distilled water and observed under a light microscope (XSP; Zhejiang Lichen Instrument Technology Co., Ltd.). Finally, the

images were captured and the colonies were quantified using ImageJ software (National Institutes of Health; version 1.53). A colony was defined as >50 cells that formed after 14 days of incubation.

Apoptosis assay. The effects of cisplatin, glycyrrhizin and their combination on apoptosis levels in A549 cells were assessed using the Annexin V Alexa Fluor™ 647/Propidium Iodide (PI) Apoptosis Assay kit (cat. no AC10862; Shanghai Acmec Biochemical Co., Ltd.) to detect early and late apoptosis. Firstly, A549 cells were cultured in 6-well plates at a density of 8x10^5 cells/ml in 1 ml of medium and incubated in a cell culture incubator for 24 h. Following incubation, cells were treated with cisplatin (20 μM), glycyrrhizin (2 mM) or both for 48 h. After treatment, cells were harvested, centrifuged at 300 x g for 5 min at 37˚C, washed twice with PBS and adjusted to a concentration of 1x10^6 cells/ml using culture medium. Subsequently, the treated cells were resuspended in 100 μl PBS, stained with 5 μl annexin V/Alexa Fluor™ 647 and 10 μl of 20 μg/ml PI solution and incubated for 15 min at room temperature in the absence of light. Controls included unstained, single-stained and double-stained samples to ensure proper gating and compensation. Subsequently, the cells were analyzed using a CytoFLEX S flow cytometer (Beckman Coulter, Inc.) and the data were processed with CytExpert software (version 2.3; Beckman Coulter, Inc.). Fluorescence intensities were measured for FITC (annexin V) and PE (PI) signals at an excitation wavelength of 488 nm and an emission wavelength of 530 nm in 400 μl PBS.

Cell cycle assay. The effects of cisplatin and glycyrrhizin administered individually or in combination on the cell cycle of A549 cells were evaluated using Cell Cycle Staining Kit (cat. no E-CK-A351; Wuhan Elabscience Biotechnology Co., Ltd.). A549 cells were seeded in 6-well culture plates in a volume of 1 ml and a density of 8x10^5 cells/ml for 24 h. They were randomly assigned to different treatment groups and treated with cisplatin (20 μM), glycyrrhizin (2 mM) or a combination of cisplatin (20 μM) and glycyrrhizin (2 mM). After 48 h incubation, the cells were collected in centrifuge tubes and centrifuged at 300 x g for 5 min at 37˚C. Subsequently, the cell pellet was washed twice with PBS, adjusted to a concentration of 5x10^5 cells/ml using PBS and resuspended in 300 μl PBS. To fix the cells, 1.2 ml ice-cold ethanol (-20˚C) was added, mixed thoroughly and the suspension was stored at -20˚C overnight. The fixed cells were centrifuged at 300 x g for 5 min at 4˚C, resuspended in 1 ml PBS, and left to stand for 15 min. After a second centrifugation at 300 x g for 5 min at 4˚C, the pellet was resuspended in 100 μl RNase A reagent and incubated in a 37˚C water bath for 30 min. Subsequently, 400 μl PI reagent (50 μg/ml) was added, mixed thoroughly and incubated at 4˚C in the dark for 30 min. Lastly, the intensity of PE signals (excitation, 488 mm; emission, 530 nm) was measured using flow cytometry (CytoFLEX S; Beckman Coulter, Inc.). The data were analyzed using FlowJo software (version 10.6.2; BD Biosciences).

Comet assay. Comet Assay kits (cat. no C2041S, Beyotime Institute of Biotechnology) were used to assess the impact of cisplatin and glycyrrhizin, both individually and in combination, on DNA damage in A549 cells. A549 cells were initially seeded in 6-well culture plates at a density of 8x10^5 cells/ml with a seeding volume of 1 ml and incubated for 24 h. Cells were then randomly assigned to different treatment groups and treated with cisplatin (20 μM), glycyrrhizin (2 mM) or a combination of both for 48 h. Subsequently, the cells were harvested, centrifuged at 300 x g for 5 min at 37˚C, washed twice with PBS and adjusted to a concentration of 1x10^6 cells/ml. The cells were then lysed using lysis buffer (Beyotime Institute of Biotechnology), DNA was unwound under alkaline conditions and gel electrophoresis was conducted at 4˚C using a 1% agarose gel at low voltage (25 V) for 30 min. Subsequently, neutral buffer (Beyotime Biotechnology) was added, followed by staining with 20 μl PI solution for 20 min at room temperature in the absence of light. Finally, DNA damage was assessed by analyzing DNA levels and comet tail length using a fluorescence microscope (excitation, 535 nm; emission, 617 nm; IX73 microscope; Olympus Corporation). The images were analyzed using ImageJ software (version 1.53u; National Institutes of Health).

Western blotting. A549 cells were cultured with cisplatin (20 μM) and/or glycyrrhizin (2 mM), after which the cell culture medium was removed and the cells were washed twice with pre-chilled PBS. The cells were then collected into sterile centrifuge tubes, lysed in 1 ml radioimmunoprecipitation assay buffer (cat. no P0013C; Beyotime Institute of Biotechnology) and subjected to sonication for 10 min at 20 kHz and on ice. Lysates were centrifuged at 12,000 x g for 10 min at 4˚C and the supernatant was collected and kept on ice. Total protein concentration was measured using the Bicinchoninic Acid Protein Assay kit (cat. no P0010S; Beyotime Institute of Biotechnology). For analysis, 20 μg total protein was mixed with sodium dodecyl sulfate loading buffer (Beyotime Institute of Biotechnology) and denatured at 95˚C for 10 min. Proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (cat. no E302-01; Vazyme Biotech Co., Ltd.) at 110 V for 1 h, with 40 μg protein loaded in each lane, and transferred onto a polyvinylidene fluoride membrane (cat. no IPVH00010; MilliporeSigma) at 90 V. The membrane was then blocked with 5% skimmed milk at room temperature for 1 h and incubated with primary antibodies overnight at 4˚C. The primary antibodies (all BIOSS) used included Bax (1:1,000; cat. no bsm-60772R), B-cell lymphoma 2 (Bcl-2; 1:1,000; cat. no bsm-33411M), cleaved-caspase-3 (1:1,000; cat. no bsm-33199M), caspase-3 (1:1,000; cat. no bsm-61071R), cyclin D1 (1:1,000; cat. no bs-20596R), cyclin-dependent kinase (CDK) 2 (1:1,000; cat. no bs-0757R), CDK4 (1:1,000; cat. no bs-0633R), γH2AX (1:1,000; cat. no bs-2560R), checkpoint kinase 1 (Chk1; 1:1,000; cat. no bs-1681R), phosphorylated-Chk1 (p-Chk1; 1:1,000; cat. no bs-13906R), p53 (1:1,000; cat. no bs-4181R), phosphorylated p53 (p-p53; 1:1,000; cat. no bs-3710R) and β-actin (1:1,000; cat. no bs-0061R). The membrane was incubated at room temperature for 1 h with goat anti-rabbit (1:5,000; cat. no bs-0295G-HRP) or anti-mouse (1:5,000; cat. no bs-0368G-HRP) secondary antibodies (BIOSS). Protein bands were visualized using enhanced chemiluminescence (SuperSignal ECL; cat. no 34580; Thermo Fisher Scientific,

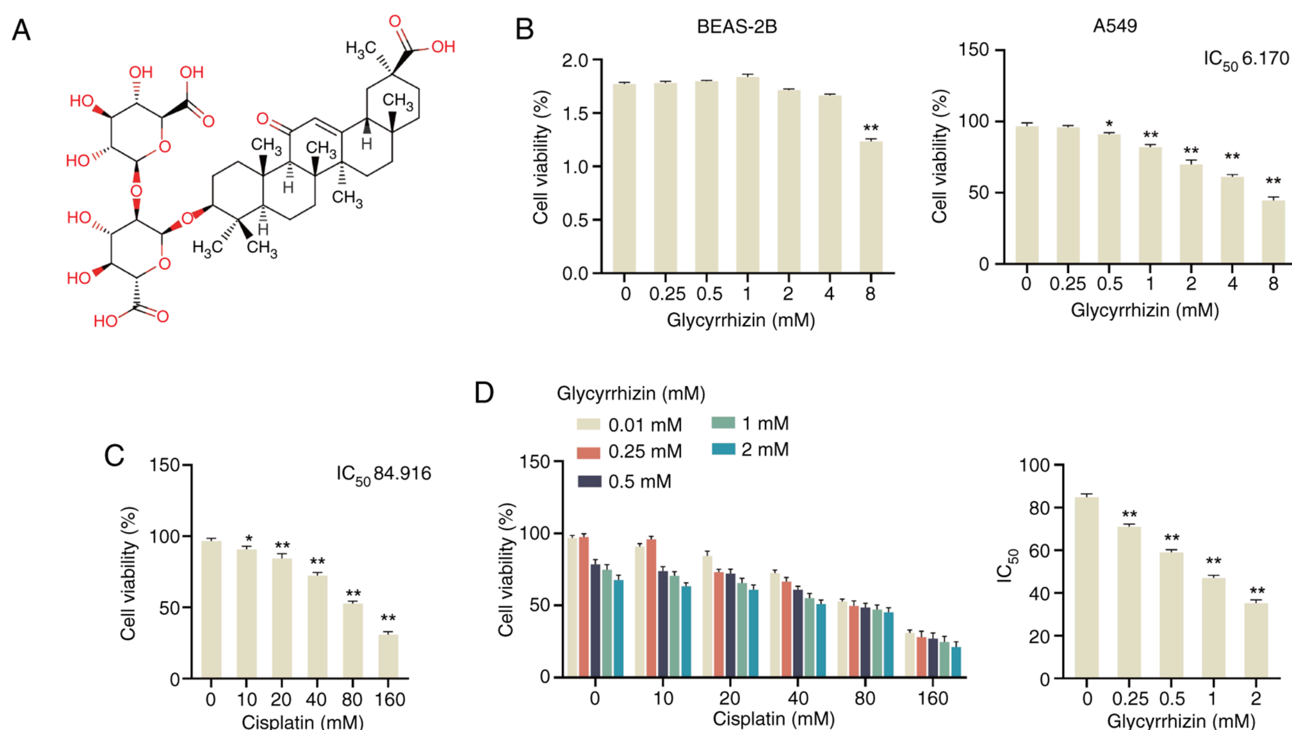

Figure 1. Inhibition of glycyrrhizin and its combination with cisplatin in A549 cells. (A) Chemical structure of glycyrrhizin. MTT was used to detect the cell viability of (B) BEAS-2B and (C) A549 cells after treatment with different concentrations of glycyrrhizin (0, 0.25, 0.5, 1, 2, 4 and 8 mM) for 48 h, and the IC_{50} was calculated for A549 cells. (D) MTT was used to detect the cell viability of A549 cells after treatment with different concentrations of cisplatin (0, 10, 20, 40, 80 and 160 μM) for 48 h, and the IC_{50} was calculated. (E) MTT was used to assess the cell viability of A549 cells after treatment with 0, 0.25, 0.5, 1 and 2 mM glycyrrhizin and 0, 10, 20, 40, 80 and 160 μM cisplatin, respectively, and the IC_{50} of cisplatin was calculated. Data are presented as mean ± SD (n=3). $^{*}P<0.05$, $^{**}P<0.01$ vs. 0 mM. IC_{50}, half-maximal inhibitory concentration.

Inc.). In addition, β-actin served as the internal control to calculate the relative expression of the target protein using Image J software (version 1.53u; National Institutes of Health).

Statistical analysis. The study findings were derived from ≥3 independent experiments and analyzed using SPSS software (version 23.0; IBM Corp.). For comparisons among multiple groups, one-way analysis of variance followed by Tukey's post hoc test was used. The normality was tested using the Shapiro-Wilk test, and P<0.05 was considered to indicate a statistically significant difference. Graphical outputs were generated using GraphPad Prism 9.2.0 (Dotmatics).

Results

Effects of glycyrrhizin and combination with cisplatin to A549 cells. Glycyrrhizin (Fig. 1A) was initially recognized for its antiviral properties, with a subsequent study reporting its anti-inflammatory and antitumor activities (27). More recently, glycyrrhizin has been demonstrated to effectively inhibit the growth of LUAD cells (25). In addition, glycyrrhizin has been identified as a compound capable of overcoming cisplatin resistance in hepatocellular carcinoma when combined with lamivudine (28). These findings suggest that glycyrrhizin not only inhibits tumor progression but also reduces cancer cell drug resistance. In the present study, the effect of glycyrrhizin combined with cisplatin on NSCLC cells was evaluated.

Initially, to assess cytotoxicity, BEAS-2B and A549 cells were treated with glycyrrhizin (0-8 mM) and analyzed using the MTT assay. Glycyrrhizin had minimal effect on BEAS-2B cell viability, with significant changes observed only at higher concentrations (8 mM) (Fig. 1B). In contrast, glycyrrhizin treatment significantly reduced the viability of A549 cells in a dose-dependent manner, with a calculated half-maximal inhibitory concentration (IC50) value of 6.17 mM (Fig. 1C). Concurrently, the impact of several concentrations of cisplatin on A549 cell viability was evaluated, revealing a significant decrease in cell viability when treated with 10, 20, 40, 80 and 160 μM of cisplatin compared with 0 mM, with the calculated IC_{50} of cisplatin for A549 cells being 84.916 mM (Fig. 1D). Subsequent investigations aimed to explore the combined effect of both compounds on A549 cell viability. The findings demonstrated a decrease in A549 cell viability with increasing concentrations of both glycyrrhizin and cisplatin. Notably, the IC_{50} of cisplatin significantly decreased with increasing glycyrrhizin concentration compared with 0 mM, with the IC_{50} of cisplatin ~35 μM at 2 mM glycyrrhizin (Fig. 1E). These results indicate that glycyrrhizin significantly diminishes A546 cell viability and enhances the cytotoxic effect of cisplatin on A546 cells.

Glycyrrhizin enhances the inhibitory effect of cisplatin on colony formation in A549 cells. In order to comprehensively assess the potential synergistic impact of glycyrrhizin in enhancing the efficacy of cisplatin against NSCLC, a colony formation assay was conducted on A549 cells treated with glycyrrhizin, cisplatin or their combination. The results of the cell colony formation assay demonstrated a significant

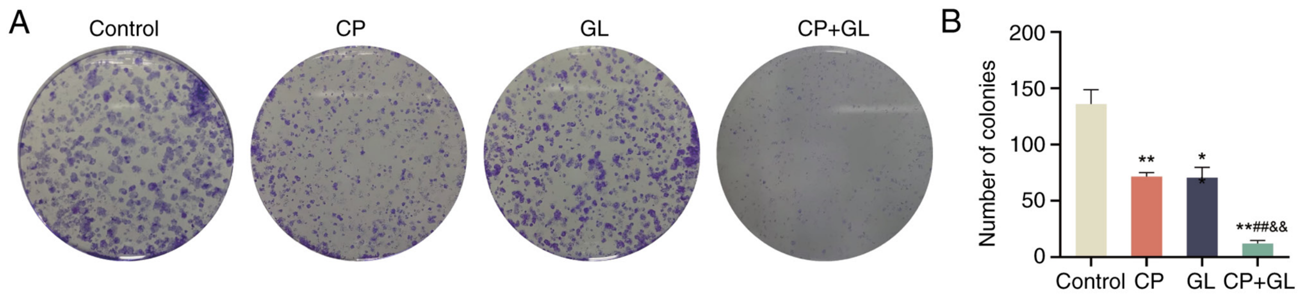

Figure 2. Glycyrrhizin enhances the inhibitory effect of cisplatin on colony formation in A549 cells. (A) Colony formation assay and (B) quantification was used to detect the colony forming ability of A549 cells in the control, CP, GL and CP + GL groups. Data are presented as mean ± SD (n=3). *P<0.05 and **P<0.01 vs. control; ##P<0.01 vs. CP; &&P<0.01 vs. GL. CP, cisplatin; GL, glycyrrhizin.

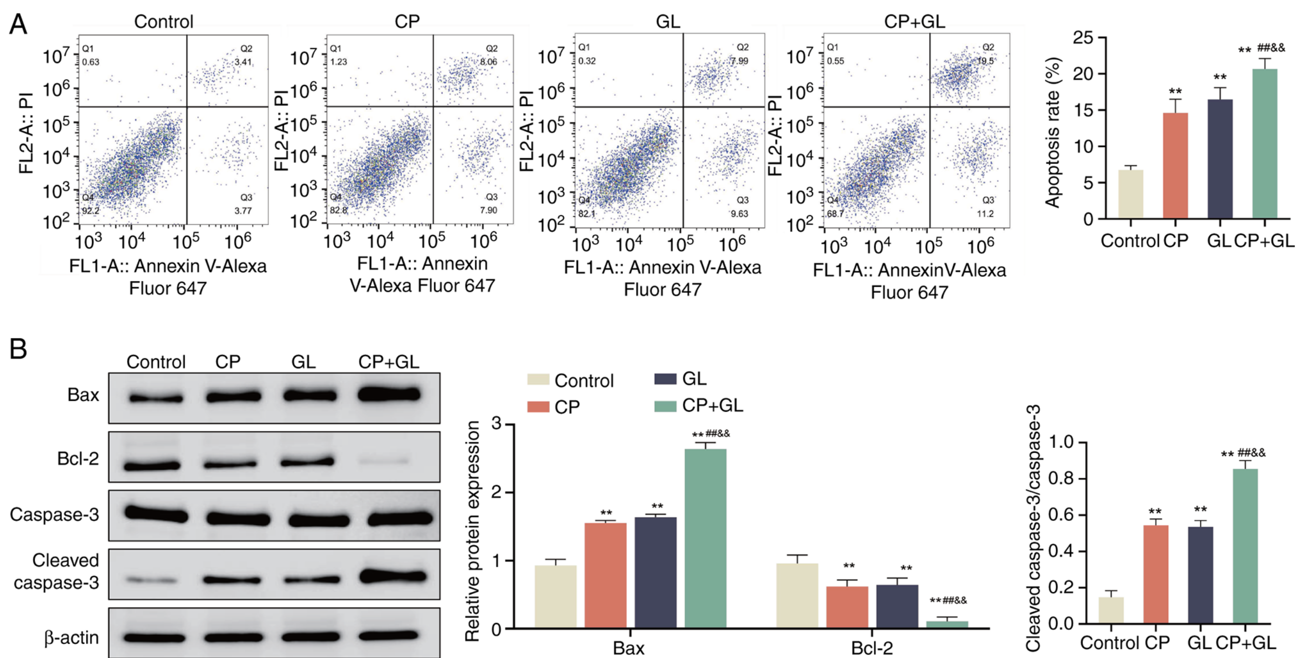

Figure 3. Glycyrrhizin enhances the promoting effect of cisplatin on A549 apoptosis. (A) Flow cytometry was used to detect the apoptosis level of A549 cells in the control group, CP group, GL group and CP + GL group. (B) Western blotting was used to assess the protein expression levels of Bax, Bcl-2 and cleaved-caspase-3/caspase-3 in A549 cells in the control group, CP group, GL group and CP + GL group. Data are presented as mean ± SD (n=3). **P<0.01 vs. control; ##P<0.01 vs. CP; &&P<0.01 vs. GL. CP, cisplatin; GL, glycyrrhizin; Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2.

reduction in the colony-forming capacity of A549 cells treated with glycyrrhizin alone, cisplatin alone or the combination of glycyrrhizin and cisplatin when compared with the control group (Fig. 2). Notably, the colony-forming ability of A549 cells treated with cisplatin and glycyrrhizin in combination was significantly lower compared with that of cells treated with cisplatin or glycyrrhizin alone (Fig. 2). These findings indicate that glycyrrhizin may potentiate the inhibitory effects of cisplatin on colony formation in A549 cells.

Glycyrrhizin enhances the effect of cisplatin on apoptosis of A549 cells. The aforementioned findings indicate that glycyrrhizin may serve as an adjuvant in NSCLC therapy. To explore the mechanism by which glycyrrhizin enhances the tumor inhibitory effects of cisplatin, further investigations were undertaken. Previous studies have demonstrated that glycyrrhizin inhibits monocytes (29), cervical cancer cells (30) and prostate cancer cells (31). Therefore, we hypothesized that glycyrrhizin may potentiate the anticancer properties of cisplatin by facilitating apoptosis in A549 cells. Initially, annexin V Alexa Fluor™ 647/PI staining analysis demonstrated that there was a significant increase in A549 cells treated with cisplatin, glycyrrhizin or their combination in comparison with the control group. Notably, the combined therapy induced a significantly higher apoptotic rate than either treatment alone (Fig. 3A). Subsequent analysis of apoptosis-related proteins indicated a significant increase in the protein levels of Bax and cleaved-caspase-3/caspase-3, along with a decrease in Bcl-2 levels, in A549 cells treated with cisplatin, glycyrrhizin or their combination compared with the control group. Furthermore, there was a significant increase in Bax and cleaved-caspase-3/caspase-3 and a decrease in Bcl-2 levels in the combined treatment group compared with cisplatin and glycyrrhizin alone (Fig. 3B). These results suggest that glycyrrhizin enhances apoptosis of A549 cells when combined with cisplatin.

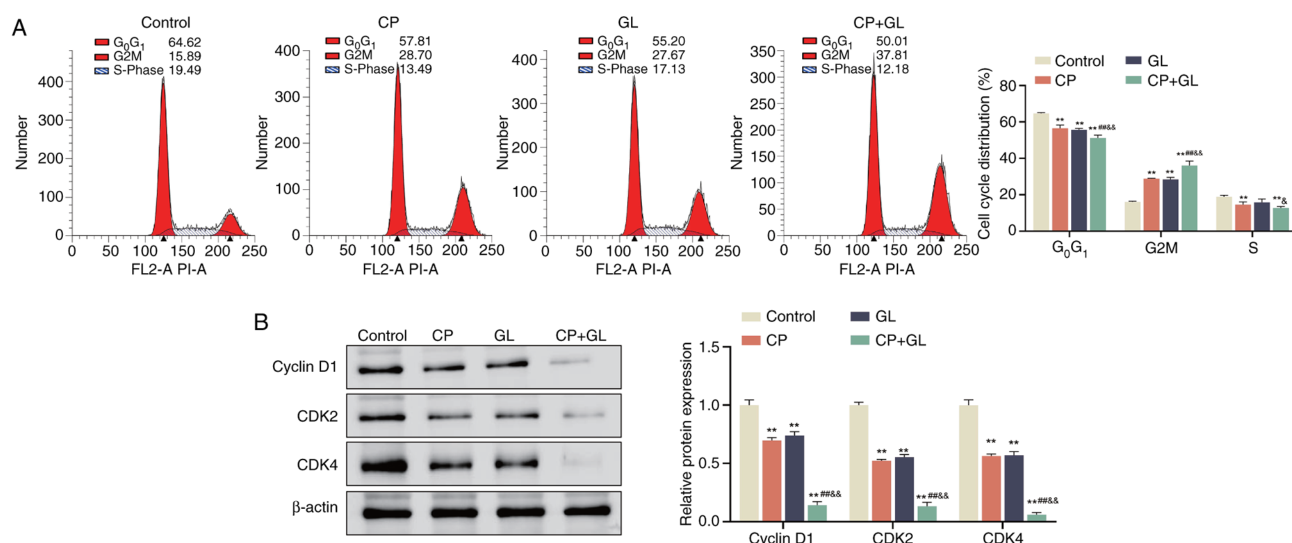

Figure 4. Glycyrrhizin enhances the effect of cisplatin on the cell cycle of A549 cells. (A) Flow cytometry was used to assess the cell cycle changes of A549 cells in the control, CP, GL and CP + GL groups. (B) Western blotting was used to evaluate the protein levels of cyclin D1, CDK2 and CDK4 in A549 cells in the control, CP, GL and CP + GL groups. Data are presented as mean ± SD (n=3). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. CP; $^{\&}$P<0.05 and $^{\&\&}$P<0.01 vs. GL. CP, cisplatin; GL, glycyrrhizin; CDK, cyclin-dependent kinase.

Glycyrrhizin enhances the effect of cisplatin on the cell cycle of A549 cells. According to a previous study, there is a close link between the cell cycle and apoptosis, and the level of apoptosis is regulated by the cell cycle (32). Therefore, the impact of the drug treatments on the cell cycle was investigated using PI staining of A549 cells. The results demonstrated alteration in the cell cycle distribution of A549 cells (Fig. 4A). Specifically, the percentage of cells in the G_0/G_1 phase decreased significantly and the proportion of cells in the G_2/M phase increased significantly upon treatment with cisplatin and glycyrrhizin, either individually or in combination, compared with the control group. Additionally, the S phase population significantly decreased when A549 cells were treated with cisplatin alone or in combination with glycyrrhizin compared with the control group. Notably, treatment with the combination of cisplatin and glycyrrhizin further significantly reduced the percentage of cells in the G_0/G_1 phase while increasing the G_2/M phase population when compared with either treatment alone (Fig. 4A).

Subsequent analysis using western blotting was conducted to assess the expression levels of cell cycle-related proteins. The analysis demonstrated a significant decrease in the protein levels of cyclin D1, CDK2 and CDK4 in A549 cells treated with cisplatin and glycyrrhizin individually or in combination compared with the control group. Furthermore, the protein levels of cyclin D1, CDK2 and CDK4 were significantly lower in A549 cells exposed to cisplatin and glycyrrhizin together compared with those treated with cisplatin or glycyrrhizin alone (Fig. 4B). These observations suggest that glycyrrhizin augments the impact of cisplatin on the cell cycle of A549 cells.

Glycyrrhizin enhances the effect of cisplatin on DNA damage of A549 cells. DNA damage has been identified in prior studies as a key factor contributing to genome instability, leading to either cell cycle arrest or apoptosis via a cascade of molecular responses (33). In the present study, treatment with cisplatin or glycyrrhizin individually significantly increased DNA damage in A549 cells compared with the control group, as demonstrated by elevated comet tail DNA and comet tail distance. Furthermore, the combination of cisplatin and glycyrrhizin resulted in a significant increase in DNA damage, comet tail DNA and comet tail distance compared with individual treatments (Fig. 5A). Subsequent western blotting analysis revealed upregulation of DNA damage-associated proteins, including γH2AX, p-Chk1/Chk1 and p-p53/p53, in cells treated with either cisplatin or glycyrrhizin alone compared with the control group, with further significant increases observed in the combination treatment group compared with the treatments alone (Fig. 5B). These results demonstrate that glycyrrhizin potentiates the DNA damage in A549 cells induced by cisplatin.

Discussion

Lung cancer is a growing threat to human health, accounting for 18% of all cancer-associated mortalities, which is markedly higher than other cancer types (34). Several treatment approaches are required for distinct subcategories of patients with lung cancer. Surgery, radiotherapy, systemic chemotherapy, interventional therapy and targeted therapy are frequently employed techniques for individuals with NSCLC (35). Chemotherapy is considered the primary choice for patients with advanced metastases or NSCLC who are unsuitable for surgical intervention. Nevertheless, resistance to chemotherapy is progressively emerging in non-small cell carcinoma, prompting concern regarding the adverse effects and toxicity associated with chemotherapy medications (36). Consequently, the exploration of potent anticancer compounds derived from natural medicinal sources is gaining importance.

Terpenoids are a category of plant secondary metabolites known for their diverse biological activities, such as anti-inflammatory, antibacterial, antiviral and anticancer properties. Glycyrrhizin, comprising two glucuronic acid molecules and one glycyrrhetinic acid molecule, is recognized

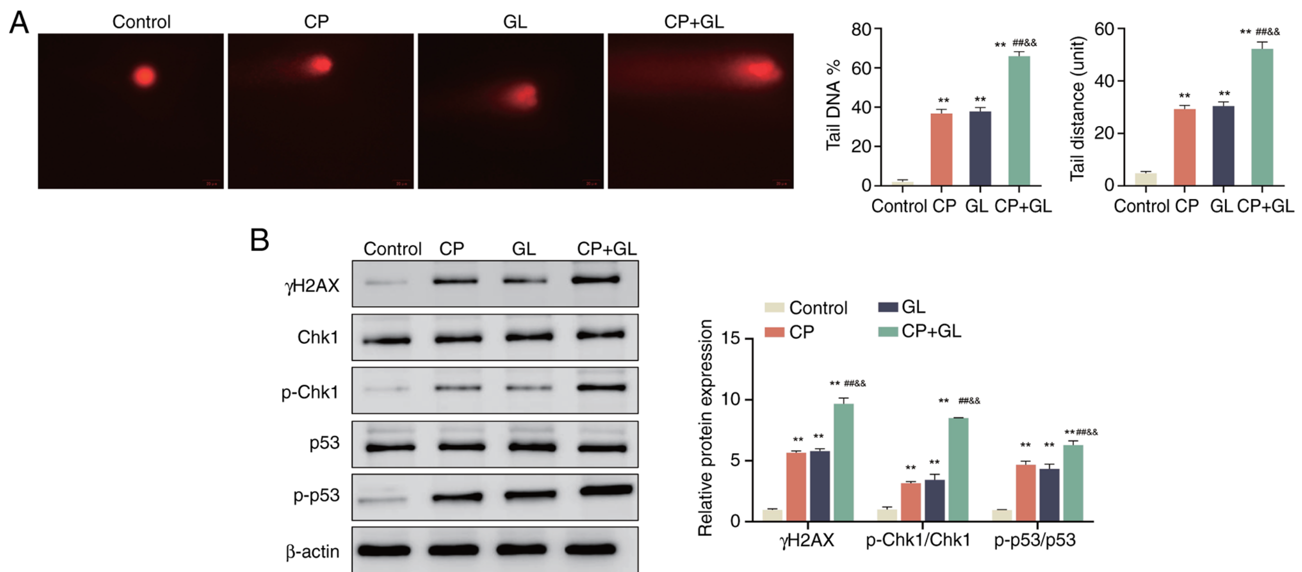

Figure 5. Glycyrrhizin enhances the effect of cisplatin on DNA damage of A549 cells. (A) DNA damage level of A549 cells in the control, CP, GL and CP + GL groups was assessed using a comet assay, and the DNA level and tail distance of the comet tail were analyzed. (B) Western blotting was used to evaluate the protein levels of γH2AX, Chk1, p-Chk1/Chk1 and p-p53/p53 in A549 cells in the control, CP, GL and CP + GL groups. **P<0.01 vs. control; $^{##}$P<0.01 vs. CP; $^{\&\&}$P<0.01 vs. GL. Data are presented as mean ± SD (n=3). CP, cisplatin; GL, glycyrrhizin; Chk1, checkpoint kinase 1; p-Chk1, phosphorylated-Chk1; p-p53, phosphorylated p53. Scale bar, 20 μm.

for its dual role in suppressing cancer cell migration and invasion while enhancing immune system function (37). In the present study, A549 cells exhibited a glycyrrhizin IC_{50} value of 6.17 mM. Conversely, cisplatin remains the conventional first-line chemotherapy for patients with NSCLC (38). The present study indicated that the IC_{50} of A549 cells for cisplatin was as high as 84.9 mM. While cisplatin can hinder cancer cell division by DNA binding, it may also form glutathione adducts with thiol-containing compounds such as glutathione within the cell, thereby reducing its cancer cell-killing efficacy (39). Notably, research suggests that glycyrrhizin can enhance drug absorption by cancer cells, impede drug resistance development (40) and inhibit the activity of the ATP-binding cassette transport system in cancer cells to prevent chemotherapy drug efflux (41). Consequently, the present study investigated the cytotoxic impact of glycyrrhizin on A549 cells when combined with cisplatin. The results demonstrated a significant reduction in the IC_{50} of cisplatin in A549 cells following glycyrrhizin supplementation. Furthermore, the combined treatment notably diminished the aggregation capacity of A549 cells, indicating the potential of glycyrrhizin as an adjunct in anticancer chemotherapy.

Cisplatin primarily exerts its anticancer effects by inducing apoptosis in cancer cells (42). Previous research has reported that glycyrrhizin serves a complex role in regulating apoptosis, depending on specific cellular and pathological conditions. For example, glycyrrhizin has been demonstrated to enhance apoptosis in hepatocellular carcinoma and tacrolimus-induced proximal tubular epithelial cell injury by activating pro-apoptotic signals (43,44). Conversely, in lung tissue and carbon tetrachloride-induced hepatocyte injury models, glycyrrhizin exhibits anti-apoptotic effects (45,46). These findings suggest that the regulatory function of glycyrrhizin in apoptosis may be influenced by variations in cellular transcription and protein levels, as well as multiple potential targets (47-49). Future research should focus on the potential of glycyrrhizin in various cell death pathways, such as the cuproptosis pathway, and explore how the combination of glycyrrhizin with nano-delivery systems can synergistically overcome chemotherapy resistance, aiming to improve the treatment outcomes of NSCLC (50).

In addition to its dual regulatory role in apoptosis, glycyrrhizin and cisplatin may exert synergistic anticancer effects by targeting multiple signaling pathways, demonstrating potential therapeutic advantages when used in combination. Cisplatin is the standard treatment for NSCLC, but its clinical application is often limited by the issue of resistance. Cisplatin resistance is primarily achieved through several mechanisms, including enhanced DNA repair capabilities, overexpression of drug efflux pumps, inhibition of apoptosis and activation of key signaling pathways such as PI3K/AKT and MAPK (7,51,52). Glycyrrhizin, as a natural extract, has been shown to modulate these mechanisms, thereby alleviating cisplatin resistance. Specifically, glycyrrhizin inhibits the PI3K/AKT pathway, reducing AKT phosphorylation and downstream survival signals, leading to G_1/S phase arrest and enhancing cisplatin-induced apoptosis (53,54). This effect may enhance the anticancer efficacy of cisplatin by reducing resistance-related anti-apoptotic signals (23,55). Additionally, glycyrrhizin and cisplatin may exhibit synergistic potential in modulating the MAPK pathway, where glycyrrhizin activates pro-apoptotic p38 signaling and cisplatin induces mitochondrial dysfunction and apoptosis through enhanced oxidative stress (55). Previous research has reported that glycyrrhizin disrupts cancer stem cell maintenance by downregulating the Notch-Hes family BHLH transcription factor 1 pathway, which helps overcome cisplatin resistance (30). The synergistic role of cisplatin in this pathway warrants further investigation, particularly in overcoming cancer stem cell-mediated resistance (30). The combination of glycyrrhizin and cisplatin not only enhances anticancer effects

but may also help optimize treatment strategies by alleviating resistance (56). Future studies could explore the combined effects of glycyrrhizin and cisplatin on these pathways using advanced molecular techniques, such as pathway-specific inhibitors and gene-editing tools, to validate their synergistic mechanisms and optimize therapeutic strategies.

To investigate the mechanism through which glycyrrhizin enhances the biological function of cisplatin, the present study initially examined the impact of glycyrrhizin combined with cisplatin on apoptosis levels. The individual administration of glycyrrhizin or cisplatin led to an increase in apoptosis levels in A549 cells. Notably, the apoptotic induction in A549 cells was increased when cisplatin was combined with glycyrrhizin. The results also demonstrated a significant reduction in the G_1 phase cell population, coupled with a concurrent increase in the G_2 phase population following glycyrrhizin treatment, reflecting alterations in the cell cycle dynamics. Glycyrrhizin has been reported to arrest gastric cancer cells at the G_2 phase, while it has also been observed to halt leukemia cells in the G_1 phase (24,57). Notably, glycyrrhizin has been found to enhance cell cycle disruption induced by cisplatin (28). Cisplatin is recognized for its capacity to trigger apoptosis and cell cycle arrest through DNA damage induction. Despite previous research demonstrating the ability of glycyrrhizin to induce DNA damage in cancer cells over a decade ago, the specific mechanisms through which glycyrrhizin and cisplatin induce DNA damage (31), as well as any potential antagonistic effects between them, remain unclear. Comet assay indicated that glycyrrhizin and cisplatin induce DNA damage, and their combined treatment may further enhance this effect. The results of the present study revealed that glycyrrhizin and cisplatin had a synergistic anticancer effect in A549 cells, marked by increased levels of DNA damage and apoptosis-related proteins (γH2AX, p-Chk1/Chk1 and p-p53/p53). Although the total levels of Chk1 and p53 remained unchanged, the upregulation of p-Chk1 and p-p53 suggested that their phosphorylation served a critical role in the DNA damage response, indicating that activation states may be more important than overall expression. To explore the synergistic anticancer mechanism of glycyrrhizin and cisplatin, future studies should adjust dosage, test other cell lines and focus on the phosphorylation response of the Chk1 and p53 pathways. Furthermore, preclinical model testing will also provide support for clinical application.

In the present study, a marked impact of the combination treatment of glycyrrhizin and cisplatin was observed on the cell cycle of A549 cells, specifically indicated by the transition of cells from the G_0/G_1 and S phases to the G_2 phase. This finding suggests that the synergistic effect of glycyrrhizin and cisplatin may serve an important role in the regulation of cell proliferation and apoptosis. To enhance the generalizability and applicability of the present research, future studies will focus on similar experiments in additional cell lines, particularly in the G_2 or S phase. Additionally, by integrating Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses, future studies should delve deeper into the clinical importance of these cell cycle changes. Understanding how alterations in the cell cycle influence cancer treatment outcomes and prognosis will potentially provide novel insights for optimizing the combined therapy of glycyrrhizin and cisplatin, ultimately offering more effective treatment options for patients with cancer.

Although glycyrrhizin is known for its broad therapeutic applications and established safety, the potential side effects of its co-administration with cisplatin have yet to be thoroughly investigated. Furthermore, differences between experimental conditions and clinical practice, such as dosing regimens and patient heterogeneity, may impact the translatability of findings. Additionally, the long-term risk of resistance to the combination therapy remains unclear. Therefore, well-designed clinical trials are crucial. Future clinical studies should adopt multicenter, randomized, double-blind designs to comprehensively evaluate the efficacy, safety and tolerability of glycyrrhizin and cisplatin combination therapy in patients with NSCLC. Moreover, optimizing dosage and administration methods will be essential for ensuring successful clinical translation. These efforts will contribute to a deeper understanding of the combined mechanisms of glycyrrhizin and cisplatin, providing stronger scientific support for their application in clinical practice.

To conclude, combining glycyrrhizin with cisplatin enhances cytotoxicity in A549 cells by inducing DNA damage, apoptosis and cell cycle arrest. This synergistic effect improves the efficacy of cisplatin in NSCLC and offers a potential strategy for overcoming chemotherapy resistance and minimizing side effects. Further research is needed to validate these findings and explore the underlying mechanisms.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ZT and ZW designed the research study. JJ, WF and SH performed the research. ZW, JJ and WF provided advice on experiments. ZT and SH analyzed the data. ZT and ZW confirm the authenticity of all the raw data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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