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Inhibitory Effects of C-phycocyanin Combined with All-transretinoic Acid on the Growth of HeLa Cells*

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ABSTRACT Objective: We focused on studying the effects of All-transretinoic acid (ATRA), C-phycocyanin (C-PC) or ATRA + C-PC on HeLa cell growth, cell cycle distribution and apoptosis. **Methods:** MTT assay was adopted to determine the effects of C-PC and/or ATRA on the growth of HeLa cells. The expressions of CDK-4 and Bcl-2 were determined by in situ hybridization and immunohistochemistry staining. TUNEL assay was used to analyze the cell apoptosis. **Results:** Both C-PC and ATRA could inhibit the growth of HeLa cells. And these two drugs showed synergistically inhibitory effects. The dosage of ATRA was reduced when used with C-PC. ATRA, combined with C-PC, was more efficient at inducing cell cycle arrest by decreasing CDK-4 expression. The combination of the two drugs could lead to down-regulation of Bcl-2 expression and thereby cell apoptosis. **Conclusion:** By combined with C-PC, ATRA can be used at lower dosage with reduced toxicity. C-PC + ATRA combination might exert the inhibitory effects on cell growth by inducing cell cycle arrest and cell apoptosis.

Key words: All-transretinoic acid; C-phycocyanin; HeLa cells; Apoptosis mechanism

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Introduction

Cancer is a class of diseases characterized by out-of-control cell growth and it's one of the leading causes of death in the world. Cancer symptoms are quite varied and depend on where the cancer is located, where it has spread, and how big the tumor is. The clinical treatments of cancer include surgery and chemotherapy. Since chemotherapy has the disadvantage of drug resistance and toxicity [1], it is necessary to find some novel therapies to reduce the toxicity of drugs.

Spirulina platensis has been widely used for 100 years as an excellent nutrient supplement for human beings. C-phycocyanin (C-PC) is extracted from Spirulina platensis and is a water-soluble protein pigment. This protein consists of α and β subunits and the natural existing form is trimeric aggregation $(\alpha\beta)3$ [2]. C-PC is a famous healthy food with its antioxidation [3], antivirus [4], anti-tumor [5] and radical scavenging effects [6].

All-trans-retinoic acid (ATRA), a derivative of vitamin A, has great effects on cell differentiation[7]. In acute promyelocytic leukemia (APL), ATRA is a potent inducer of APL cell differentiation. However, the therapeutic use of this drug is often limited by its toxicity [8]. To solve this problem, several chemotherapeutic drugs such as arsenic trioxide (As2O3) [9] and fludarabine [10] were used in combination with ATRA.

For the same purpose, we focused on studying the effects of ATRA, C-PC or ATRA + C-PC on the growth, cell cycle distribution and apoptosis of HeLa cells, and further revealing the anticancer mechanism of the drug combination.

1 Materials and Method

1.1 Materials

Henrietta Lacks strain of cancer cells (HeLa cells) were kindly provided by Affiliated Hospital of Medical College of Qingdao University. Spirulina platensis tablets were purchased from Ocean University of China. All-transretinoic acid was purchased from Sigma-Aldrich Group. MTT were purchased from Beijing solarbio science & technology. Biotinylated rabbit anti-human bcl-2 monoclonal antibody was from santa Co., Ltd; Cdk-4 in situ hybridization kit, in situ cell apoptosis detection kit, DAB staining kit and immunohistochemistry staining kit were purchased from Boster Biological Engineering Co., Ltd.

1.2 Cell culture

HeLa cells were incubated in RPMI 1640 supplemented with 10% fetal calf serum and cultured in a humidi atmosphere with 5% CO_2 at 37 ℃. Cells with exponentially growing were used for all experiments.

1.3 Drugs

C-phycocyanin (C-PC) was extracted from Spirulina platensis [11] and was dissolved in phosphate buffer saline (PBS) and stored at -20 ℃. All-transretinoic acid (ATRA) was kept at a concentration

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of 10 mM in ethanol at -20 ℃ and diluted before use.

1.4 Cell proliferation and clonogenic assay [12]

MTT (methyl thiazolyl tetrazolium) assay was used to measure the effect of C-PC and ATRA on HeLa cell proliferation. The HeLa cells (1×10^4 cells/well) were seeded into a 96-well plate in 100 μL medium and cultured overnight. Twenty-four hours later, six different concentrations of drugs were added to the plates at a volume of 100 μL per well and the plates were incubated for 48h with the drugs. Then, 20 μL of MTT was added to each well and incubated for 4h at 37 ℃. The medium was carefully discarded. The formazan crystals were dissolved in 100 μL dimethylsulphoxide (DMSO) and the absorbance was read at 490 nm by spectrophotometer. Absorbance values were expressed as a percentage of drug treated group and untreated control group. The half maximal inhibitory concentration (IC50) of each drug was calculated and used as the optimum concentration of the drug of each group. Half of the IC50 value of each group was the drug concentration used in the following experiments.

1.5 The groups of the experiment

HeLa cells were divided into four groups: (1) control group: HeLa cells were not treated with any drugs. (2) C-PC treated group: HeLa cells were treated with C-PC alone. (3) ATRA treated group: HeLa cells were treated with ATRA alone. (4) C-PC + ATRA synergy group: HeLa cells were treated with C-PC and ATRA at the same time.

1.6 Detection of CDK4 mRNA by in situ hybridization (ISH)

A 96 bp of oligonucleotide probe for CDK4 mRNA of human (5'-ACCTT TAACC CACAT AAGCG AATCT CTGCC TT-3', labeled with digoxin at 5' end) was synthesized. The cells were seeded on the cover slip (treated by Poly-L-lysine) in a 6-well plate, added the drugs for 48 hours and fixed with freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30min at room temperature. Endogenous peroxidase were inactivated by 3% H_2O_2. Binding sites were exposed by pepsase (diluted by 3% citric acid) for 2 min at 37 ℃. Prehybridization was carried out at 38 ℃ for 4 h and hybridization was at 38 ℃ for 12 h. Then, the slips were washed sequentially by 2*SSC for 5min, 0.5*SSC for 15min and 0.2*SSC for 15min at 37 ℃. Blocking buffer was added for 30min and mouse anti-digoxin IgG were added for 60min at 37 ℃. After washed by PBS, SABC were added for 20min and biotinylated peroxidase was added for 30min at 37 ℃. The slides were stained by DAB and observed under light microscope.

1.7 Apoptosis determination by TUNEL assay

HeLa cells were washed with PBS and fixed in 4 % paraformaldehyde. DNA breaks were labeled sequentially by TdT, bromodeoxyuridine triphosphate (BrdUTP) and DIG -labeled anti-BrdU antibody. Then the cells were incubated in blocking buffer, biotinylated anti-digoxin IgG, SABC and DAB in turns. Integrated optical density (IOD) of each group was calculated by Image-Pro Plus (IPP) and used as index for apoptosis levels.

1.8 The expression of bcl-2 protein by immunohistochemistry staining

HeLa cells were washed with PBS, fixed in 4 % paraformaldehyde and treated with 3% H_2O_2 at room temperature for 10 min. After washing with distilled water, antigen was restored by heat. The expressions of proteins (bcl-2) were examined by immunohistochemical staining. Briefly, after culturing in blocking buffer, the slides were incubated with biotinylated rabbit anti-human bcl-2 monoclonal antibodies, followed by streptavidin-biotin complex (SABC) and diaminobenzidine (DAB, for color development). The slides were observed under light microscope.

1.9 Statistical analysis

The results were expressed as mean + SD. Data were obtained from three independent experiments. Student's t-test was used for comparison between means. The difference was considered statistically significant when $P<0.05$. SPSS software was used.

2 Results

2.1 Inhibitory effects of C-PC and ATRA on the growth of HeLa cells in vitro

In order to determine the effects of C-PC and ATRA on the growth of HeLa cells in vitro, MTT assay was adopted. The optical density (OD) values were negatively associated with numbers of dead cells. The results demonstrated that inhibition ratio was enhanced by increasing the concentration of each drug (Fig.1). IC50 of each drug was calculated and showed in Table 1. For the combination experiments, the cells were treated by two drugs. The concentration of the drugs and inhibition ratio were showed in Fig 2. The IC50 value of ATRA combined with C-PC at 40 and 80 μg/L was calculated and showed in Table 1. When cells treated with ATRA combined with C-PC, the IC50 value was lower than that in single ATRA group (0.158 mM). But under the same inhibition concentration (IC50), the more C-PC was used, the less ATRA was needed. The results demonstrated that the dosage of ATRA could be reduced by cooperating with C-PC so as to reduce the toxicity.

2.2 Screening of experimental drug dose

Based on the results of MTT assay (Table 1), the half of IC50 value was sett as the experimental drug dose. HeLa cells were randomly divided into 4 groups: (1) Control group. HeLa cells were only cultured in serum-free medium. (2) C-PC treated group. HeLa cells were treated with 96 μg/L C-PC. (3) ATRA treated group. HeLa cells were treated with 0.079 mM ATRA. (4) C-PC+ATRA synergy group. HeLa cells were treated with 96μg/L C-PC together with 0.079 mM ATRA.

2.3 In situ hybridization (ISH) for CDK4 mRNA

The results showed that positive cells were stained brownish

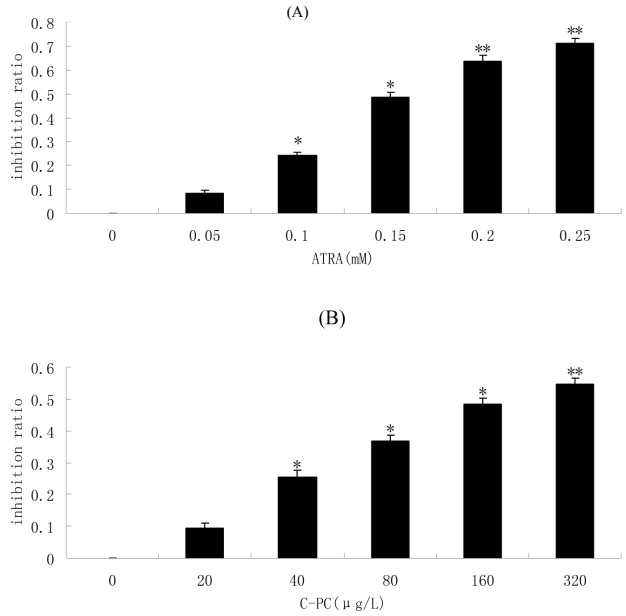

Fig.1. Inhibition ratio of HeLa cells treated with ATRA or C-PC. (A) HeLa cells were treated with ATRA at 0, 0.05, 0.1, 0.15, 0.2, 0.25 mM for 48 h. (B) HeLa cells were treated with C-PC at 0, 20, 40, 80, 160, 320 μg/L for 48 h. The cell apoptosis was positive correlated with C-PC concentration. Data was expressed as mean ± S.D from three individual experiments. *P < 0.05, **P < 0.01 compared with control

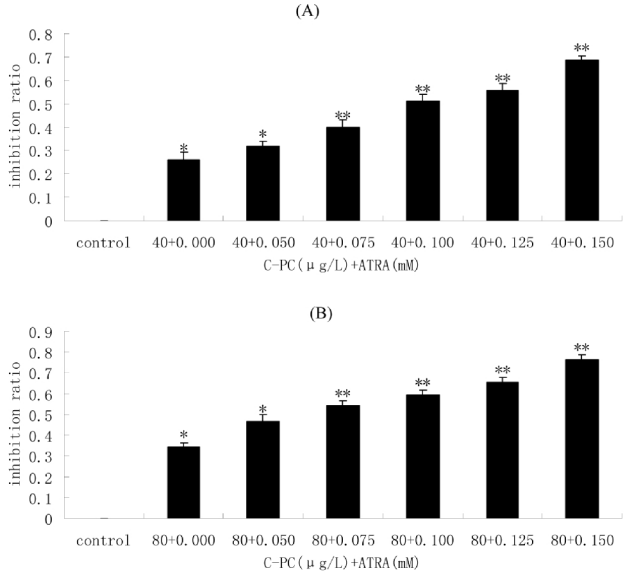

Fig.2 Inhibition ratio of HeLa cells treated with combination of C-PC and ATRA. (A) HeLa cells were treated with 40 μg/L C-PC and different concentraions of ATRA (0, 0.05, 0.075, 0.1, 0.125, 0.15 mM) for 48 hours. (B) HeLa cells were treated with C-PC at 80 μg/L combined with ATRA at 0, 0.05, 0.075, 0.1, 0.125, 0.15 mM for 48 hours. Data was expressed as mean $\bar{x}$± s.D from three individual experiments. *P< 0.05, **P< 0.01 compared with control

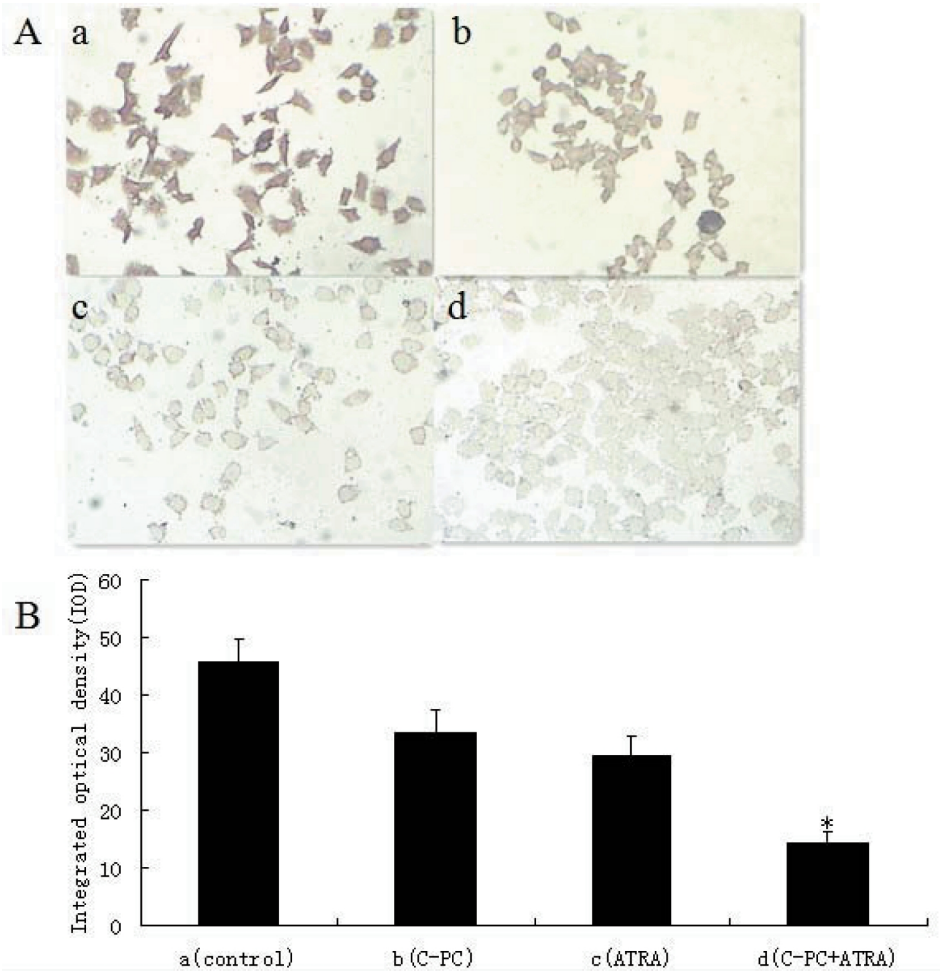

Fig.3 Screening of CDK-4 mRNA levels. (A) In situ hybridization. (a) Control group. (b) C-PC treated group. (c) ATRA treated group. (d) C-PC+ATRA synergy group. (B) IOD value.. (a) Control group. IOD 45.83± 4.02; (b) C-PC treated group. IOD 33.49± 3.91; (c) ATRA treated roup. IOD 29.39± 3.42; (d) C-PC+ATRA treated group. IOD 14.4± 2.07. *P < 0.05 compared with control

Table 1 IC 50 of ATRA and C-PC single used and the IC50 values of ATRA when combined with C-PC at 40 and 80μg/L

	ATRA(mM)	C-PC(μg/L)	ATRA(mM)	
			C-PC(40 μg/L)	C-PC(80 μg/L)
IC50	0.158 ± 0.036	192.75 ± 5.79	0.095 ± 0.007	0.062 ± 0.004

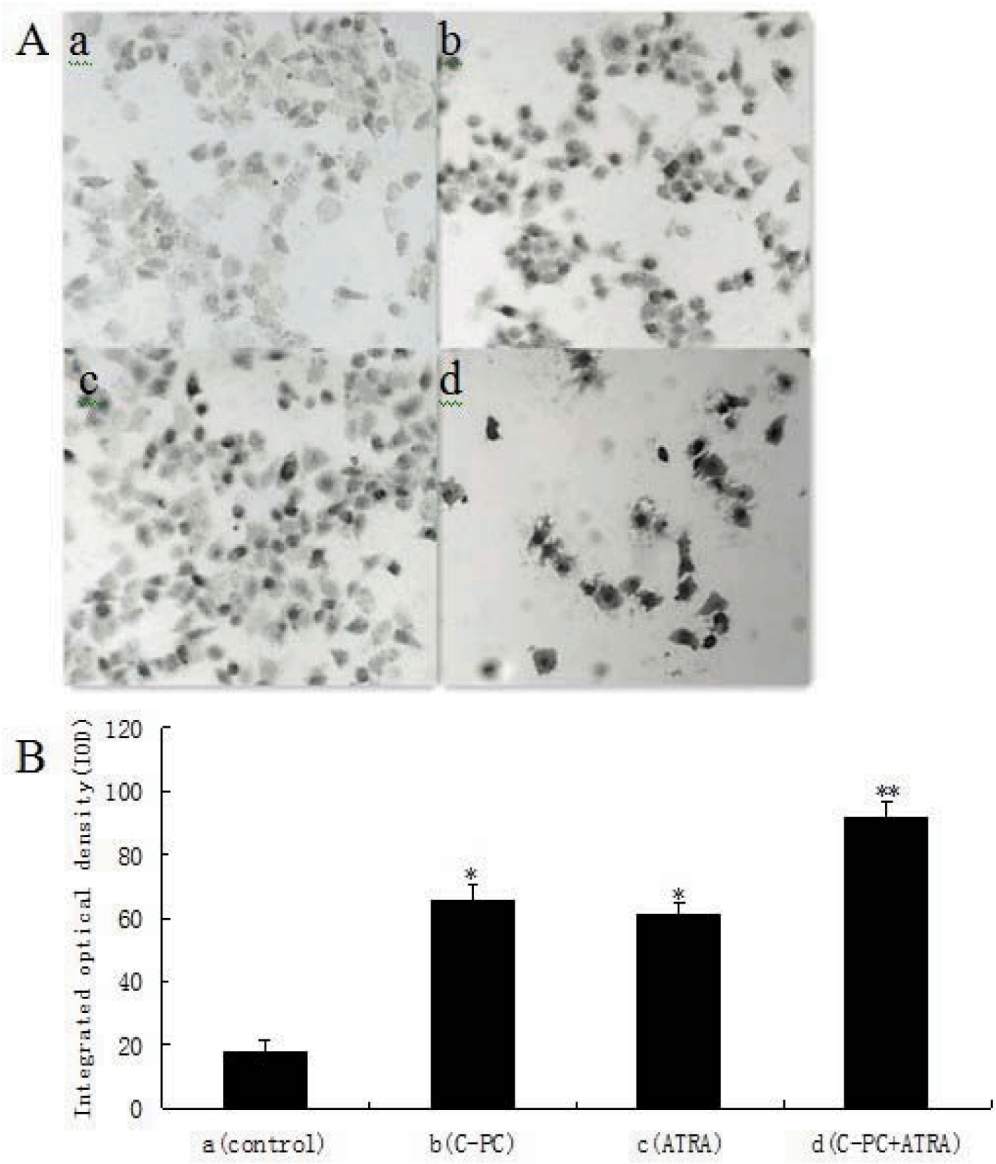

Fig.4 Apoptosis analysis of HeLa cells of four groups. (A) TUNEL. (a) Control group. (b) C-PC treated group. (c) ATRA treated group. (d) C-PC+ATRA synergy group. (B) IOD of each group. (a) Control group. (b) C-PC treated group. (c) ATRA treated group. (d) C-PC+ATRA synergy group. *P < 0.05 , **P<0.01 compared with control.

yellow. Integrated optical density (IOD) was positively associated with the CDK4 mRNA levels. The level of CDK-4 mRNA in combination group was obviously lower than that in single drug group or control group (Fig.3). These results suggested that C-PC combined with ATRA could significantly decrease the expression of CDK-4 mRNA (P<0.05).

2.4 Apoptosis analysis by TUNEL assay

The effect of C-PC and ATRA on HeLa cells apoptosis was determined by TUNEL assay. Integrated optical density (IOD) of each group was calculated by Image-Pro Plus (IPP) and used as index for apoptosis levels (Fig.4). Compared with control HeLa cells, the IOD in C-PC or ATRA treated group was increased, and the differences were statistically significant (P<0.05) and the differences were further increased in combination group (P<0.01). These results indicated that either ATRA or C-PC could induce apoptosis of HeLa cells in vitro, and when HeLa cells were treated with ATRA combined with C-PC, the apoptosis was obviously increased.

2.5 Determination of Bcl-2 protein expression by immunohistochemistry

The expression of bcl-2 protein was determined by immunohistochemistry staining. As shown in Fig. 5, the expression of bcl-2 in C-PC group were less than in the control group but was similar to that in ATRA group, whereas the expression in combination

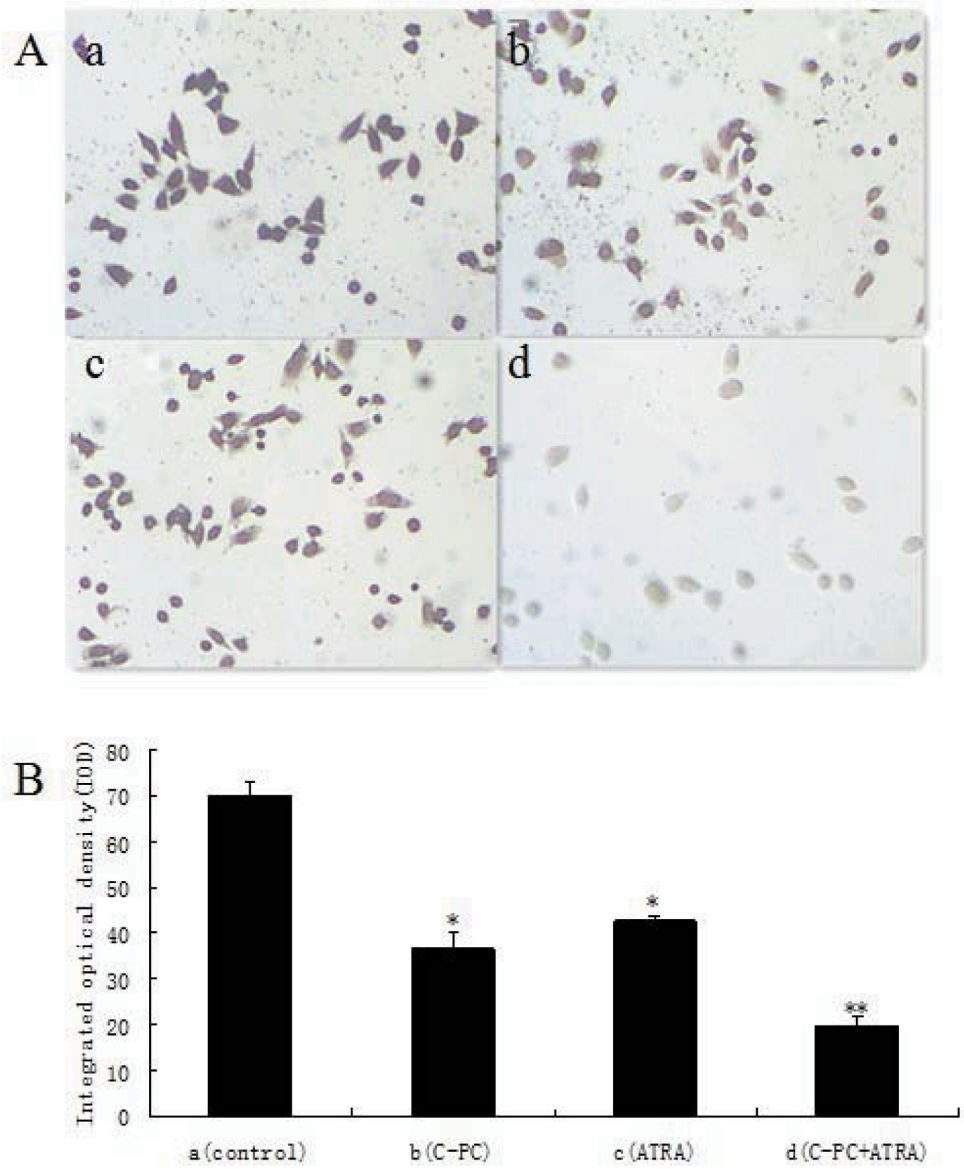

Fig.5 Effects of C-PC and ATRA on bcl-2 protein expression. (A) Immunohistochemical staining. (a) Control group, (b) C-PC treated group, (c) ATRA treated group, (d) C-PC+ATRA combination group. (B) IOD values. (a) Control group. IOD was 70.04± 3.23; (b) C-PC treated group. IOD was 36.43± 4.05; (c) ATRA treated Group. IOD was 42.66± 1.08; (d) C-PC+ATRA treated group. IOD was 19.39± 2.29. *P < 0.05, **P < 0.01 compared with control

group was least. These results indicated that both C-PC and ATRA could inhibit the bcl-2 expression of HeLa cells, and when C-PC combined with ATRA, this effect was further enhanced.

3 Discussion

Due to strong antiproliferative activity, ATRA represent an attractive option for chemoprevention and treatment of human malignancies. However, the clinical applications of ATRA were often limited by severe dose-limiting side effects and development of acquired drug resistance. The detailed anti-tumor mechanisms of ATRA and other retinoids are still unclear [13]. C-PC is a natural component of edible Spirulina platensis and has no toxic side effects. C-PC is widely used as an excellent nutrient supplement for human beings. From the results of our previous study, we confirm that C-PC could inhibit the growth of cancer cells both *in vivo* and *in vitro*. So C-PC may become a good substitute for highly toxic conventional chemotherapeutic anticancer drugs in the future. In the present study, we tried to take C-PC as an ideal nutrient-based agent for cancer therapy in combination with other chemotherapeutic agents such as ATRA in order to reduce the dosage and toxity of ATRA. In addition, the inhibitory effects and mechanisms of C-PC combined with ATRA on the growth of HeLa cells in vitro were explored.

Firstly, MTT assay was adopted to determine the effects of C-PC and ATRA on the growth of HeLa cells. It is obviously that both C-PC and ATRA could inhibit the proliferation of HeLa cells. The half maximal inhibitory concentration (IC50) of each drug was calculated to find the optimum concentration which would be used in different groups in the following experiments. Moreover, for the combination experiment, 40 μg/L and 80 μg/L of C-PC

were combined with different concentration of ATRA. IC50 of ATRA in the two groups was calculated and the results showed that when C-PC combined with ATRA, the dosage of ATRA could be decreased so as to reduce the toxicity.

One of the aims of carcinogenesis research is to identify the precise molecular alterations responsible for neoplastic transformation, in particular inducing cell proliferation. An increasing number of alterations in the normal expression pattern of proteins in these pathways, including pRb, cyclinD1 and p53 have been reported in resectable tumors[14]. Early results from other studies also give some indication that alteration of cell cycle regulating proteins may play an important role also in advanced diseases [15]. The molecular events controlling the G1 phase of the cell cycle are determined by a series of phosphorylation events that are regulated by the expression of specific cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors [16]. In our study, we tested the expression of CDK-4. Results suggested compared with control group and single drug groups, the expression of CDK-4 in C-PC+ATRA combination group were obviously decreased. This confirmed that ATRA combined with C-PC could induce more cell cycle arrest than single drug (ATRA or C-PC) by decreasing CDK-4 expression, and then inhibit the proliferation of HeLa cells.

Apoptosis has been shown as a significant mode of cell death following cytotoxic drug treatment in a variety of tumor types. A correlation between induction of apoptosis and chemosensitivity has been documented in some preclinical models. Bcl-2, is a family of apoptosis regulating proteins [17] which are classified into two categories [18]. One is pro-apoptotic (Bax/Bad proteins) and another is anti-apoptotic (Bcl-2, Bcl-XL, Bcl-W). The ratio between these two categories will determine whether or not a cell undergoes apoptosis [19]. Anti-apoptotic proteins such as Bcl-2 take effects by preventing the release of cytochrome c from mitochondria [20]. In present study, the occurrence of apoptosis as a mechanism of ATRA- or C-PC-mediated cell death in vitro was discussed. Firstly, TUNEL assay was adopted to determine the levels of cellular apoptosis. Analytic results showed that compared with single drug group and control group, C-PC combined with ATRA was apt to induce apoptosis. Moreover, in the combination group, HeLa cell apoptosis was accompanied by the down-regulation of Bcl-2, and compared with control group and single drug group, there was a statistically significant difference.

In summary, C-PC combined with ATRA was proved to be an effective inhibitor for HeLa cell proliferation. By combined with C-PC, the dosage of ATRA was effectively reduced so as to reduce the toxicity. C-PC+ATRA combination might take effects by inhibiting the progress of cell cycle and inducing cell apoptosis So the novel therapies may be exploited to function as a kind of novel potential anti-tumor therapeutic agent and used in the future.

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藻蓝蛋白联合全反式维甲酸对 HeLa 细胞生长抑制作用的研究 *

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摘要 目的:探讨全反式维甲酸和藻蓝蛋白单独及联合用药对 HeLa 细胞生长的影响,并揭示两者联合用药对细胞周期和细胞凋亡影响的分子机制。**方法:** MTT 法检测全反式维甲酸和藻蓝蛋白单独及联合用药对 HeLa 细胞生长的影响,原位杂交法检测用药前后细胞内 CDK-4 基因 mRNA 的表达情况,免疫组化法检测用药前后 bcl-2 基因的表达情况,TUNEL 法检测用药前后细胞凋亡情况。**结果:** 全反式维甲酸和藻蓝蛋白均具有抑制 HeLa 细胞生长的作用,当达到相同的抑制率时,联合藻蓝蛋白使用可以显著降低全反式维甲酸的使用剂量从而达到降低毒副作用的目的。两者联合用药可以显著降低 CDK-4 的表达量从而对 HeLa 细胞的细胞周期产生影响。两者联合用药可以显著下调 bcl-2 的表达水平从而引发细胞凋亡。**结论:** 通过联合藻蓝蛋白,可以显著降低全反式维甲酸的使用剂量从而降低毒副作用。全反式维甲酸和藻蓝蛋白联合用药抑制 HeLa 细胞生长的分子机制可能是通过抑制 CDK-4 和 bcl-2 的表达来影响细胞周期并最终导致细胞凋亡。

关键词:全反式维甲酸;藻蓝蛋白;HeLa 细胞;凋亡机制

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