

Original Research Article

Effect of chloroquine on the apoptosis of tongue squamous cell carcinoma SCC25 cells induced by bleomycin and nedaplatin

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Abstract: Objective: To study the effect and mechanism of chloroquine-induced chemotherapy drugs bleomycin and nedaplatin on apoptosis of tongue squamous cell carcinoma SCC25 cells. **Methods:** The inhibitory effect of different drugs on the growth of SCC25 cells was screened by MTT assay. MTT, Hoechst33258 staining and FCM were used to observe the changes of morphology, cell cycle and apoptosis of SCC25 cells after different treatments. The quantitative analysis of chloroquine induced by Western blot The effect of chemotherapy drugs on the expression of Bax, Bcl-2 and NFkB apoptosis- related proteins in SCC25 cells. **Results:** The proliferation inhibition rate of bleomycin and nedaplatin SCC25 cells induced by chloroquine was significantly higher than that of bleomycin and nedaplatin alone ($p < 0.05$); Hoechst33358 fluorescence staining and flow cytometry showed In the bleomycin group, the apoptotic rate of the chloroquine plus bleomycin group was significantly increased, and the apoptosis rate of the chloroquine + nedaplatin group was significantly higher than that of the nedaplatin group ($p < 0.05$). The proportion of G0/G1 phase in the chloroquine + bleomycin group was higher than that in the bleomycin group ($p < 0.05$), and the ratio of cells in the S phase to the G2/M phase was decreased; the same chloroquine + nedaplatin group G0/G1 The proportion of cells in the period was higher than that in the nedaplatin group ($p < 0.05$), and the proportion of cells in the S phase and G2/M phase decreased. WB results showed that the expression levels of Bax and NFkB in chloroquine + bleomycin group were significantly higher than those in bleomycin group alone, and the expression level of Bcl-2 was lower than that in bleomycin group alone. Bax and NFkB in chloroquine + nedaplatin group The expression level was significantly higher than that of the nedaplatin group alone. The expression level of Bcl-2 was lower than that of the nedaplatin group alone, and the difference was significant ($p < 0.05$). **Conclusion:** Chloroquine induced bleomycin and nedaplatin can significantly inhibit the proliferation of tongue squamous cell carcinoma SCC25 cells, and its proapoptotic effect may be related to the activation of mitochondrial apoptosis-related proteins and nuclear transcription factors.

Keywords: Chloroquine; bleomycin; nedaplatin; tongue squamous cell carcinoma scc25 cells; proliferation; apoptosis cycle

Introduction

Bleomycin (BLM) was produced by Japanese scientist Umezawa from *Streptomyces verticillatus* in 1966 (*Streptomyces verticillus*) is a natural glycopeptide antibiotic first isolated. Studies have shown that bleomycin has anti-cancer activity and is widely used clinically as a class of chemotherapeutic drugs in head and neck squamous cell carcinoma, nasopharyngeal carcinoma, esophageal carcinoma, malignant lymphoma, breast cancer, choriocarcinoma,

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etc. Nedaplatin (NDP) is a platinum derivative synthesized by Shionogi Pharmaceutical Company and 1983 to solve the problem of cisplatin resistance, aiming to provide similar therapeutic effect to cisplatin and nephrotoxicity therapy with less gastrointestinal toxicity^[2]. Chloroquine (CQ) was first synthesized in 1934 by Bayer AG of Germany. Because of its low price, it has become a natural substitute for quinine^[3]. Chloroquine can be used as chemosensitizer to improve anticancer effect, inhibit cell growth, differentiation and induce cancer cell apoptosis. The purpose of this study is to study the effects of chloroquine-induced bleomycin and nedaplatin on the proliferation of human tongue squamous cell carcinoma SCC25 cells in vitro and the expression changes of apoptosis-related proteins, thus providing certain experimental basis for the application of chloroquine combined with commonly used chemotherapeutic drugs in enhancing the sensitivity of clinical efficacy of tongue squamous cell carcinoma.

1. Materials and methods

1.1 Major reagents and instruments

-80°C refrigerator (Thermo Scientific, US). HR-150AZ Precision Electronic Balance (AND, Japan); CX31 Inverted Phase Difference Microscope (Olympus, Japan). HF90 Heal Force Carbon Dioxide Incubator (Shanghai Lishen Scientific Instruments Co., Ltd., China); Fluorescence Inverted Microscope (Olympus, Japan); Countstar full automatic cell counter (Shanghai Ruiyu Biotechnology Co., Ltd., China); BD FACSCanto II Flow Cytometer (BD Company of the United States); Western blot membrane transfer instrument (Bio-rad company); Western blot electrophoresis apparatus (Bio-rad Company); L420 centrifuge table low speed (Chang Sha Xiang Yi Centrifuge Instrument Co., Ltd., China); R PMI 1640 culture solution (Gibco Company of the United States); Fetal bovine serum (FBS, Biological Industries, BI); Penicillin-streptomycin solution (Shanghai biyun biotechnology Co., Ltd); Trypsin (Sigma, USA); Hoechst33258 (Shanghai biyun biotechnology Co., Ltd); Rabbit anti-human Bcl-2, Bax, NFkB antibodies (United States Abcam company); Bleomycin (Thermophilic), Nedaplatin (Jiangsu sincere Co., Ltd.) and Chloroquine (Shanghai McLean Biochemical Technology Co., Ltd.; Human tongue squamous carcinoma cell SCC25 (available from ATCC, CRL-1628).

1.2 Cell culture

Using RPMI1640 culture solution containing 10% fetal bovine serum, 1% cyan/streptomycin, SCC25 cells of human tongue squamous cell carcinoma were cultured in an incubator of 37 °C and 5% CO₂ according to standard instructions, and the cells of logarithmic growth phase were taken for subsequent experiments.

1.3 MTT experiment

1.3.1 MTT method was used to determine the effect of chloroquine, bleomycin and nedaplatin on SCC25 cells after different time impact of survival

Inoculate SCC25 cell suspension with a concentration of 1×10^4 /ml into a 96 well plate, each well being 100ul. After the cells adhere to the wall for 12h, respectively add the culture solution containing 0, 0.01, 0.1, 1, 10, 100 ug/ml chloroquine, bleomycin and nedaplatin, and chloroquine continuously culture for 24 and 48h, After bleomycin and nedaplatin continue to be cultured for 24, 48 and 72 hours, add 15ul MTT solution, incubate for 4 hours, carefully discard the supernatant, add 150ul DMSO, shake with a horizontal shaker for 15min, and perform absorbance detection at 570 nm wavelength. For the above concentrations, 6 multiple wells were set and the experiment was repeated 3 times. Cell growth inhibition rate = (control group a value-administration group a value)/control group a value $\times 100\%$. The half effective inhibitory concentration (IC₅₀) of bleomycin and nedaplatin was selected as the experimental dose, and the concentration of chloroquine should be the dose that can inhibit cell growth and has no killing effect on SCC25 cells.

1.3.2 Effect of bleomycin and nedaplatin after chloroquine induction SCC25 cells 48 h changes in cell survival rate

Inoculate SCC25 cell suspension with concentration of 1×10^4 individuals, mL in 96 Orifice plate, each well 100 ul,

12 h Adherence to wall. After that, they were divided into groups according to experiments (blank zero adjustment group, control group, chloroquine group, bleomycin group, nedaplatin group, chloroquine + Laimycin group, chloroquine + nedaplatin group). Among them, the treatment method of chloroquine+bleomycin group is: adding chloroquine with concentration of 5 mg/L and 100 ul for pretreatment 24 h, discarding chloroquine solution and adding 5 mg/L bleomycin solution 100 ul. The treatment method of chloroquine +nedaplatin group is as follows: adding chloroquine with concentration of 5 mg/L and 100 ul for pretreatment for 24 h, discarding chloroquine solution, adding 7 mg/L nedaplatin solution 100 ul, the holes without MTT solution are blank zeroing group, and each group is provided with 6 multiple holes. After the drug is added, it is placed in an incubator with 37 °C, 5% CO₂ and 95% relative humidity for 48 hours. After each well is added with 15 ul MTT solution, and after the culture is continued for 4 hours, the supernatant is carefully discarded, the supernatant is added with 150ul DMSO, the shaking table is placed for 15min, the absorbance value is detected at 570 nm wavelength, and the experiment is repeated 3 times.

1.4 Hoechst33258 fluorescent staining method

Inoculate 1×10^4 /ml cell suspensions into 6 well plates, with each well being 1ml. After the cells adhere to the wall 12h, divide them into groups according to established experiments and carry out drug treatment. After 48 hours of continuous culture, PBS was washed 2 times, Hoechst33258 dye solution 300ul was added in turn in the dark. After incubation in an incubator 15min, PBS was washed 3 times, each time 3min. Morphological observation of apoptosis of SCC25 cells was performed under a fluorescence inverted microscope.

1.5 Flow cytometry to detect apoptosis and cell cycle

1.5.1 Flow cytometry detects apoptosis of scc25 cells in different treatment groups

Inoculate 5.0×10^4 /ml cell suspensions into a 25 cm² culture flask, after adhering to the wall for 12 hours ,after adding drugs, each group is placed in an incubator with 37 °C, 5% CO₂ and 95% relative humidity for 48 hours ,and then PBS is washed twice, 0.25% trypsin digestion ,

1000 rpm/min, 5 min centrifugation ,discard supernatant, readjust cell density to $5-10 \times 10^5$ cells/ml. Take 1 ml cell suspension 1000 rpm/min, centrifuge 5 min, discard the supernatant, add 100 ul Binding Buffer for cell resuspension, add FITC labels AnnexinV 5 ul and PI 5 ul in turn, avoid light for 15min, transfer to flow-type on-board detection tube, add 400 ul PBS for on-board apoptosis detection.

1.5.2 Flow cytometry detects cell cycle of SCC25 in different treatment groups

Inoculate 5.0×10^4 /ml cell suspensions into 25 cm² culture flasks, after 12 h adherence, grouping as above. After the drug is added, it is placed in an incubator with 37 °C, 5% CO₂ and 95% relative humidity for 48 hours. PBS is washed twice, digested, and cell density is adjusted to $5-10 \times 10^5$ cells/ml. Take 1 ml cell suspension 1000 rpm and centrifuge for 5 min, discard supernatant, precool at 4 °C, fix with 75% ethanol overnight, centrifuge at 1000 rpm and 5 min, discard supernatant, add 100 ul Binding Buffer for cell resuspension, add PI dye night 1 ml(1g/L) in turn, leave at room temperature in the dark for 30min, and carry out cell cycle detection on the machine.

1.6 Western blot protein detection

The cells of each experimental group were collected and treated. The cells were lysed on ice with RIPA lysate, and the total protein was extracted. The protein content was quantified by BCA. 40 ug of each sample was taken for electrophoretic separation by 12% SDS-PAGE, then transferred to PVDF membrane, sealed with 5% skim milk powder (prepared with TBST) for 2 h, respectively added with rabbit anti-human (Bcl-2, Bax, NFkB) primary antibody, 4 °C incubation overnight, TBST membrane washing 3 times, each time for 10 min, Add Dylight800 to mark goat anti-rabbit secondary antibody, and incubate 2 h on decolorizing shaker under dark shaking at room temperature, 3 times TBST membrane washing for 10 min each time. The Odyssey two-color infrared fluorescence scanning imaging system was used to collect strip images, and the Image J software was used for quantitative analysis, with β -actin as the internal reference.

2. Experimental results

2.1 MMT experiment

2.1.1 MTT tests the effects of chloroquine (0, 0.01, 0.1, 1, 10, 100 mg/L) on the proliferation of SCC25 cells in different concentration groups

After chloroquine of different concentrations was applied to SCC25 cells of tongue squamous cell carcinoma for different times, the cell survival rate gradually decreased with the increase of drug concentration. Compared with the control group, chloroquine group with concentration of 10 mg/L could obviously inhibit cell proliferation within 24 hours.

2.1.2 MTT test the effect of bleomycin on the proliferation of SCC25 cells in different concentration groups

MTT assay was used to detect the effect of bleomycin (0, 0.01, 0.1, 1, 10 and 100 mg/L) on the proliferation of SCC25 cells. The IC₅₀ of 24 h, 48h and 72 h were 26.5 mg/L, 4.88 mg/L and 1.29 mg/L, respectively. The inhibition rate of bleomycin at the same concentration for 72 h is higher than that for 48h and higher than that for 24h. At the same time, with the increase of drug concentration, the inhibition rate of cells increased significantly. Therefore, the effect of bleomycin on the proliferation of SCC25 cells is time and concentration dependent (**Figure 1**. $p < 0.05$).

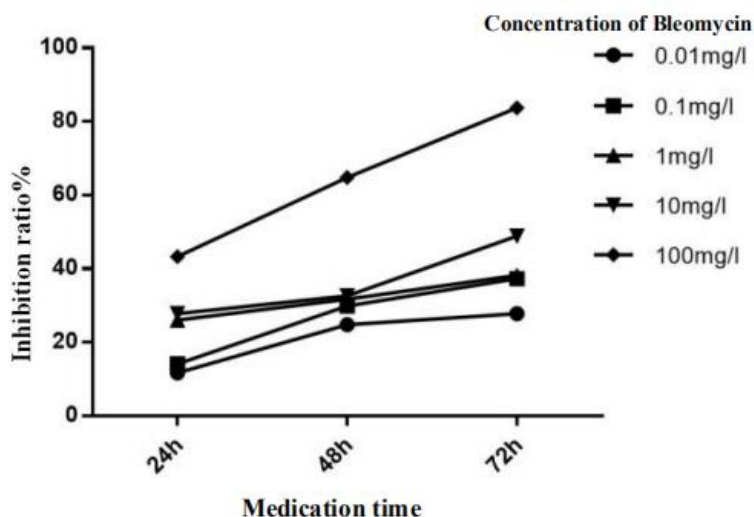


Figure 1. Effect of different concentrations of bleomycin on the inhibition of proliferation of SCC25 cells at 24 h, 48 h and 72 h ($*p < 0.05$).

2.1.3 MTT Test Effect of Nedaplatin on Proliferation of SCC25 Cells in Different Concentration Groups;

MTT was used to detect the effects of nedaplatin (0, 0.01, 0.1, 1, 10, 100mg/L) on the proliferation of SCC25 cells in different concentration groups. The IC₅₀ of 24h, 48h and 72h were 15.6mg/L, 6.98mg/L and 1.47 mg/L, respectively. The inhibition rate of nedaplatin with the same concentration for 72h is higher than that for 48h and higher than that for 24h. At the same time, with the increase of drug concentration, the inhibition rate of cells increased significantly. Therefore, the effect of nedaplatin on the proliferation of SCC25 cells is time and concentration dependent. (**Figure 2** $p < 0.05$).

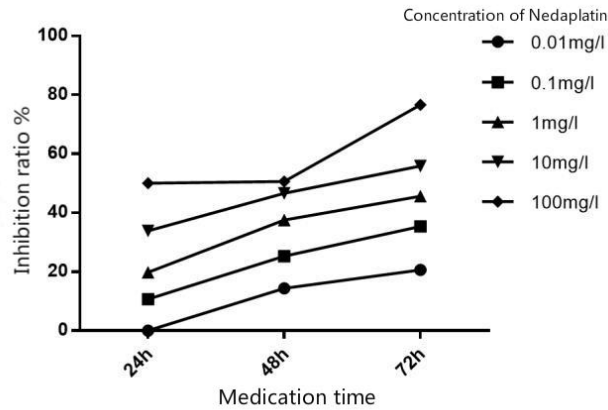


Figure 2. Effect of different concentrations of nedaplatin on the inhibition rate of SCC25 cell proliferation at 24 h, 48 h and 72 h (* $p < 0.05$).

2.1.4 MTT, determination of the effect of bleomycin and nedaplatin induced by chloroquine, SCC25, cell, 48 h, post-cell survival rate and Inhibition rate

All experimental groups are higher than that of control group. The inhibition rate of chloroquine+bleomycin group (36.3%) is higher than that of bleomycin group production rate (24%), chloroquine +nedaplatin group inhibition rate (35.7%) is greater than nedaplatin group inhibition rate (26.2%). The bleomycin or nedaplatin induced by chloroquine can increase the inhibitory effect of bleomycin or nedaplatin alone on SCC25 cells (Figure 3. $p < 0.05$).

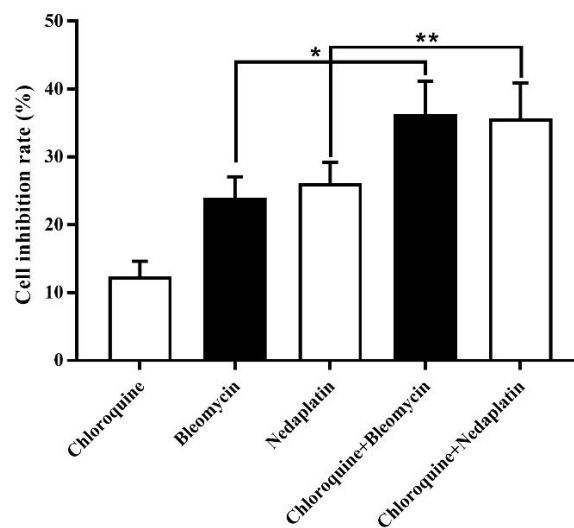


Figure 3. Cell inhibition rate of the SCC25 cells in different drug groups at 48h.

2.2 Hocchest-33258 fluorescence staining to observe apoptosis

Hocchest-33258 fluorescence staining was used to observe the changes of nuclear morphology. Compared with the control group, apoptosis in each drug group had obvious changes. The percentage of apoptosis in chloroquine+bleomycin group was significantly higher than that in bleomycin group, while the percentage of apoptosis in chloroquine+nedaplatin group was significantly higher than that in nedaplatin group alone. It is indicated that bleomycin or nedaplatin induced by chloroquine can increase the apoptosis ratio of cells compared with bleomycin and nedaplatin alone. Compared with the control group, the other groups can cause typical morphological changes of apoptosis such as nuclear concentration and consolidation in different degrees of SCC25 cells (Figure 4).

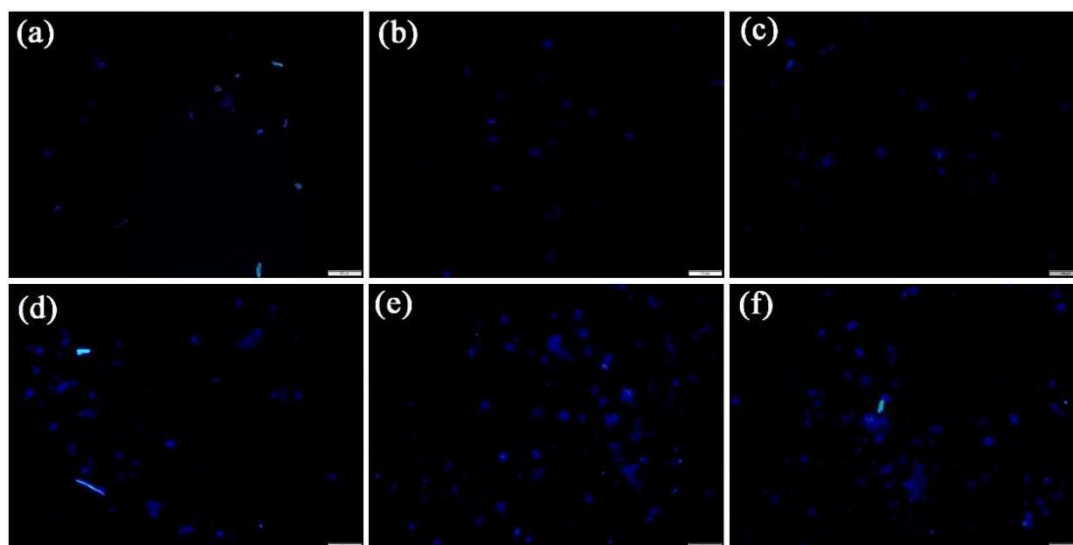


Figure 4. SCC25 cell morphology of each drug group Hoechst33258 fluorescent staining. (a. control group; b. CQ group; c. BLM group; d. NDP group; e. CQ + BLM group; f. CQ + NDP group).

2.3 Flow cytometry to detect effects of different drugs 48 h effect on SCC25 apoptosis and cell cycle

2.3.1 Flow cytometry to detect the effects of different drugs 48 h effect on SCC25 apoptosis

The proportion of total apoptosis is larger than that of bleomycin group, and the proportion of early apoptosis, late apoptosis and total apoptosis in chloroquine + nedaplatin group is larger than that in nedaplatin group, and the apoptosis rate of each group has significant statistical difference (**Figure 5**).

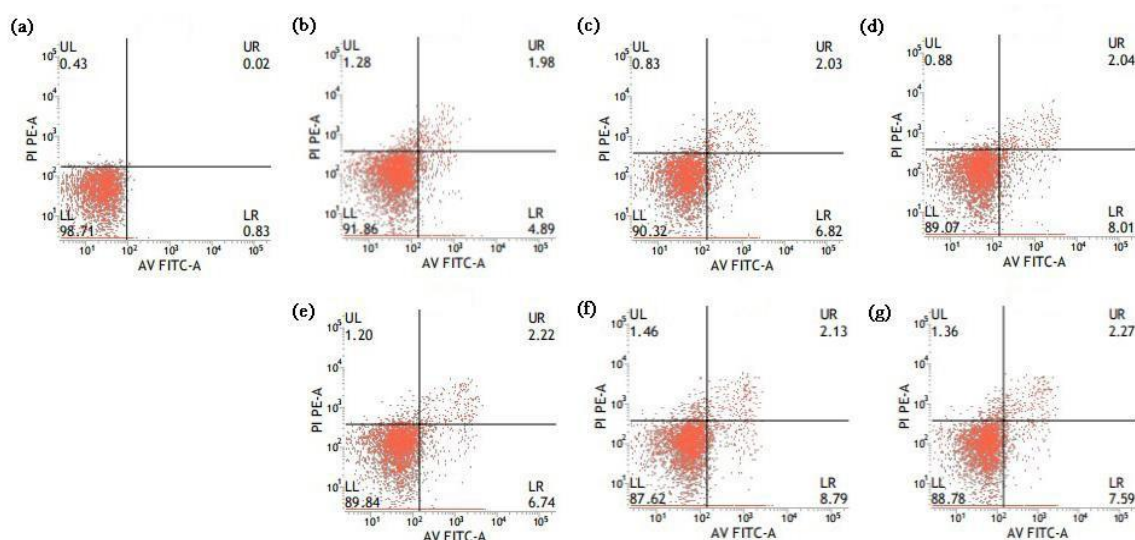


Figure 5. Detect the effect of different drugs on the apoptosis of SCC25 cells for 48 h by flow cytometry. (a. blank group; b. control group; c. CQ group; d. BLM group; e. NDP group; f. CQ + BLM group; g. CQ + NDP group).

2.3.2 Flow cytometer detects the effect of different drugs 48 h on cell cycle of SCC25

After different drugs acted on SCC25 cells for 48 h, the results of flow cytometry showed that each drug group could induce the cell cycle of SCC25 to change. Compared with bleomycin group, the proportion of G0/G1 phase in chloroquine + bleomycin group increased, and the proportion of S phase and G2/M phase decreased. Compared with nedaplatin group, the proportion of cells in G0/G1 phase of chloroquine + nedaplatin group increased, and the proportion of cells in S phase and G2/M phase decreased. Therefore, both bleomycin and nedaplatin can block G0/G1

phase of SCC25 cells. The combination of chloroquine and bleomycin or nedaplatin causes the G0/G1 phase retardation of SCC25 cells to be more severe than the single effect of bleomycin or nedaplatin on SCC25 cells (**Figure 6**).

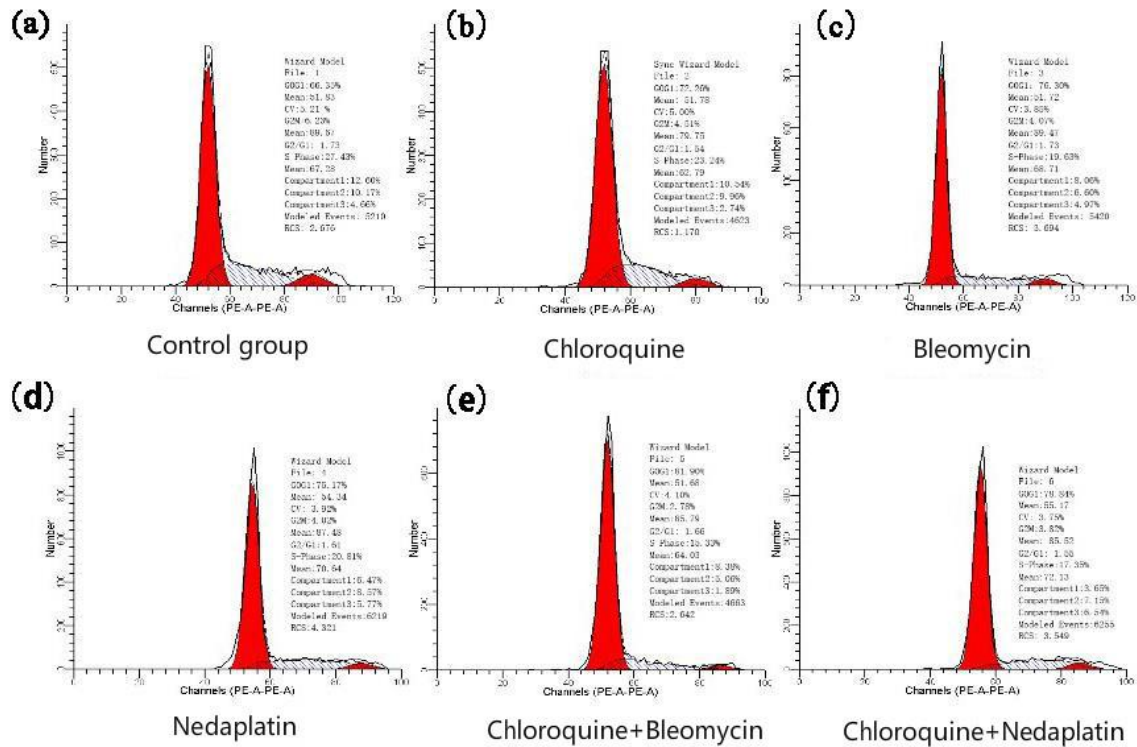


Figure 6. Changes in cell cycle of SCC25 induced by different drugs for 48h by flow cytometry.

2.4 Effects of different drugs on protein expression of SCC25 cells Bcl-2, Bax and NFkB

The protein expression level of SCC25 cells in different drug groups changed. Compared with the control group, the expression levels of Bax, NFkB and Bcl-2 in all drug groups increased and decreased. The expression level of Bax and NFkB in chloroquine +bleomycin group is higher than that in bleomycin group, while the expression level of Bcl-2 is lower than that in bleomycin group. The expression level of Bax and NFkB in chloroquine +nedaplatin group is higher than that in nedaplatin group, while the expression level of Bcl-2 is lower than that in bleomycin group (**Figure 7**. $p < 0.05$).

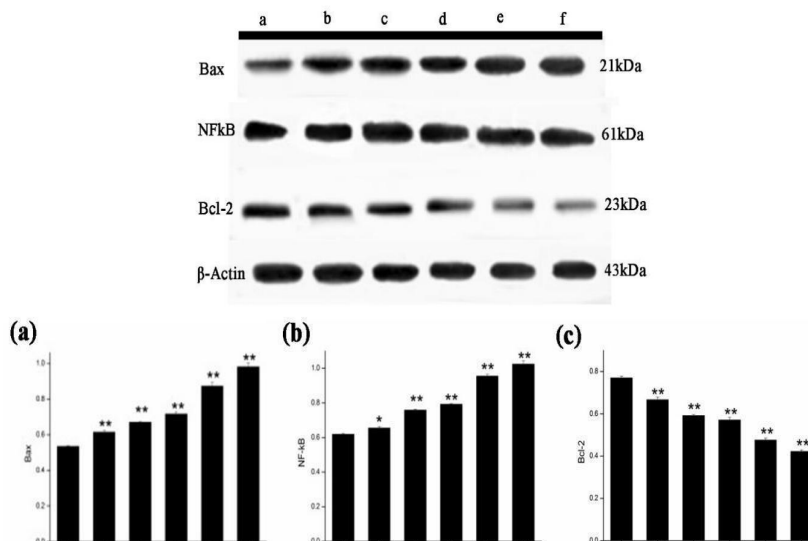


Figure 7. Effect of different drug groups on protein expression of SCC25 cells ($p < 0.05$, $p < 0.01$ vs blank group).

3. Discussion

The incidence of tongue squamous cell carcinoma is the highest among oral cancers. Tongue cancer has high malignant degree, high local recurrence rate and high cervical metastasis rate, which endangers the life of patients, thus requiring radical surgery. At present, the treatment method is mainly surgical operation, supplemented by chemotherapy or radiotherapy combined treatment^[4]. As the tongue is a heavy functional organ such as pronunciation and chewing, the patient should be cured on the basis of reducing dysfunction as much as possible. Chemotherapy, as one of the important methods for treating tongue cancer, is crucial for the treatment of advanced cases, auxiliary before and after surgery and some patients with distant metastasis, and can effectively improve the prognosis of patients. However, conventional chemotherapy drugs at present are easy to cause a series of side effects^[5-9] such as loss of appetite, alopecia, vomiting, nausea, body immunity decline, bone marrow suppression, etc. At present, scholars at home and abroad believe that the combination of drugs can effectively improve the curative effect of tumor and survival rate of patients, but these treatments often increase drug toxicity and cause the risk of cross drug resistance^[10-11]. The development of new anti-cancer drugs requires huge human and material resources. According to James Black, a famous Nobel Prize laureate in pharmacology, the most effective way to find new drugs is to start with existing drugs^[12-13]. Therefore, the way to solve drug resistance is to find out the new functions of the existing drugs and to reduce the side effects of the drugs.

Chloroquine can be used as chemosensitizer to improve anticancer effect, inhibit cancer cell growth, differentiation and induce cell apoptosis. It can be combined with various chemotherapeutic drugs to improve the therapeutic effect of cancer^[14-17]. The effect of bleomycin and nedaplatin of different concentrations on the proliferation of SCC25 cells was detected by MTT method in this experiment. The results showed that with the increase of different drug concentrations, the in vitro inhibitory effect on SCC25 cells also gradually increased, and the survival rate of SCC25 cells significantly decreased, showing a time-dose dependent relationship. At the same time, the in vitro inhibitory effect of bleomycin or nedaplatin induced by chloroquine on SCC25 cells was greater than that of single drug, and the inhibitory rate of SCC25 cells increased significantly. Preliminary results show that the use of bleomycin and nedaplatin after chloroquine induction has better in vitro inhibitory effect on SCC25 cells. The key mechanism of chemotherapeutic drugs acting on tumor cells is inducing apoptosis of fine cells^[18]. In this study, the occurrence rates of early apoptosis, late apoptosis and necrosis of cells were detected by flow cytometry. The results showed that with the increase of concentration of bleomycin or nedaplatin, the cells of human tongue squamous cell carcinoma SCC25 had obvious apoptosis induction effect, while the bleomycin and nedaplatin induced by chloroquine had higher apoptosis induction effect on the cells of human tongue squamous cell carcinoma SCC25, and the occurrence rates of apoptosis among various drug groups were significantly different. Cell cycle is a process of cell division and proliferation into progeny, of which G1 to S and G2 to M are the two most important stages, which are in a period of complex and active molecular level changes. Being susceptible to environmental conditions, cell cycle is often used as the key^[19] for in-depth understanding of biological growth and development and controlling tumor growth. At the same time, the changes of cell cycle were detected by PI single staining in each drug group. The results showed that each drug group could cause G0/G1 phase retardation of SCC5 cells, while bleomycin or nedaplatin could cause more G0/G1 phase retardation of SCC25 cells after chloroquine induction. Further Western blot experiment was used to quantitatively analyze the protein expression changes of apoptosis-related egg white Bcl-2, Bax and NFkB after 48h culture of SCC25 cells treated by different drug groups. The results showed that compared with the control group, all protein expression levels in each drug group were activated. The expression level of Bax in chloroquine +bleomycin group was significantly higher than that in bleomycin group alone, and the expression levels of Bcl-2 and NFkB were significantly lower than that in bleomycin group alone. However, the expression level of Bax in chloroquine +nedaplatin group is higher than that in nedaplatin group alone, and the expression levels of Bcl- 2 and NF-kB are significantly lower than that in nedaplatin group alone, with significant difference ($p < 0.05$). Relevant research findings are similar to this^[20-22].

To sum up, this study preliminarily shows that the combined action of chloroquine, bleomycin and nedaplatin can

significantly inhibit the proliferation and induce apoptosis of tongue squamous cell carcinoma SCC25 cells in vitro, and its effect of promoting apoptosis may be related to the activation of mitochondrial apoptosis-related proteins and nuclear transcription factors. This provides a certain empirical basis for chloroquine-induced application of bleomycin and nedaplatin in clinical treatment of tongue squamous cell carcinoma. The author believes that although chloroquine has been widely recognized as a chemosensitizer to improve the anti-cancer effect, there is still a lack of in vivo experimental research, which is also the deficiency of this topic. Therefore, in the future, we should further explore from the aspects of in vivo test, drug toxicity, safe and effective dose and deeper molecular mechanism.

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