

Panax Notoginseng Saponins Exert Anti-osteosarcoma Effect by Inhibiting G₀ / G₁ Phase and Affecting p53-mediated Autophagy and Mitochondrial Apoptosis

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Research article

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Abstract

Background

Osteosarcoma is the most common primary bone malignancy. Chemotherapy for osteosarcoma often induces severe complications to the patients. Thus, the identification of new effective antineoplastic agents with fewer side effects remain a necessity. Panax notoginseng saponins (PNS) were therapeutic active components of panax notoginseng and were reported taking the capability to inhibit the growth of several tumors in vitro and in vivo. However, its effect on osteosarcoma has not been studied. This study first investigated the effect of PNS on osteosarcoma cells.

Methods

CCK-8 assay used to determine the appropriate working concentration of PNS on osteosarcoma,annexV-FITC/PI experiment used to measure the apoptosis of PNS on osteosarcoma, wound healing assay was used to detect the migration of PNS on osteosarcoma, cell invasiveness was measured by transwell assay,cell cycle was measured by PI,the expression of relative protein was shown by western blot.

Results

Our result indicated that PNS inhibited osteosarcoma cells' proliferation, invasion and migration, promoted their apoptosis. Besides, PNS also increased mitochondrial membrane potential and the level of reactive oxygen species. Cell cycle of osteosarcoma was arrested in G₀ / G₁ phase after treatment with PNS. The expression of p53, and mitochondrial related apoptosis proteins were promoted; however, decreased autophagy in osteosarcoma cells with PNS treatment were observed.

Conclusion

Taking the above effect of PNS on osteosarcoma, PNS were of the potential therapeutic value for treatment of osteosarcoma.

1. Introduction

Osteosarcoma is a rare malignancy that occurs in children and adolescents and is diagnosed about 10 patients in per million people [1]. It is highly malignant and tends to metastasize at the early stage [2]. For patients with osteosarcoma, most current treatments mainly depend on neoadjuvant chemotherapy followed by surgical resection [3]. However, chemotherapy often introduces severe complications to the patients [4]. Thus, the identification of new effective antineoplastic agents with fewer side effects remain a necessity.

Autophagy is a process in which aging or damaged organisms were surrounded by autophagosomes, which fused with lysosomes and form autosomes for degradation [5, 6]. Autophagy can prevent accumulation of damaged cell debris and can help keeping the micro-environment homeostasis [7].

Apoptosis is a classical mechanism of inducing cell death [8]. Generally, autophagy is considered to be a protective response to stress, and take the opposite effect on apoptosis: that is, autophagy inhibits the generation of apoptosis [9]. A well-balanced autophagy and apoptosis will be beneficial for limitation the development of tumor.

Panax notoginseng saponins (PNS) are the extract from panax notoginseng, its pharmaceutical effects are widely studied in recent years [10–15]. Based on the published literatures, PNS can facilitate both autophagy and apoptosis in several tumors [16, 17]. However, different cells may take different biological characteristics when facing the same chemical treatment. Taking the reported autophagy and apoptosis regulation function of PNS on some other cells of the previous study, we explored for the first time to know whether PNS influence the growth of osteosarcoma and investigated its possible regulation mechanism related to cell apoptosis and autophagy. We found that PNS can inhibit osteosarcoma cell proliferation and migration, increase mitochondrial membrane potential, inhibit osteosarcoma in G₀ / G₁ phase. Different from the results of other tumor treatment studies, PNS enhance mitochondrial apoptosis but inhibit autophagy in osteosarcoma cells.

2. Materials And Methods

2.1 Antibodies and reagents

DMEM/F12 high glucose was provided by Hyclone (Utah, USA), fetal bovine serum (FBS), trypsin, penicillin-streptomycin, Annex-V/PI kit, Cell Counting Kit-8 (CCK-8), JC-1 kit and ROS kit were supplied by Gibco (New York, USA), transwell chambers with 8 µm-pore-sized membranes were bought from Corning Life Science (New York, USA), Hoechst 33258 was purchased from Sigma-Aldrich (St Louis, MO, USA), PI was purchased from MultiSciences (Zhejiang Province, China), primary antibodies against p53, p27, cleaved caspase-9, cleaved caspase-3, Bcl-2, Bax, cyclinD1, cyclin-dependent kinase 2 (CDK2), cytochrome c, apoptotic protease activating factor 1 (Apaf-1), LC3-I, LC3-II, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were provided by Cell Signaling Technology (Beverly, MA, USA), horseradish peroxidase (HRP)-conjugated secondary antibodies were provided by Huabio (Hangzhou, Zhejiang, China), pifithrin-α (PFT-α) was provided by Selleck Chemicals (Shanghai, China), PNS was provided by Kunming Pharmaceutical Company (Kunming, China).

2.2 Cell culture

Osteosarcoma 143B and HOS cells were obtained from China Centre for Type Culture Collection (Wuhan, China). Cells were cultured in DMEM/F12 medium containing 1% penicillin/streptomycin and 10% FBS in a conventional incubator at 37 °C with 5% CO₂.

2.3 Cell viability assay

Cell viability was measured by CCK-8 kit according to the manufacturer's instructions. Briefly, in a nutshell, cells were seeded in a 96-well plate with 5×10³ cells/well. After 24 hours, the medium was

replaced by the new medium containing 0, 100, 200, 300µg /ml PNS. After incubation for 24 hours or 48 hours, 10µL CCK-8 solution was added to each well. Cell viability was measured at 450 nm using a micro tablet reader (Tecan Sunrise, Salzburg, Austria) 1.5 hours later. All the experiments were carried out in triplicate.

2.4 Cell apoptosis determination

Cells were collected for apoptosis determination by using AnnexV-fluorescent isothiocyanate (FITC)/sodium propanodide (PI) cell apoptosis detection kit. In short, cells were resuspended in 1×binding buffer and added with 5 µL FITC solutions and 5 µL PI solutions. After incubated at 37°C for 15 minutes, cell apoptosis were analyzed by flow cytometry.

2.5 Wound-healing assay

Cells (10^6 cells) were seeded into six-well plates and allowed to fusion. The osteosarcoma cells monolayer was mechanically scratched using a 200 µL sterile pipette tip. After washing twice with phosphate-buffered saline(PBS), cells in DMEM/F12 medium (without FBS) were treated with different concentrations of PNS, and then cultured for a period of time. A series of images of cell migration were captured with a microscope (Olympus IX51) 0 and 24 hours after scratching.

2.6 Colony-formation assay

Osteosarcoma cells were treated in different concentrations of PNS. Cells were placed in a six-well plate with the density of 5×10^2 cells / well, and incubated for another 2 weeks with no PNS in control group and various concentrations of PNS in the treatment groups. Methanol was used to fix the colonies and was stained with Wright-Giemsa solution. Plates were taken and the number of colonies were counted. All experiments were carried out in triplicate.

2.7 Nuclear staining analysis by Hoechst 33258

After 24 hours treatment with PNS, osteosarcoma cells were washed twice with PBS and incubated with Hoechst 33258 at room temperature in the dark for 15 minutes. After washing with PBS twice, cell photographs were taken under an inverted fluorescent microscope (Olympus BX51; Olympus, Tokyo, Japan) (×200), apoptotic cells showed a bright-blue fluorescence. All experiments were repeated in triplicate.

2.8 Measurement of mitochondrial membrane potential (MMP)

JC-1 was performed to evaluate mitochondrial membrane potential. 5×10^5 Cells were rinsed with PBS and trypsinized at 37 ° C for 2 min, and then were incubated with 0.5 mL JC-1 staining working solution at 37 ° C for 20 min. After staining, MMP was measured using flow cytometry.

2.9 Mitochondrial permeability transition pore (MPTP)

Osteosarcoma cells were seeded in the density of 5×10^5 cells / mL, 3 μ L working solution and 5 μ L fluorescence quencher were added to each sample, then be placed in a dark environment at 37 ° C for 15 minutes. Next, after washing with 3 mL MPTP buffer, osteosarcoma cells were resuspended with 400 mL Hanks' balanced salt solution (HBSS). The change of fluorescence intensity of each sample was measured by flow cytometry. The decrease in relative fluorescence units was used to evaluate the openness of MPTP.

2.10 Reactive oxygen species (ROS)

Following the manufacturer's instructions, 5×10^5 cells/mL cells were detected by dichlorodihydrofluorescein diacetate (DCHF DA). Briefly, 10 μ M DCHF DA was stained in medium containing cells at room temperature for 2 hours. After staining, cells were collected and were washed twice with pre-chilled PBS, the amount of reactive oxygen was determined by flow cytometry.

2.11 Transwell essay

Transwell assay was used to evaluate the cell migration ability. Chambers were first washed with serum-free medium, and then 600 μ L of treatment medium containing 10% FBS (control, 100 μ g / ml PNS, 200 μ g / ml PNS) was added to the lower chamber. Cells (200 μ L; 10^6 cells / mL) were planted in the upper chamber. After incubating the cells for 8 hours, the chamber was fixed with 4% paraformaldehyde for 15–20 minutes and then was stained with crystal violet for 15 minutes. Finally, wiping the cells off the upper membrane, and counting the lower membrane cells through an inverted microscopy.

2.12 Cell cycle

Firstly, about $2-3 \times 10^5$ osteosarcoma cells per well were planted into 6-well plate, and were incubated at 37°C for 24 hours after adding PNS of different concentrations (0, 100 μ g / ml, 200 μ g / mL). Then, osteosarcoma cells were washed with PBS and were collected. After that, cells were immobilized in the 75% cold ethanol and kept at -20°C for at least 2 hours. After centrifugation at 12000 rpm for 5 minutes, the cells were hydrated with PBS for 15 minutes, and then the supernatant was aspirated. Finally, the cells were centrifuged at a speed of 12000 rpm for 5 minutes and then stained with 50 μ g / mL PI (MultiSciences, Zhejiang Province) for 30 minutes. The samples were analyzed by flow cytometry (Becton-Dickinson, New Jersey, USA).

2.13 Western blot

Protein expression levels of p27, cyclin D1, CDK2, Bcl-2, P53, Bax, Cytochrome c, Cleaved caspase 9, Cleaved caspase 3, Apaf-1, LC3-I, LC3-II in osteosarcoma 143B and HOS cells were analyzed by Western blot. After treating with different concentrations of PNS, osteosarcoma cells were centrifuged and were washed twice with phosphate buffer solution (PBS). Cells were lysed and centrifuged at 4 ° C for 12 min at 12000 r / min to collect total protein. A certain amount of protein loading buffer was added and boiled at 100 ° C for 15 min. After that, BCA protein assay kit was used to calculate the amount of protein. The

equal protein was denatured for polyacrylamide gel electrophoresis (12%, SDS-PAGE). After electrophoresis, the protein was transferred to a 0.45 μm polyvinylidene fluoride membrane (PVDF). The PVDF membrane was blocked with 5% skimmed milk powder for 1 hour at room temperature, and primary antibody was added to incubate overnight. After the incubation, the PVDF membrane was washed with TBST buffer (TRIS-HCL balanced salt buffer + Tween) 3 times, and then incubated with a secondary antibody at room temperature in the dark for 1 h. Then washing the band twice with TBST buffer, using the Odyssey infrared laser imaging system to scan the film, and utilizing the gel image processing system to analyze the optical density value of the target band.

2.14 Statistical analyses

SPSS 20.0 statistical software package (SPSS Inc, Chicago, Illinois) was used to process the results. The experimental results were expressed in the form of mean \pm standard deviation (SD), and all experiments were repeated three times. One-way analysis of variance was used for statistical analysis, followed by least significant difference (LSD). $P < 0.05$ indicates a significant difference.

2.5 Ethical statement

All the data of this paper was obtained from cell lines, we did not get these data from patients or animals directly, nor intervene these patients. So the ethical approval was not necessary.

3. Results

3.1 PNS inhibit the proliferation of osteosarcoma

Firstly, in order to determine the appropriate working concentration of PNS (chemical structure in figure 1A) on osteosarcoma, CCK-8 test was carried out with PNS treatment at different concentrations. As shown in figure 1B, 1E, 6A and 6B, the optimal concentrations of PNS in osteosarcoma 143B cells at 24h and 48h were $(182.6 \pm 2.3) \mu\text{g/ml}$ and $(193.6 \pm 3.5) \mu\text{g/ml}$ respectively, and the optimal concentrations of PNS in osteosarcoma HOS cells at 24 hours and 48 hours were $(293.8 \pm 3.5) \mu\text{g/ml}$ and $(299.1 \pm 5.2) \mu\text{g/ml}$ respectively. According to the above results, the 143B cells were sub-divided into three groups, namely control ($0 \mu\text{g/ml}$), low concentration group ($100 \mu\text{g/ml}$) and normal concentration group ($200 \mu\text{g/ml}$) respectively; and the HOS cells were sub-divided into three groups, namely nc group ($0 \mu\text{g/ml}$), low concentration group ($150 \mu\text{g/ml}$) and normal concentration group ($300 \mu\text{g/ml}$) respectively. And secondly, we found that the proliferation of osteosarcoma decreased with the increasing concentration of PNS, as shown in figure 1C, D.

3.2 PNS promote the apoptosis of osteosarcoma

AnnexV-FITC/PI experiment indicated that with the increased concentration of PNS, the effect of PNS on apoptosis of osteosarcoma is more obvious, The difference between $100 \mu\text{g/ml}$ group and nc group was statistically significant ($P < 0.05$), the difference between $200 \mu\text{g/ml}$ group and nc group was also statistically significant ($P < 0.05$) (figure 2A, B). Hoechst 32258 was used to measure the apoptosis of

osteosarcoma. Bright blue nuclei indicated apoptotic cells. The results showed that PNS promoted the production apoptosis in a concentration-dependent way (figure. 2C). The difference between 100µg/ml group and nc group was statistically significant ($P < 0.05$), the difference between 200µg/ml group and nc group was also statistically significant ($P < 0.05$). PNS increase the protein expression levels of p53, cytochrome c, cleaved caspase 9, cleaved caspase 3, Apaf-1 and Bax in dose-dependent manner, but decrease the expression levels of Bcl-2 in dose-dependent manner by WB (Figure. 2D, 2E, 6C, 6D). To further verify the above conclusions, PFT- α (p53 inhibitor) was used to detect the apoptosis level of osteosarcoma. The results showed that 30 µm PFT- α could effectively inhibit the apoptosis of osteosarcoma (Figure. 2F, 6G). These results indicated that PNS could promote the apoptosis of osteosarcoma by activating the P53 mitochondrial pathway.

3.3 PNS affect the expression of P53 and autophagy related gene proteins of osteosarcoma

To investigate the effect of PNS on p53 on autophagy, expression of p53 and autophagy related proteins LC3-I and LC3-II were studied. The results showed that PNS increased the expression of P53 and reduced the proportion of LC3-II/LC3-I in a concentration-dependent manner (Figure. 2D, 2E, 6E, 6F). To confirm the above conclusion, PFT- α (p53 inhibitor) was used to detect the autophagy level of osteosarcoma. The results showed that 30µm PFT- α could effectively promote autophagy of osteosarcoma (Figure. 2G, 2H, 6H, 6I). These results confirmed that PNS could affect the expression of autophagy related gene proteins by P53 pathway.

3.4 PNS inhibit the migration and invasion of osteosarcoma

Wound healing assay was used to detect the migration of tumor cells. The experiment indicated that with the increasing concentration of PNS, the migration rate of osteosarcoma was lower (figure 3A, B). The difference between 100µg/ml group and nc group was statistically significant ($P < 0.05$), the difference between 200µg/ml group and nc group was also statistically significant ($P < 0.05$). Cell invasiveness was measured by transwell assay. PNS can significantly inhibit the invasiveness of osteosarcoma (figure 3C, D). The difference between 100µg/ml group and nc group was statistically significant ($P < 0.05$), the difference between 200µg/ml group and nc group was also statistically significant ($P < 0.05$).

3.5 PNS affected the G₀/G₁ phase of osteosarcoma

Cell cycle assay results showed that PNS take significant effect on the G₀/G₁ phase of osteosarcoma cells (figure 4A, B). The difference between 100µg/ml group and nc group was statistically significant ($P < 0.05$), the difference between 200µg/ml group and nc group was also statistically significant ($P < 0.05$). Proteins involved in cell cycle were studied, and the results showed that PNS reduced the expression of cyclinD1 and CDK2, but increased the expression of P27 (figure 4C, 4D, 6E, 6F).

3.6 PNS increase the ROS level, decrease mitochondrial membrane potential and promote the opening of MPTP on osteosarcoma

ROS levels were a sign of early apoptosis. The increasing level of ROS will result in loss of mitochondrial membrane potential and change of MPTP. After 24hours treatment with PNS, flow cytometry was used to measure the level of ROS. PNS increased the ROS level of osteosarcoma (figure 5C, D).The difference between 100µg/ml group and nc group was statistically significant ($P < 0.05$),the difference between 200µg/ml group and nc group was also statistically significant ($P < 0.05$). MMP and MPTP were used to verify the effect of PNS on osteosarcoma. PNS promoted the opening of MPTP and the decrease of mitochondrial membrane potential in a dose-dependent manner (figure 5A,5B,5E).The difference between 100µg/ml group and nc group was statistically significant in MPTP($P < 0.05$),the difference between 200µg/ml group and nc group was also statistically significant in MPTP($P < 0.05$).The difference between 100µg/ml group and nc group was statistically significant in MMP($P < 0.05$),the difference between 200µg/ml group and nc group was also statistically significant in MMP($P < 0.05$).

4. Discussion

Compounds from medicinal plants may take pharmacological function. PNS play an important role in the clinical application in myocardial ischemia, cerebral infarction, atherosclerosis and several metabolic diseases [18-21]. Recently, PNS have been reported to have anti-tumor activity. P Wang et al. pointed out that the PNS can inhibit the metastasis of breast cancer [22]. ZG Yang et al. believed that PNS treated cervical cancer by inducing Hela cell apoptosis [23]. CZ Wang demonstrated that PNS could inhibit the proliferation of colon cancer [24]. Q Yang et al. pointed out that PNS could slow down the proliferation of lung cancer [25]. Some early tumor researches results indicated PNS can inhibit tumor cell proliferation with both increased cell apoptosis and autophagy [16, 17]. However, chemicals may take different function in different cells; the therapeutic effect of PNS on osteosarcoma has not been studied yet.

In the present study, we first demonstrate that PNS inhibited the proliferation of osteosarcoma and determined the appropriate treatment concentration by CCK-8 essay, and such inhibitory effect was in a time-dependent and concentration-dependent way. After PNS treatment, cell apoptosis was promoted. These results suggested that PNS may be of potential medical value for osteosarcoma therapy.

P53 is the guardian human genes and plays as an important tumor suppressor gene [26]. It is involved in several cellular processes, including cell cycle control, apoptosis and autophagy regulation [27-29]. P53 can induce cell cycle arrest and can promote apoptosis related gene expression; it can induce mitochondrial pathway by affecting Bcl-2 and Bax, which result in the loss of MMP and openness of MPTP, and lead to mitochondrial dysfunction. As a result, cytochrome C is leaked into the cytoplasm, which activates the expression of Apaf-1, cleaved caspase 9 and cleaved caspase 3, and eventually lead to the activation of apoptotic pathways. But p53 can also inhibit autophagy by inhibiting the expression of LC3-II. In the present study, different from the early research, osteosarcoma cells showed an increased apoptosis and decreased autophagy after PNS treatment.

Cell metastasis is a sign of malignancy in tumor cells [30]. Malignant metastasis of tumor cells will lead to the failure of treatment in osteosarcoma, and will cause death of about 90% patients. In the present

experiment, PNS could effectively reduce the invasion and migration of osteosarcoma cells in a concentration-dependent manner.

Cell cycle is mainly composed of G_1 , S, G_2 and M phase, which plays a role in inhibiting the growth and development of cells [31]. We observed a significant increase in the ratio of the G_0/G_1 phase in osteosarcoma with the treatment of PNS, which indicating that PNS inhibited the growth and development of the osteosarcoma by inducing G_0/G_1 arrest of the osteosarcoma. Different cell cycles are influenced by different cell cycle regulatory proteins. The combination of CDK and Cyclin D promotes the formation of G1 phase. P27 inhibits this process by binding to the D-CDK4 and E-CDK2 complexes. In our experiment, we found that PNS could promote the expression of p27 and reduce the expression of cyclin D1 and CDK2 to arrest osteosarcoma in the G_0/G_1 phase and inhibit the growth and development of osteosarcoma.

Increasing apoptosis is often accompanied with proliferation inhibition in tumors [32], and osteosarcoma is not an exception. Apoptosis can be divided into death receptor-mediated and mitochondrial-mediated pathways. This study just focused on the effect of PNS on mitochondria-mediated apoptosis. Loss of MMP will increase the mitochondrial membrane permeability and increase the production of intracellular ROS. This study indicated that PNS can cause MMP loss by promoting intracellular ROS production and increasing mitochondrial membrane permeability, together with promoted MPTP opening and up-regulated expression of apoptosis-related proteins, which eventually increase mitochondrial apoptosis in osteosarcoma cells.

Mitochondrial function, autophagy and apoptosis are critical in maintaining the normal physiological function [33, 34]. On the one hand, excessive autophagy can induce apoptosis. However, it should not be ignored that inhibiting autophagy can also increase apoptosis [35]. In the present study, PNS treatment reduced the autophagy in osteosarcoma cells which lead to a significant increase in the adaptability of tumor cells to the neighbor micro-environment, leading to damage in normal cells, such as abnormal metabolism of normal cells, and cause more death of normal cells. In this context, our results are in consistent with some early researches on osteosarcoma [36-38].

For the first time, our study demonstrated that PNS can cause G_0/G_1 arrest, apoptosis increment, but can inhibit autophagy via the p53 pathway in the osteosarcoma 143B and HOS cells. Although the current research hasn't proved the PNS effect in some other osteosarcoma cell lines and hasn't be verified by in vivo experiment, and the death receptor-mediated pathway was not fully investigated; these encouraging findings still open a new way of taking PNS as a potential anti-tumor drug for further investigation.

Abbreviations

PNS=panax notoginseng saponins, FBS=fatal bovine serum, PI=propidium iodide, PBS=phosphate-buffered saline, FITC=fluorescent isothiocyanate, DCHF DA=dichlorodihydrofluorescein diacetate, PVDF=polyvinylidene fluoride membrane, TBST=TRIS-HCL balanced salt buffer + Tween,

MMP=measurement of mitochondrial membrane potential, MPTP=mitochondrial permeability transition pore, ROS=reactive oxygen species

Declarations

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Contribution

Guangtao Han and Ting Liu performed the experiment, and Guangtao Han was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Ethics approval

This article is based on cell lines,so we don't need any ethics approval.

Avalibility of data and materials

All data generated or analysed during this study are included in this published article.

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Figures

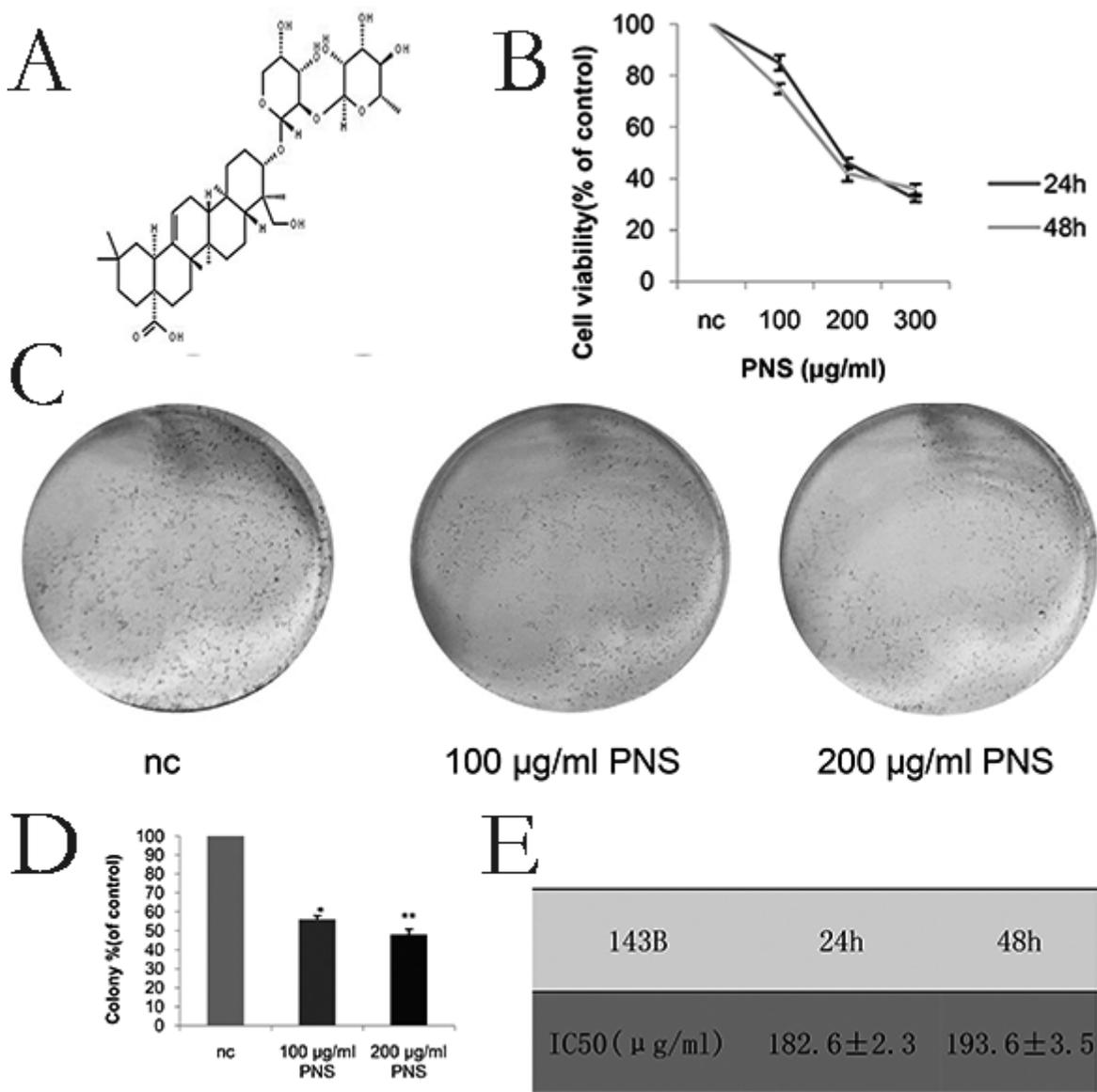


Figure 1

PNS increase the cytotoxicity of osteosarcoma 143B cells and inhibit the proliferation of osteosarcoma in vitro. A. The chemical structure of PNS. B. CCK-8 test to determine the cell viability of PNS on

osteosarcoma 143B cells after 24h and 48h. C-D. Plate cloning assay results and data analysis. The cells were incubated with different concentrations of PNS (0, 100 $\mu\text{g} / \text{ml}$, 200 $\mu\text{g} / \text{mL}$) for 24 hours, and allowed to place osteosarcoma in the medium for 14 days. E. Analysis of IC50 of PNS in osteosarcoma 143B cells. Data are expressed as mean \pm standard deviation. * P means $P < 0.05$ vs nc, ** P means $P < 0.01$ vs nc. All experiments were performed three times.

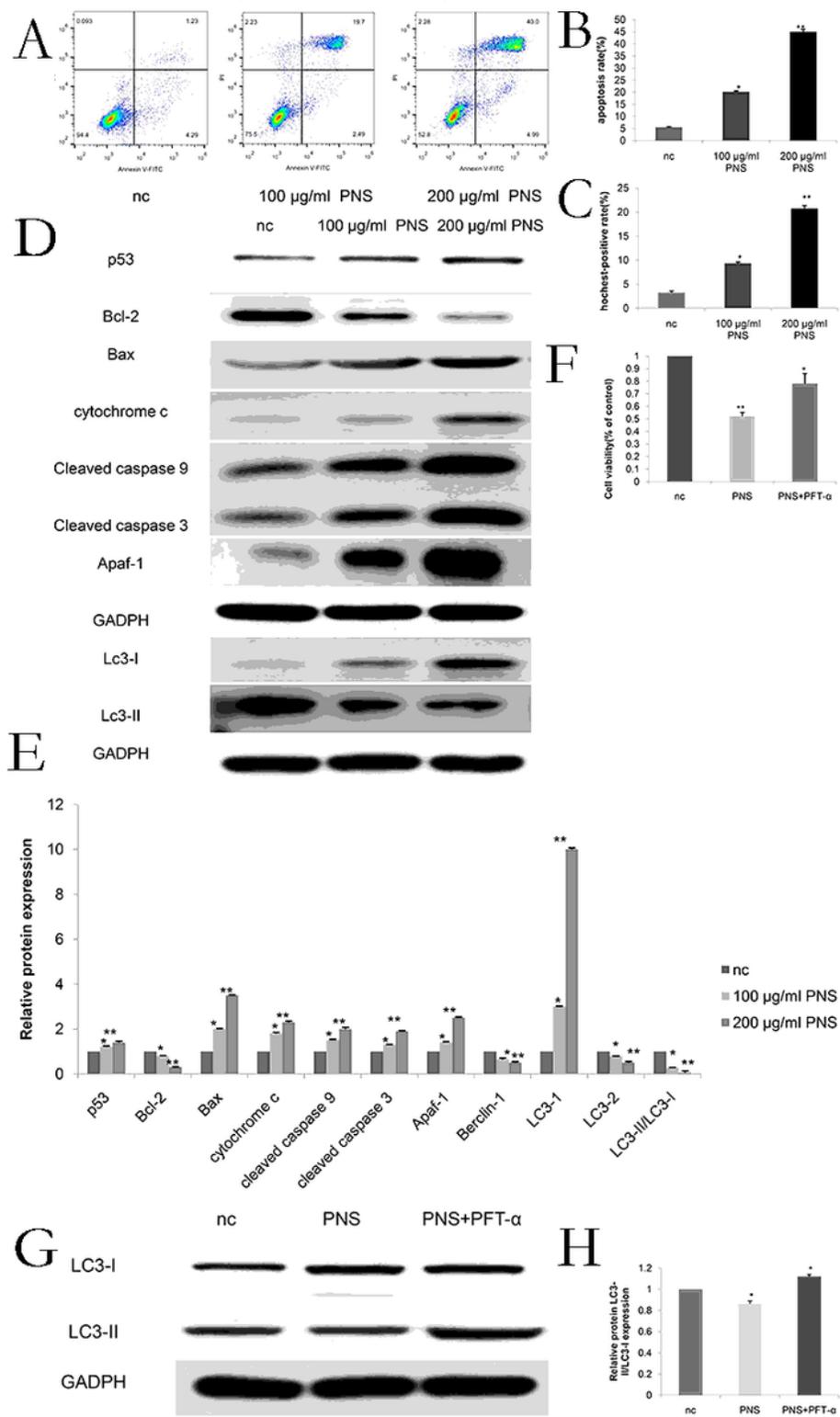


Figure 2

PNS promote the apoptosis of osteosarcoma 143B cells in vitro. A-B. Annex V / PI double staining method was used to detect and analyze the apoptosis level of osteosarcoma 143B cells. As the concentration of PNS increased, the apoptosis rate of osteosarcoma gradually increased. C. Hoechst 32258 analyzes and quantitatively analyzes the morphology of osteosarcoma. The bright blue fluorescent nuclei represent apoptotic osteosarcoma. D-F. Western Blot detected and analyzed p53, apoptosis-related proteins and autophagy-related proteins. Before treating osteosarcoma 143B cells with PNS, PTF- α was used for treatment, and the cell proliferation rate was analyzed by CCK-8. G-H. Western Blot detects and analyzes autophagy-related proteins. Before treating osteosarcoma with PNS, PTF- α was used for treatment. The expression of autophagy-related genes was analyzed by Western Blot. Data were expressed as mean \pm standard deviation. * P means $P < 0.05$ vs nc, ** P means $P < 0.01$ vs nc. All experiments were performed three times.

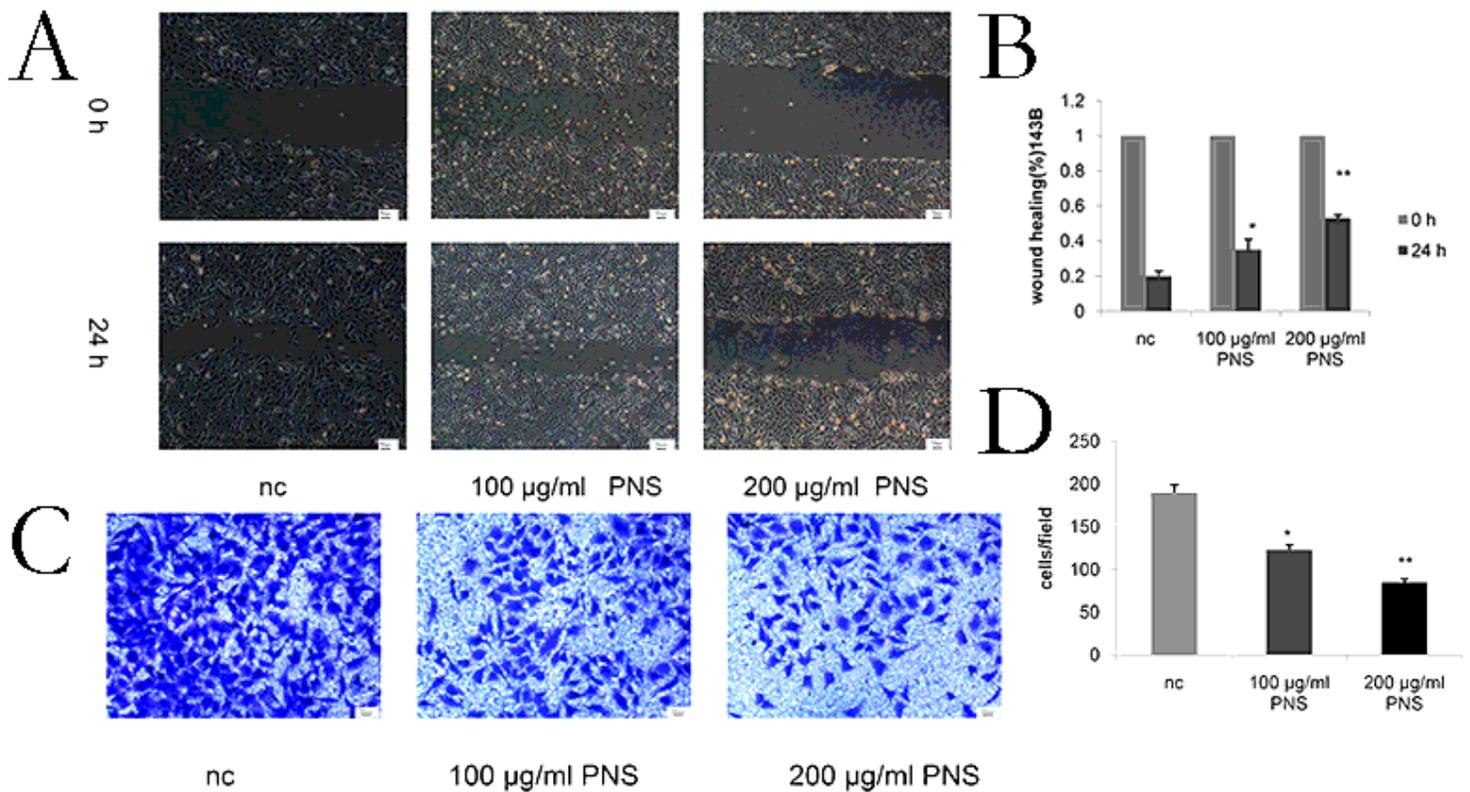


Figure 3

PNS inhibit the invasion and migration of osteosarcoma 143B cells in vitro. A-B. The wound healing experiment indicated that PNS had measured and quantitatively analyzed the migration level of osteosarcoma. The C-D. Transwell assay showed that the invasion level of PNS on osteosarcoma was determined and quantitatively analyzed. Data are expressed as mean \pm standard deviation. * P means $P < 0.05$ vs nc, ** P means $P < 0.01$ vs nc. All experiments were performed three times.

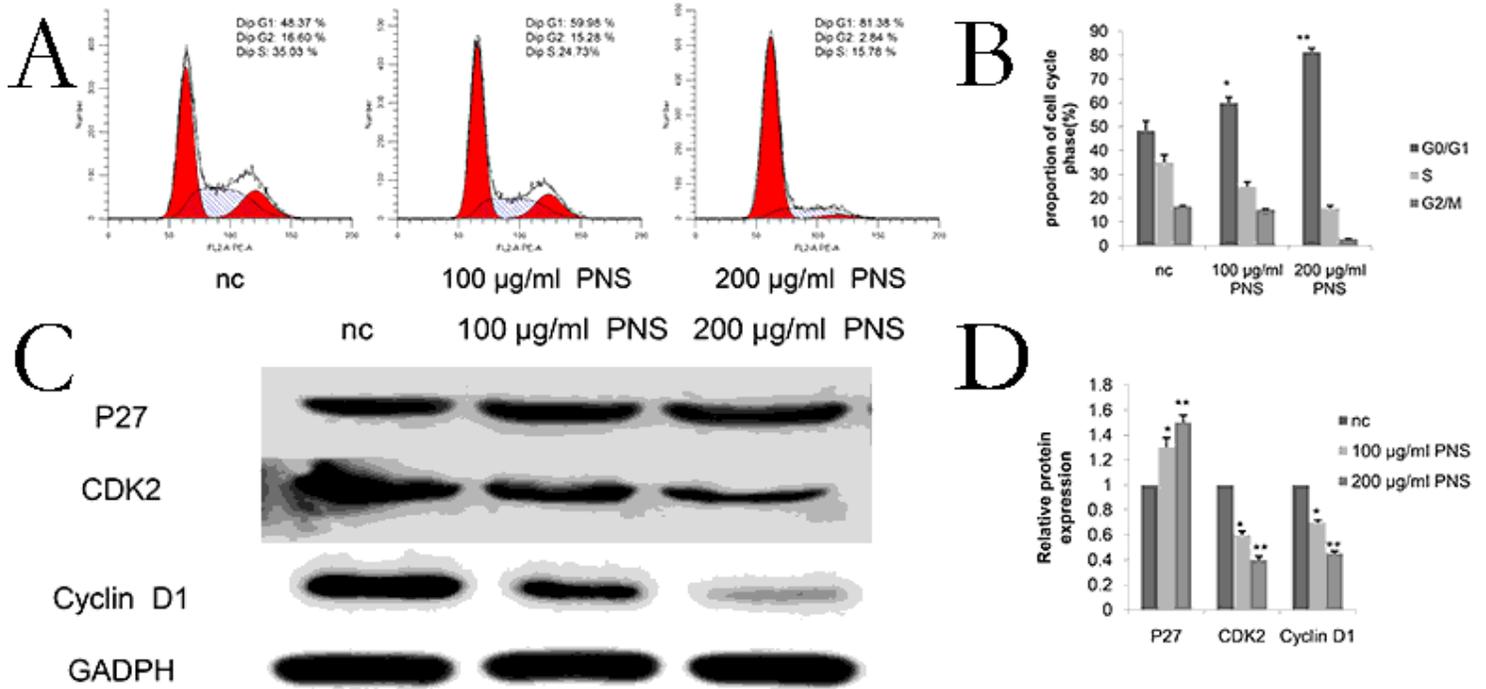


Figure 4

PNS inhibit osteosarcoma 143B cells in G0 / G1 phase in vitro. A-B. PNS were treated with osteosarcoma 143B cells for 24 hours, and the flow cytometry was used to distribute and quantify the DNA content of each stage. The results indicate that PNS block osteosarcoma 143B cells in G0 / G1 phase. C-D, Western blot is used to indicate the detection and quantitative analysis of PNS along with cell cycle related proteins. Data were expressed as mean \pm standard deviation. * P means $P < 0.05$ vs nc, ** P means $P < 0.01$ vs nc. All experiments were performed three times.

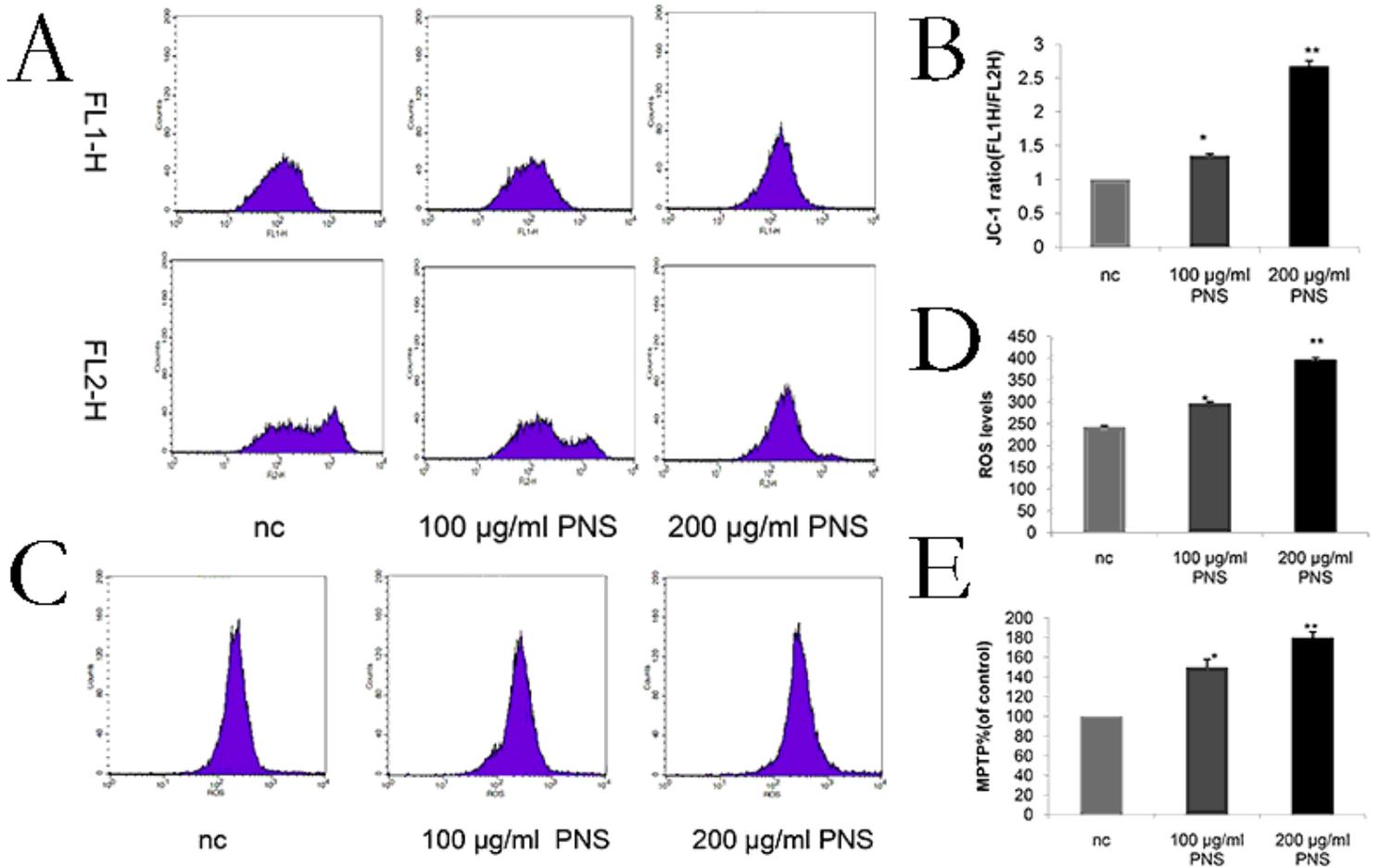


Figure 5

Effect of PNS on mitochondrial membrane potential and reactive oxygen levels in osteosarcoma 143B cells. A-B. Effect and quantitative analysis of PNS on mitochondrial membrane potential level of osteosarcoma. F1L-H represents apoptotic cells, and F2L-H represents normal cells. C-D. The effect and quantitative analysis of PNS on active oxygen level of osteosarcoma. Data were expressed as mean \pm standard deviation. E. MPTP of PNS on osteosarcoma 143B cells.* P means $P < 0.05$ vs nc, ** P means $P < 0.01$ vs nc. All experiments were performed three times.

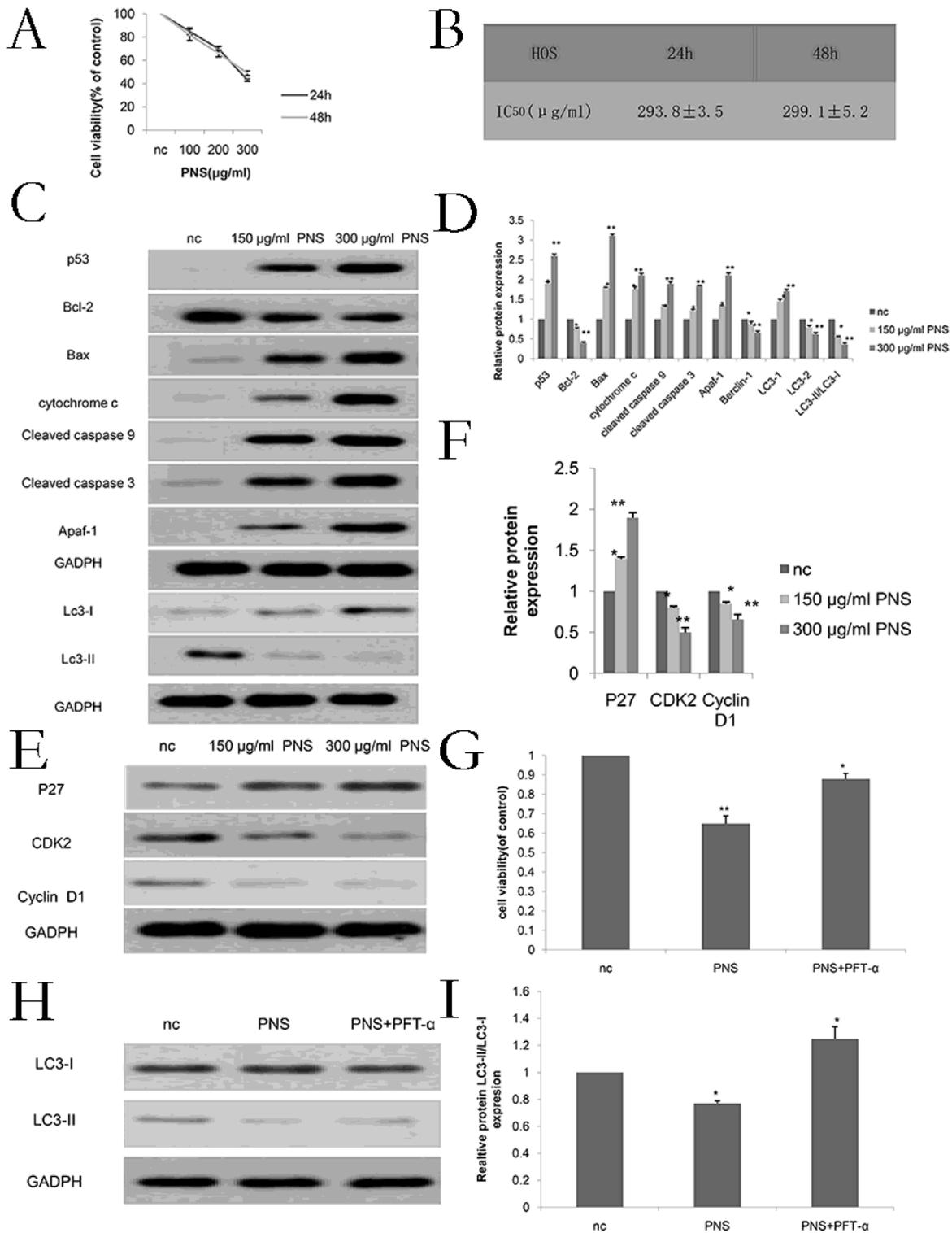


Figure 6

PNS increase the cytotoxicity of osteosarcoma HOS cells, promote the apoptosis of osteosarcoma and inhibit osteosarcoma in G₀ / G₁ phase in vitro. A-B.CCK-8 was used to determine the cell viability of PNS on osteosarcoma HOS cells after 24h and 48h. C-F. WB detected and analyzed p53, apoptosis-related proteins, cell cycle related genes and autophagy-related proteins. G-I, Before treating osteosarcoma with PNS, PTF- α was used for treatment. The expression of autophagy-related genes was analyzed by Western

Blot. Before treating osteosarcoma HOS cells with PNS, PTF- α was used for treatment, and the cell proliferation rate was analyzed by CCK8.* P means $P < 0.05$ vs nc, ** P means $P < 0.01$ vs nc. All experiments were performed three times.