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Original Article

Effects of okadaic acid combined with cisplatin on the proliferation and apoptosis of human lung adenocarcinoma A549 cells

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Abstract: Objective: This study aimed to investigate the effects of okadaic acid (OA) combined with cisplatin (DDP) on the proliferation and apoptosis of human lung cancer A549 cells and to explore the potential mechanism. Methods: A549 cells were independently treated with cisplatin and OA at different concentrations. Combined treatment group was treated 1 μg/ml DDP with 20, 40 or 60 ng/ml OA. Cell viability was detected at 24 h, 48 h, 72 h after treatment By MTT assay. Giemsa staining and acridine orange fluorescence staining were used to detect cell apoptosis. Results: Cell viability was reduced at 24 h, 48 h, 72 h after treatment with OA and DDP. Conclusion: OA or DDP inhibits the proliferation of A549 cells in a time and concentration dependent manner, and both OA and DDP may exert synergistic inhibitory effects on the proliferation of A549 cells.

Keywords: Okadaic acid, cisplatin, A549 cells, combination, apoptosis

Introduction

Lung cancer is a malignancy significantly threatening human health. It has high morbidity and high mortality word wide, and its therapeutic efficacy remains still unsatisfactory. Lung cancer has become the most common malignancy since twentieth century. It was estimated by the International Cancer Research Center that there were more than 1.3 million new lung cancer cases and nearly 1.2 million deaths from lung cancer in 2002 [1]. In 2014, there were about 0.65 million new lung cancer cases and 0.52 million deaths related to lung cancer, and lung cancer had been the leading cause of death world wide [2]. In past 3 decades, the mortality of lung cancer patients in China had risen sharply to 46.5% and lung cancer had become the leading cause of death in China, followed by liver cancer. There are now more than one million patients with lung cancer in China, suggesting that China has the largest population of lung cancer patients in the world. Thus, effective measures are necessary for the prevention and treatment of lung cancer.

Chemotherapy is one of the main strategies for the treatment of lung cancer. Cisplatin (DDP) is one of the most commonly used chemotherapeutics in lung cancer patients. In late 1980s, DDP combined other platinum containing compounds had been employed for the treatment of lung cancer achieving favorable efficacy. However, lung cancer in some patients is progressively resistant to the platinum based chemotherapy, resulting in a poor prognosis. Therefore, it is imperative to develop new chemotherapeutics or new strategies with low toxicity and high efficiency for the treatment of lung cancer. As a marine toxin, okadaic acid (OA) can induce the apoptosis of human lung adenocarcinoma cells and human liver cancer cells, and the anti-tumor effect of OA has been observed at a low concentration [3]. In this study, the effects of OA and/or DDP on the proliferation and apoptosis of human lung adenocarcinoma A549 cells were investigated, and the potential mechanism was further investigated, in order to provide a theoretical basis for the treatment of lung adenocarcinoma.

Materials and methods

Materials

A549 cells were purchased from the Academy of Military Medical Sciences (Beijing, China).

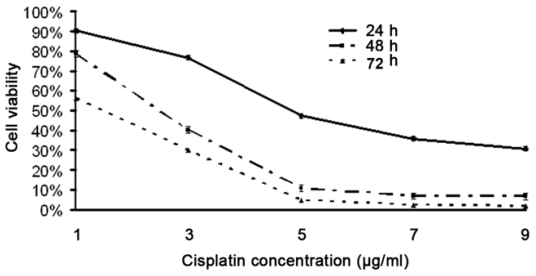

Figure 1. Effect of cisplatin on A549 cells proliferation (MTT assay). The survival rate of A549 cells decreased gradually with the increase in cisplatin concentration and the prolongation of cisplatin treatment.

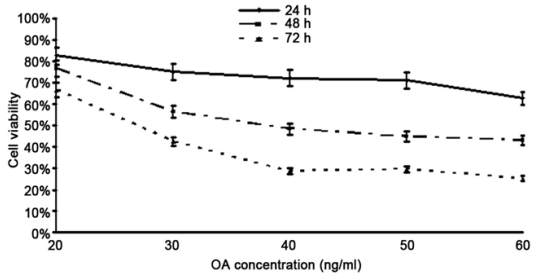

Figure 2. Effect of OA on A549 cells viability (MTT assay). The survival rate of A549 cells decreased gradually with the increase in OA concentration and the prolongation of OA treatment.

RPMI Medium 1640 (Gibco, Grand Island, N.Y.147072, USA), OA (Sigma, St. Louis, MO, USA), cisplatin (Qilu pharmaceutical), fetal bovine serum (FBS), newborn calf serum (Thermo), Giemsa, Acridine Orange (Sinopharm Chemical Reagent Co, Ltd), DMSO and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Solarbio) were used in the present study.

Cell culture

A549 cells were seeded in a 100-ml flask and maintained in RPMI-1640 containing 10% FBS, 10000 U/L penicillin and 0.1 g/L streptomycin at 37°C in a humidified environment with 5% CO_2. When cell confluence reached 80%-90%, cells were digested with 0.05% trypsin in 0.02% EDTA. After centrifugation, cells were harvested, counted and passaged.

Cytotoxicity assay

The cytotoxicity of OA was detected by MTT assay. Cells in logarithmic growth phase were re-suspended in RPMI-1640 to prepare single cell suspension at a density of 4×10^4 cells/ml. Cell suspension was seeded into a 96-well plate (100 μl/well; seven wells per group). Then, the medium was refreshed 24 h later. Cells were treated with OA (20, 30, 40, 50 and 60 ng/ml) or DDP (1, 3, 5, 7, and 9 μg/ml) (100 μl/well) for 20 h, 44 h, and 68 h. MTT solution (20 μl, 5 g/L) was added to each well, followed by incubation for 4 h. The medium was removed, and DMSO (150 μl/well) was added. The absorbance was measured at 570 nm using a microplate reader.

The inhibitory effect of 1 μg/ml DDP combined with 20, 40 and 60 ng/ml OA on the proliferation of A549 cells was investigated with the method abovementioned.

The growth inhibition rate was calculated as follow: 1-(A-B)/(C-B)×100% where A is the absorbance of experiment group, B is the absorbance of blank control group, and C is the absorbance of solvent control group.

The combined effect of DDP and OA was evaluated with following formula [4]. Q=R(A+B)/[R(A)+R(B)-R(A)×R(B)], R(A+B) is the inhibition rate of both drugs; R(A) and R(B) refer to the inhibition rate of A and B, respectively. Q=0.85-1.15 suggests additive effect, Q>1.15 suggests synergistic effect and Q<0.85 suggests antagonistic effect.

Morphological observation of A549 cells after Giemsa staining

Cells in logarithmic growth phase were re-suspended in RPMI-1640 at a density of 2×10^4 cells/ml. Cells were seeded into a 6-well plate (2 ml/well) (two wells per group). The medium was refreshed 24 h later. Cells were treated with 20 ng/ml OA and/or 1 μg/ml DDP. After 48-h incubation, the medium was removed, and cells were then washed twice with PBS. Cells were fixed for 10 min and then stained with Giemsa for 15 min. After washing in ddH_2O twice, cells were observed under a light microscope.

Acridine orange fluorescence staining of A549 cell nuclei

Cells in logarithmic growth phase were re-suspended with RPMI-1640 to prepare single

cell suspension at a density of 2×10^4 cells/ml. Cells were seeded into 6-well plate (2 ml/well) (two wells per group). The medium was refreshed 24 h later. Then, cells were treated with 20 ng/ml OA and/or 1 μg/ml DDP. After 48-h incubation, the medium was removed. Cells were washed twice with PBS and then fixed in 95% ethanol for 20 min. Subsequently, acridine orange staining solution was added to each well. Following washing with PBS, cells were observed under a fluorescence microscope.

Statistical analysis

Statistical analysis was performed with SPSS version 10.0. Data are expressed as mean ± standard deviation (SD). A value of $P<0.05$ was considered statistically significant. Experiments were repeated at least three times. Comparisons were done with one way analysis of variance (ANOVA).

Results

Proliferation of A549 cells was significantly inhibited by OA and/or DDP

MTT assay showed DDP inhibited the proliferation of A549 cells in a time and dose dependent manner. Within a certain range of concentration, DDP significantly inhibited the proliferation of A549 cells (**Figure 1**). As an antitumor drug, OA also inhibited the proliferation of A549 cells at low concentrations in a time and dose dependent manner (**Figure 2**).

OA and DDP had synergistic inhibitory effects on cell proliferation

Cells were treated with DDP at different concentrations for 24, 48 and 72 h, and the proliferation was determined. Results showed that the proliferation of A549 cells was significantly inhibited when compared with control group ($P<0.01$), and the inhibition rate was significantly increased at 24 h when compared with that at 48 h and 72 h ($P<0.05$) (**Table 1**).

After treatment with OA at different concentrations, the growth of A549 cells was significantly inhibited at different time points as compared to control group. Moreover, the inhibition rate was significantly increased at 24 h when compared with that at 48 h and 72 h ($P<0.01$). The inhibition rate was significantly increased at 24 h when compared 48 h and 72 h DDP groups ($P<0.05$) (**Table 2**).

In addition, when A549 cells were treated with 1 μg/ml DDP combined with OA at different concentrations, and the growth inhibition rate was significantly higher than in cells treated with DDP or OA alone (**Table 3**). In order to clarify the relationship between two drugs, the formula of king was used. Results showed two drugs had additive or synergistic inhibitory effects on the proliferation of A549 cells (**Table 4**). Synergistic effect was observed with 20 ng/ml OA and DDP 1 μg/ml at 48 h (**Table 4**). Thus, this treatment was used in the following experiments.

OA combined with DDP had more potent capability to induce cell apoptosis

Cells in control group were spindle-shaped, contacted with each other and had complete nucleus (**Figure 3A**). DDP treated cells become round or oval, and detached from surrounding cells, and cells had a trend of lysis (**Figure 3B**). Apoptotic bodies were found in OA treated cells. Cell apoptosis was more obvious in OA group than in DDP group (**Figure 3C**). Cells treated with both OA and DDP showed diffused distribution, and a large number of apoptotic bodies were observed. Cell morphology changed significantly (**Figure 3D**).

Under a laser scanning confocal microscope, cells in control group displayed strong green fluorescence, their nuclei were complete with uniform distribution (**Figure 4A**). In DDP group, the nuclear fluorescence intensity reduced and the number of nuclei decreased (**Figure 4B**). In OA group, chromosome condensation was observed, and cells became pea or cap-like (**Figure 4C**). The nuclei of cells treated with both OA and DDP were further concentrated and became crescent (**Figure 4D**).

When compared with control group, the apoptosis of cells treated with both OA and DDP was more obvious, and no chromatin fragmentation was observed in the apoptotic bodies (**Figure 5A**, **5B**).

Discussion

OA is a derivative of polycyclic polyether fatty acids and separated from Prorocentrum lima

Table 1. Inhibition rate of A549 cells treated with cisplatin at different concentrations and for different durations ($\bar{x}$±s, n=8)

Dose (μg/ml)	OD (24 h)	IR (24 h)	OD (48 h)	IR (48 h)	OD (72 h)	IR (72 h)
0	1.138±0.110	0	1.383±0.219	0	1.295±0.449	0
1	1.100±0.199	0.097▲	1.116±0.184	0.214Δ,☆	0.787±0.103	0.439▲,★
3	0.902±0.533	0.233▲	0.638±0.143	0.598▲,★	0.486±0.137	0.699▲,★
5	0.607±0.104	0.526▲	0.272±0.049	0.892▲,★	0.195±0.023	0.951▲,★
7	0.492±0.071	0.641▲	0.222±0.043	0.931▲,★	0.168±0.014	0.975▲,★
9	0.439±0.074	0.692▲	0.222±0.033	0.932▲,★	0.158±0.012	0.983▲,★

Notes: ΔP<0.05, ▲P<0.01 vs. control group; ☆P<0.05, ★P<0.01 vs. 24 h group. OD: Optical density, IR: Inhibition rate.

Table 2. Inhibition rate of A549 cells treated with OA at different concentrations and for different durations ($\bar{x}$±s, n=8)

Dose (μg/ml)	OD (24 h)	IR (24 h)	OD (48 h)	IR (48 h)	OD (72 h)	IR (72 h)
0	0.894±0.131	0	1.164±0.138	0	2.032±0.525	0
20	0.888±0.021	0.174	1.240±0.075	0.234▲,★	1.317±0.080	0.334▲,★
30	0.865±0.048	0.250	0.952±0.033	0.437▲,★	0.902±0.061	0.575▲,★
40	0.795±0.083	0.278	0.839±0.032	0.516▲,★	0.664±0.040	0.713▲,★
50	0.784±0.037	0.290Δ	0.788±0.026	0.552▲,☆	0.675±0.033	0.706▲,★

Notes: ΔP<0.05, ▲P<0.01 vs control group; ☆P<0.05, ★P<0.01 vs 24 h group. OD: Optical density, IR: Inhibition rate.

Table 3. Effect of 1 μg/ml cisplatin combined with OA at different concentrations on the proliferation of A549 cells at three time points ($\bar{x}$±s, n=8)

Groups	OD (24 h)	IR (24 h)	OD (48 h)	IR (48 h)	OD (72 h)	IR (72 h)
Control group	1.119±0.733	0	1.149±0.134	0	1.036±0.157	0
DDP (1 μg/ml)	1.101±0.199	0.097	1.116±0.184	0.214	0.787±0.103	0.439▲,★,#
DDP+OA (20 ng/ml)	0.930±0.071	0.257	0.649±0.067	0.543▲,★,#	0.289±0.029	0.786▲,★,#
DDP+OA (40 ng/ml)	0.886±0.357	0.298	0.515±0.032	0.659▲,★,#	0.282±0.037	0.797▲,★,#
DDP+OA (60 ng/ml)	0.662±0.037	0.505▲,★	0.382±0.025	0.774▲,★,#	0.262±0.031	0.826▲,★,#

Notes: ▲P<0.01 vs control group. ★P<0.01 vs cisplatin group. #P<0.01 vs 24 h group. OD: Optical density, IR: Inhibition rate.

Table 4. Growth inhibition rate after OA treatment at different concentrations and Q value in A549 cells

Time	20 ng/ml	Q	40 ng/ml	Q	60 mg/ml	Q
24 h	25.69%	1.01	29.81%	0.86	50.52%	1.16
48 h	54.34%	1.36	65.94%	1.06	77.40%	1.16
72 h	78.60%	1.25	79.73%	0.95	82.64%	0.96

Note: Q=R(A+B)/[R(A)+R(B)-R(A)×R(B)], R(A+B) is the inhibition rate of both drugs; R(A) and R(B) refer to the inhibition rate of A and B, respectively. Q<0.85: antagonistic effect, Q=0.85-1.15: additive effect; Q>1.15: synergy effect.

[5]. OA is rich in the digestive gland of the marine shellfish. It may not only cause economic loss of aquaculture, but threaten human health. Reversible protein phosphorylation, mediated by protein kinases and protein phosphatases, occurs in about one-third of all proteins in human cells, and controls almost every aspect of cell activities [6]. Abnormal protein phosphorylation has been identified in many human diseases, including cancer [7]. OA is a potent cell permeable serine/threonine phosphatase inhibitor [8], and can specifically inhibit protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) [9]. At very low concentrations, OA can induce apoptosis of many cell types including hepatocytes [10], nerve cells [11], 3T3 cells [12], amnion cells [13] and lung adenocarcinoma cells [3].

DDP is an "old" chemotherapeutic. In as early as 1845, it was synthesized by M. Peyrone.

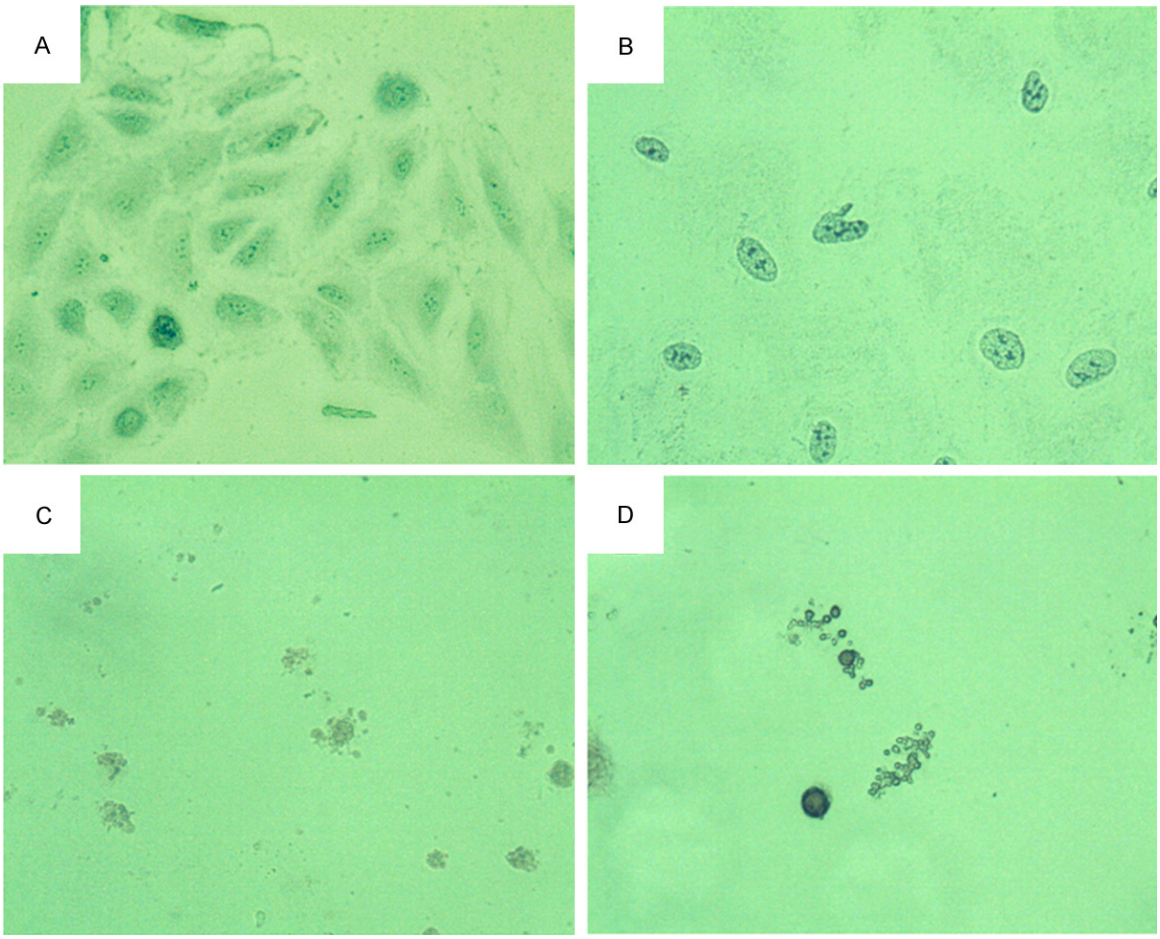

Figure 3. Apoptosis of A549 cells after different treatments (Giemsa staining). A: Control; B: DDP (1 μg/ml); C: OA (20 ng/ml); D: DDP (1 μg/ml) + OA (20 ng/ml).

Food and Drug Administration (FDA) of the United States approved its clinical use in 1978. DDP can be regarded as a magic chemical drug, even though its side effects have been criticized. The cure rate of testicular cancer is almost 100% with DDP, and DDP also has the possibility to cure small cell lung cancer, testicular cancer, osteosarcoma and early ovarian cancer [14]. DDP has a very simple structure and the mechanism of its anti-tumor effect has been elucidated. After DDP enters the human body, it can cross the cell membrane and bind to DNA single strand or double strands [15]. The DNA replication in cancer cells is inhibited by DDP, resulting in cell apoptosis [16]. However, DDP has a high toxicity and may cause resistance to chemotherapy. Thus, it is imperative to develop new anti-cancer drugs with low toxicity and high efficiency. With the pharmacological development, a variety of new anti-cancer drugs have been developed.

Currently, increasing studies employ DDP based combined chemotherapy for the treatment of cancers. Peng et al [17] found that melatonin combined with DDP was able to exert synergistic inhibitory effects on the growth of A549 cells. Yu et al [18] found that dexamethasone combined with DDP promoted the apoptosis of A549 cells, induced cell cycle arrest, and then increased the sensitivity of A549 cells to DDP based chemotherapy.

However, few studies have been conducted to investigate the combined therapy of cancers with OA and DDP. In this study, A549 cell was treated with OA and/or DDP at different concentrations, and the cell viability and apoptosis were detected. Results showed the survival rate of A549 cells was gradually decreased with the increase in the concentration of OA or DDP. Treatment with OA at different concentrations (20 ng/ml, 40 ng/ml and 60 ng/ml) and

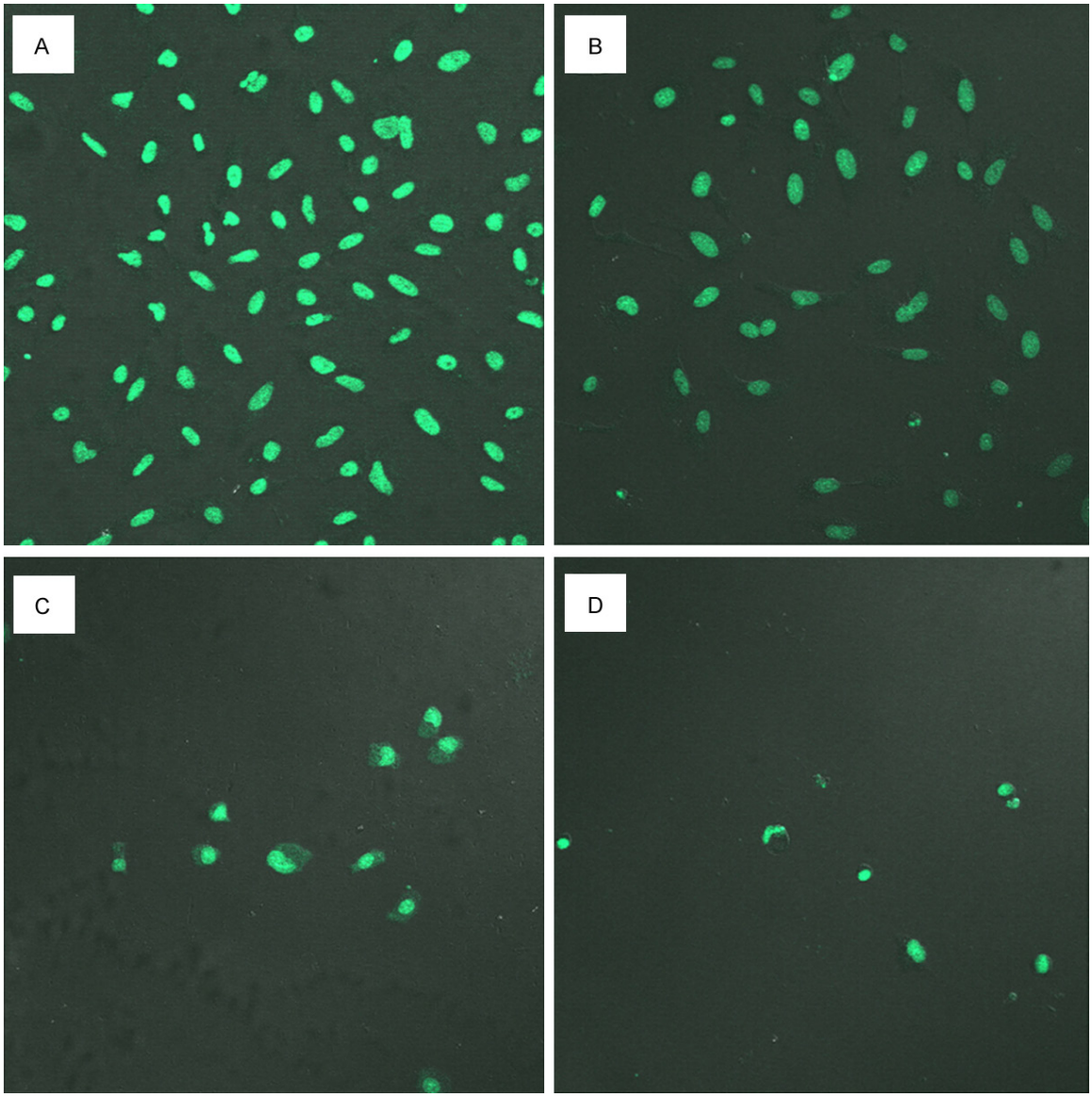

Figure 4. Morphology of A549 cells after different treatments (acridine orange staining, 400×). A: Control group; B: DDP (1 μg/ml) group; C: OA (20 ng/ml); D: DDP (1 μg/ml) + OA (20 ng/ml).

DDP (1 μg/ml) for 24 h, 48 h, and 72 h significantly inhibited the proliferation of A549 cells in a time and dose dependent manner. The Q value of combined treatment was great than 0.85 as shown by the King formula. Moreover, the Q value of OA (20 ng/ml) combined with DDP (1 μg/ml) was higher than 1.15 at 48 h and 72 h, suggesting their additive or synergistic effect.

The proliferation inhibition rate was 52.6% in A549 cells treated with 5 μg/ml DDP for 24 h, and 51.52% in cells treated with 1 μg/ml DDP and 60 ng/ml OA. The proliferation inhibition rate was 59.8% in A549 cells treated with 3 μg/ml DDP for 48 h and 54.34% in cells treated with 1 μg/ml DDP and 20 ng/ml OA. These findings showed that the combined use of OA and DDP reduces the dose of DDP without compromising the anti-tumor effect, which also avoids the side effects of DDP at a high dose [19]. The anti-tumor effect was further confirmed by Giemsa staining and acridine orange staining. The growth inhibition rate of A549 cells treated with OA (20 ng/ml) and DDP (1 μg/ml) was significantly higher than that after monotherapy.

In summary, OA not only inhibits the proliferation of lung adenocarcinoma cells, but enhances the inhibitory effect of DDP on their prolifera-

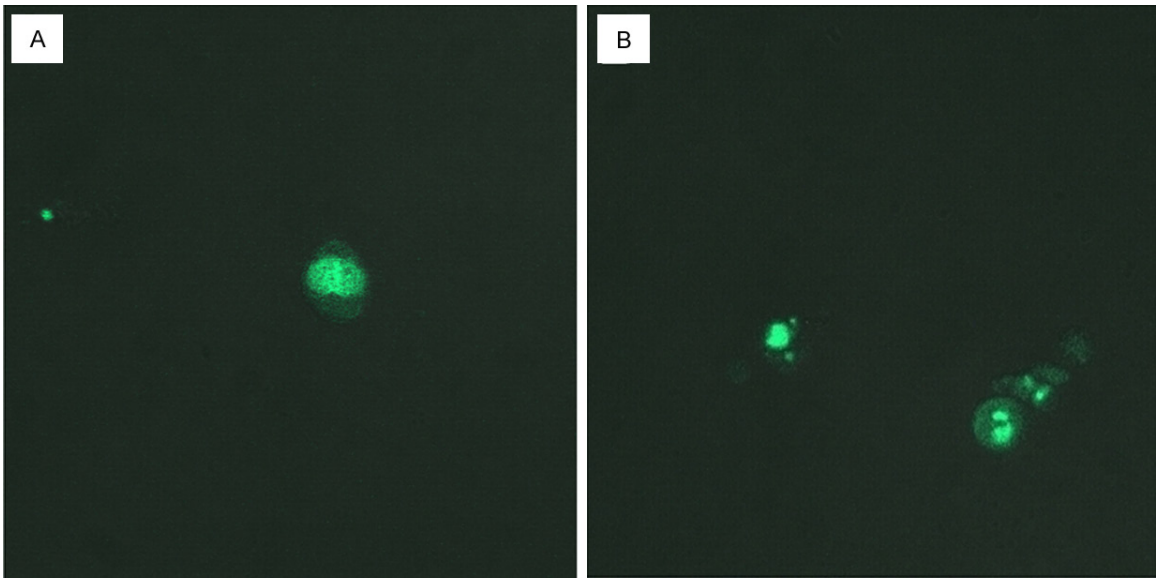

Figure 5. Morphology of A549 cells after different treatments (acridine orange staining; 600× oil lens). A: Control group; B: DDP (1 μg/ml) + OA (20 ng/ml) group.

tion. The effective concentration of OA is relatively low and may be used in combination with DDP. However, these findings are required to be confirmed in animal models before the clinical application.

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Disclosure of conflict of interest

None.

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