Int J Clin Exp Med 2016;9(7):12772-12779
www.ijcem.com /ISSN:1940-5901/IJCEM0022024

Original Article

Emodin combined with cisplatin inhibits proliferation and promotes apoptosis in esophageal carcinoma cell line EC-9706 via increasing reactive oxygen species

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Received December 16, 2015; Accepted May 4, 2016; Epub July 15, 2016; Published July 30, 2016

Abstract: Context: Currently, many efforts have focused on finding new and more effective anticancer drugs through Traditional Chinese Medicine (TCM) investigation with both low side effect and high toleration. Emodin (1,3,8-trihydroxy-6-methylanthraquinone) can inhibit proliferation and induce apoptosis in different kinds of cancer cells. Objective: This study demonstrated whether emodin combined with cisplatin (DDP) can afford more effective therapeutics against esophageal carcinoma EC-9706 cells and its possible molecule mechanisms. Materials and methods: The EC-9706 cells were treated with emodin, cisplatin and the combination of the two agents for 12 h, 24 h and 48 h. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Apoptosis and reactive oxygen species (ROS) were evaluated by flow cytometry. Results: Both emodin and cisplatin inhibited cell proliferation and promoted apoptosis ($P<0.05$), and the combination of emodin and cisplatin significantly enhanced these effects ($P<0.05$). After treatment with Emodin, DDP and the combination of the two drugs, the intracellular ROS levels in the cell were 35.57±3.16%, 29.44±2.43% and 43.23±3.68% respectively, with the control group 4.72±2.12%, $P<0.05$). *Discussion and conclusion:* We found that the combination of emodin and cisplatin may be anti-cancer drug against EC9706 cells by inhibiting the proliferation and inducing apoptosis via generating cellular ROS.

Keywords: Emodin, cisplatin, esophageal cancer, proliferation, combination

Introduction

In the process of carcinoma cell apoptosis, Reactive Oxygen Species (ROS), plays as a nonage signal role, which induces cell apoptosis through acting on mitochondrion then changes the mitochondria membrane permeability and dysfunction [1, 2]. Damaged mitochondria also can generate ROS further more, which accelerates cell to apoptosis. Nowadays ROS is considered as the most common induced bioactive molecule in the process of apoptosis. Emodin acting as a safe ROS stimulus which enhance ROS levels in the cancer cell due to the special molecular structure that similar to bi-methylnaphthoquinone and ubiquinone in mitochondria can buildup cancer cell's sensibility to chemotherapeutics [3-7]. We have previously demonstrated that emodin, a kind of natural anthraquinone, enriched in the traditional Chinese herbal medicines, facilitates arsenic trioxide-induced apoptosis in human esophageal cancer cell line EC109 cell line, both in vitro and in vivo via the mechanism of generation of reactive oxygen species (ROS) [8].

Cisplatin (cis-diaminodichloroplatinum, CDDP/DDP) was the highly effective chemotherapeutic agent, which was with obvious effect of ototoxic side [9-11]. From 1970s, DDP, as the well-known DNA-damaging agent, was used in treatment of cancers. According to Eastman et al. [12], the complex of DDP-DNA adducts might kill tumor cells by induction of programmed cell death or apoptosis, as well as some other mechanisms descripting that DDP could induced apoptosis [13, 14]. Santos et al. [15] explained that DDP exposure could result in the intracellular ROS increase in normal cells, while treatment with antioxidants could ameliorate the

toxic effects of DDP on the organs [16, 17]. The similar results were also found other researches on the DDP and ROS [18, 19].

Plentifully previous reports descripted about the relationship of ROS and emodin or DDP, but there was less literature showing the combined treatment of two on cancer by regulating ROS.

This experiment is to investigate the proliferation of human esophageal EC-9706 treated by emodin, a kind of endogenous stimulus of ROS, combined with cisplatin on purpose to set up primary theory on emodin combined with anticarcinoma drugs on caner cells.

Materials and methods

Drugs and reagents

Emodin was purchased from China Drugs Appreciation Department, purity >98%, Cisplatin was obtained from Qilu tragacanth (Shandong, China), Methyl thiazolyl tetrazolium (MTT), Dimethyl sulfoxide dimethyl sulphoxide (DMSO) were prepared from Sigma company. Tyrisin, RPMI-1640 medium were acquired from Gibco company. Fetal bovine serum (FBS) from Sijiqing campany (Hangzhou, China), Annexin V-FITC/PI detection box from Kaiji (Nanjing, China), Reactive Oxygen Species Assay Kit (ROS) from Biyuntian (Jiangsu, China).

Cell culture and drugs preparation

Human esophageal squamous cell carcinoma (ESCC) cell line EC-9706 was purchased from the cell bank of Shanghai Institute of Cell Biology (Shanghai, China). EC-9706 cells were cultured in RPMI-1640 medium containing 10% heat-inactivated FBS and 100 U/ml penicillin and 100 ug/ml streptomycin. Cells were grown in 37°C incubator supplied with 95% room air and 5% CO_2, growing to 80-90% were trypsinized with 0.25% trypsin, counted and placed down at the desired density for further treatment. For the drug treatment experiments, the EC-9706 cells were harvested at the exponential growth phase, and seeded into multi-well culture plates at 5×10^4-1×10^5 cells/ml in fresh medium. Emodin was dissolved in dimethylsulfoxide (DMSO) and diluted in complete medium. The final DMSO concentration in medium was 0.1% and did not affect cell function and assay systems.

Emodin was dissolved in DMSO, adjusted the concentration at 4 mg/ml, and put into -20°C ice-box away from light, and diluted working concentration at 30 μg/ml with complete medium. Use the same way dissolved DDP at 1 mg/ml in 4°C refrigerator in duck environment, dilute working concentration at 5 μg/ml with the same medium.

Fluorescence morphological examination

Apoptotic morphology was studied by staining the cells with a combination of the fluorescent DNA-binding dyes acridine orange (AO) and ethidium bromide (EB). Cells were harvested and washed three times with PBS, after being incubated with different groups for 24 h, and were then stained with 100 ug/ml AO and EB for 5 min. Then the color and structure [20] of the different cell types were observed under a fluorescence microscope (Olympus, IX70, Japan).

MTT assay of cell viability

At the end of the drugs treatment, 20 ul of 5 mg/ml MTT solution in PBS (PBS without MTT as the blank) was fed to each well of the culture plate (containing 100 ml medium). After 4 h incubation, the formazan crystal formed in the well, which was solubilized with 150 ml DMSO for optical density reading at 492 nm. Combination index analysis for combined treatment with emodin and cisplatin Cytotoxicity induced by combined treatment with emodin and cisplatin was compared with the cytotoxicity induced by each drug individually with use of a combination index (CI): CI<0.9, CI=0.9-1.1, and CI>1.1 indicated synergistic, additive, and antagonistic effects, respectively. The combination index analysis was performed using CalcuSyn software (Biosoft, Oxford, UK). The combination index analysis is a quantitative measure of the degree of drug interaction and CI values at fraction affected (FA) of 0.5, 0.75, and 0.9 were averaged for each experiment, and the value was used to calculate the mean between three independent experiments.

Annexin V-FITC/PI assay of apoptotic cells

Apoptosis was determined by staining cells with Annexin V-FITC and PI labeling, because Annexin V can identify externalization of phosphatidylserine during the progression of apoptosis, and can detect cells in early stages of apoptosis. To quantitate apoptosis, prepared

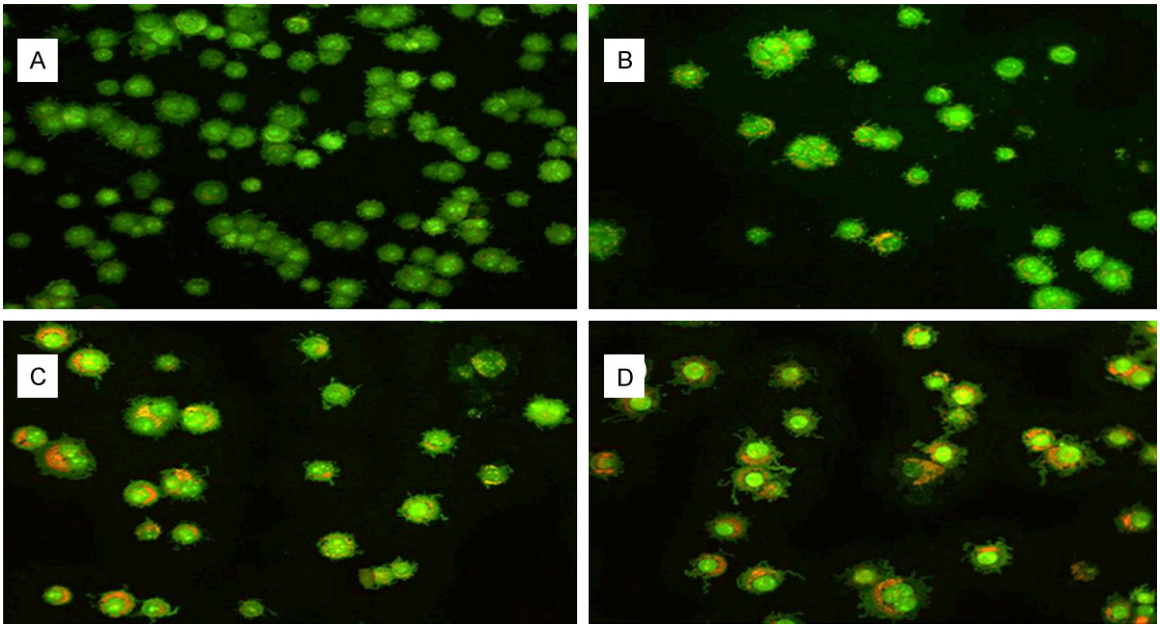

Figure 1. The morphological changes in EC-9706 cells after treatment with different drugs for 48 h (magnification 400×). A: Control; B: Treated with Emodin (30 μg/ml); C: Treated with DDP (5 μg/ml); D: Treated with Emodin (30 μg/ml) combined with DDP (5 μg/ml).

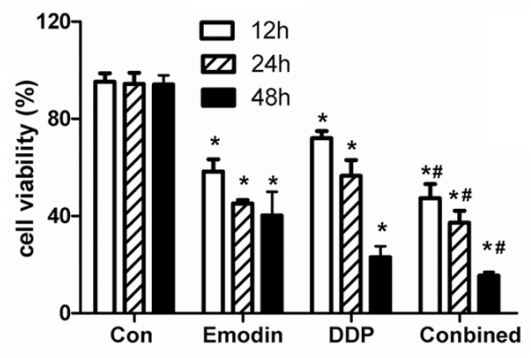

Figure 2. T he survival rate of EC-9706 cell treated with Emodin (30 μg/ml), DDP (5 μg/ml) and Emodin combined with DDP at 12 h, 24 h and 48 h respectively ($\bar{x}$±SD, n=3). *P<0.05, vs control, #P<0.05, vs Emodin and DDP.

cells were washed twice with cold PBS and then re-suspended in 500 ul binding buffer at a concentration of 1×10^6 cells/ml. Five microliters Annexin V-FITC and 10 ul PI (1 ug/ml) were then added to these cells, analyzed by FACScalibur flow cytometry (Becton Dickinson), and calculated by CellQuest software. Viable cells were negative for both PI and Annexin V and apoptotic cells were positive for Annexin V and negative for PI, while late apoptotic dead cells displayed both high Annexin V and PI labeling. Non-viable cells, which underwent necrosis, were positive for PI and negative for Annexin V.

ROS detection by FCM

2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma) was used as ROS capturing reagent with the method described previously [21]. DCFH-DA was deacetylated intracellularly by nonspecific esterase, which was furthered oxidized by ROS to the fluorescent compound 2,7-dichlorofluorescein (DCF). Cells were incubated with 10 AM DCFH-DA at 37°C for 15 min. DCF fluorescence was detected by flow cytometry.

Statistical analysis

SPSS19.0 software was used for statistical analysis. Data are reported as mean ± S.D. All experiments were done at least three times in the same condition. Statistically significant values were compared using Student's t-test for single comparison and *p*-values less than 0.05 were considered statistically significant.

Result

Emodin induced cell morphological changes

To determine whether the growth inhibitory activity of Emodin and DDP was related to the induction of apoptosis, morphological assay of cell death was investigated by the AO/EB staining for fluorescence microscopy. Uniformly

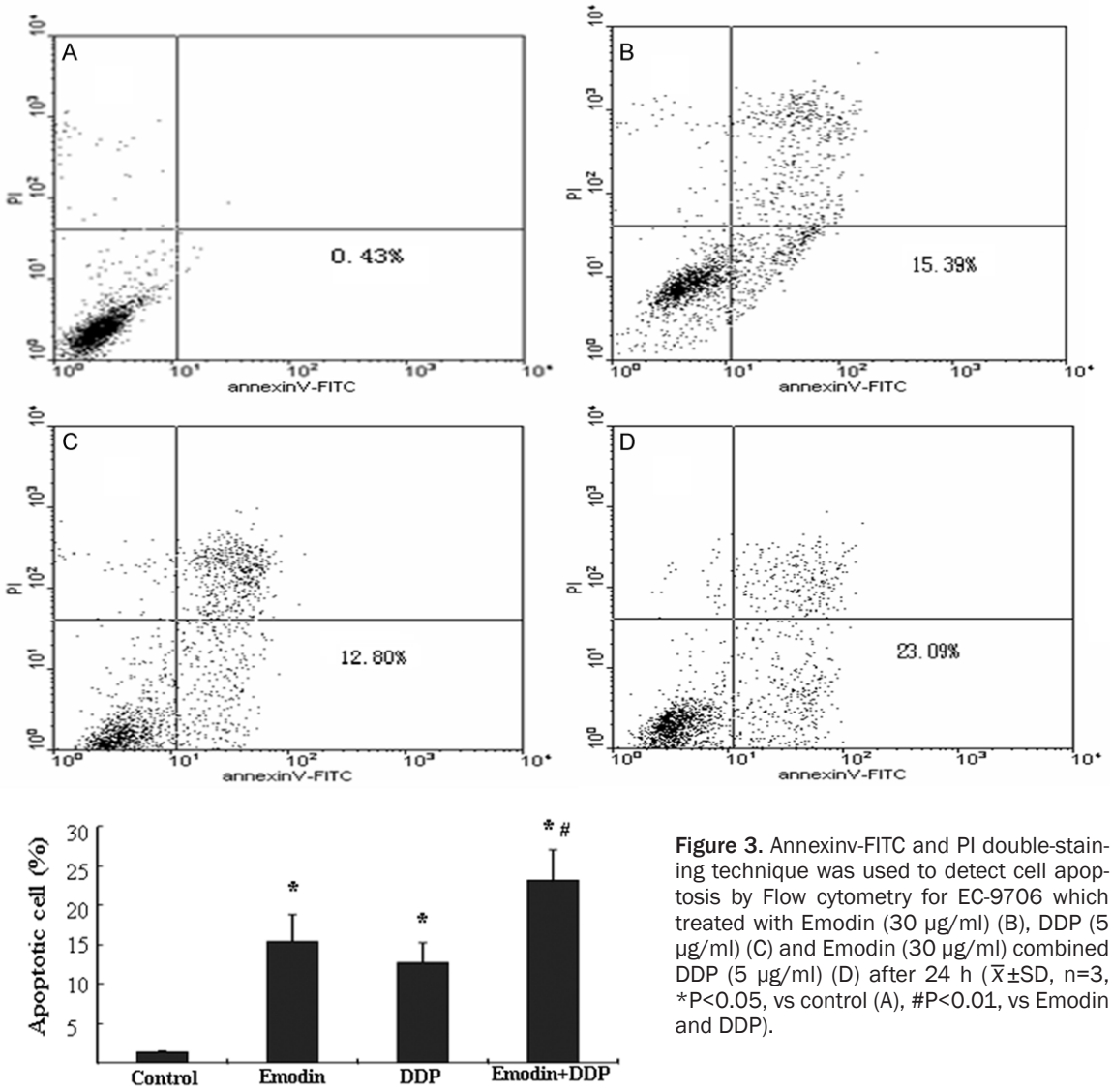

Figure 3. Annexinv-FITC and PI double-staining technique was used to detect cell apoptosis by Flow cytometry for EC-9706 which treated with Emodin (30 μg/ml) (B), DDP (5 μg/ml) (C) and Emodin (30 μg/ml) combined DDP (5 μg/ml) (D) after 24 h ($\bar{x}$±SD, n=3, *P<0.05, vs control (A), #P<0.01, vs Emodin and DDP).

green live cells with normal morphology were seen in the control group (**Figure 1A**), whereas green early apoptotic cells with nuclear margination and chromatin condensation occurred in the experimental group with Emodin and DDP groups (**Figure 1B** and **1C**), orange apoptotic cells with fragmented chromatin and apoptotic bodies were seen in the DDP and combined group obviously (**Figure 1C** and **1D**). The results suggested that Emodin and DDP could induce marked apoptotic morphology in EC-9706 cells.

Effect of Emodin and DDP on cell proliferation

MTT assay was used to assess the effect of Emodin and DDP on cell proliferation. A typical time-dependent inhibition of cell growth was observed in EC-9706 cells with an IC50 of approximately 30 μg/ml following treatment with Emodin for 48 h (**Figure 2**). The result demonstrated that Emodin had a significant inhibitory effect on proliferation of EC-9706 cells.

Analysis of cell apoptosis by FCM

To further confirm that Emodin and DDP induced cell apoptosis, EC-9706 cells were stained with Annexin V-FITC and PI and analyzed by flowcytometry. This method affords the detection of viable, necrotic, early apoptotic, and late apoptotic cells based on distinct double-staining. As **Figure 3** showed, Emodin increased the number of early apoptotic cells of EC-9706 cells, The number of early apoptotic cell (%) of EC-9706 was elevated to approximately 15.39%±3.40% at 24 h following the

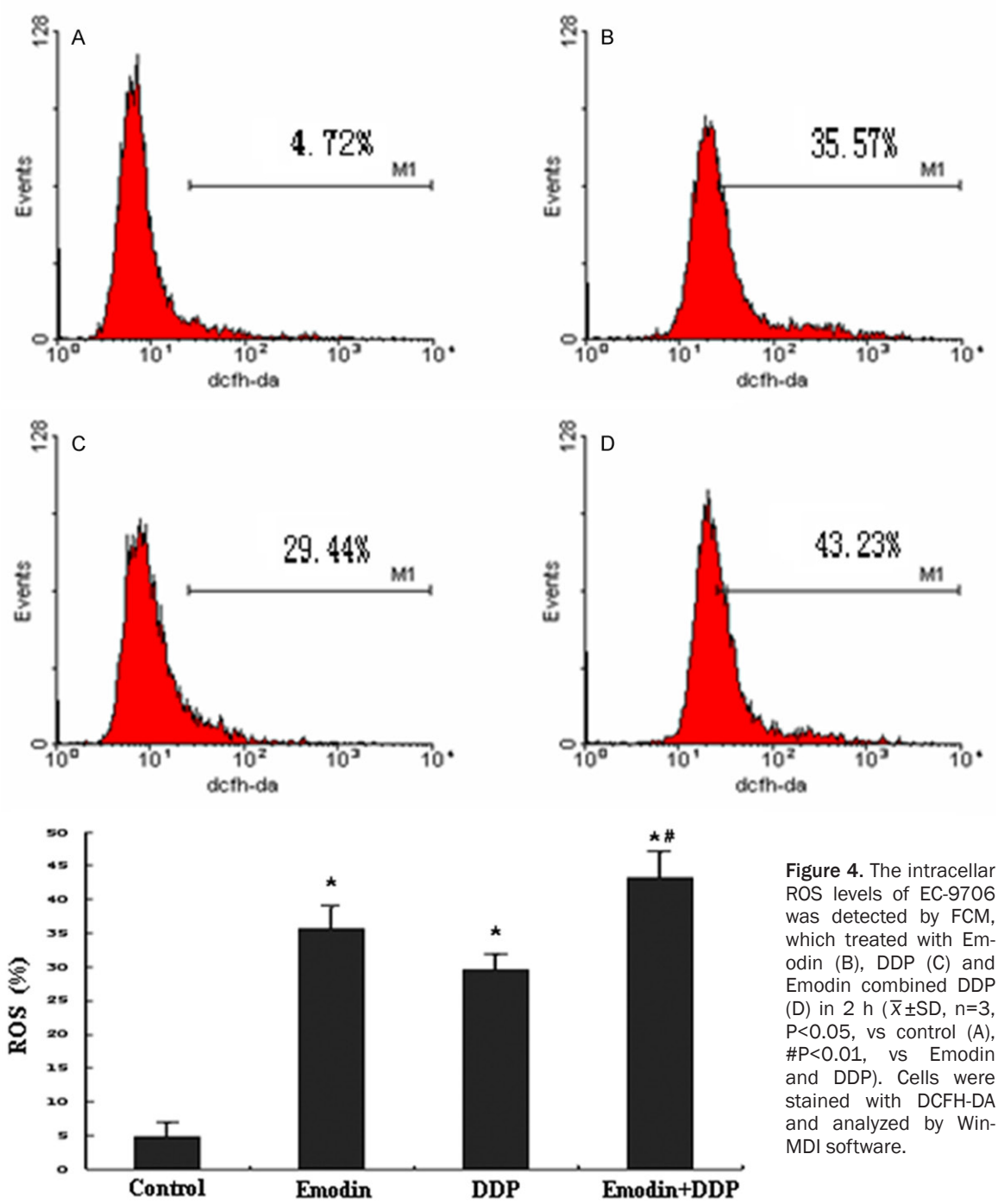

Figure 4. The intracellar ROS levels of EC-9706 was detected by FCM, which treated with Emodin (B), DDP (C) and Emodin combined DDP (D) in 2 h ($\bar{x}$±SD, n=3, P<0.05, vs control (A), #P<0.01, vs Emodin and DDP). Cells were stained with DCFH-DA and analyzed by WinMDI software.

treatment with Emodin, and DDP was 12.80%± 2.41%, combined group was 23.09%±3.97%, as the control group was only 1.34%±1.04%.

The intracellar ROS level by FCM

According to a previous study from our laboratory [8], Ros generation in another kind of esophageal carcinoma cell line EC-109 peaked at 2 h after exposure to emdion, hence we detected the ROS level after 2 hours treatment. As the **Figure 4**. showed after the treatment with Emodin, DDP and the combination of the two drugs, the ROS levels in the cell were 35.57±3.16%, 29.44±2.43% and 43.23± 3.68% respectively, with the control group was only 4.72±2.12%. Statistical analysis revealed that 3 groups were significantly different form the control group. From the data we sur-

prised that the two drugs combination may exist more power which induced cell to death through increasing the intracellar ROS level.

Discussion

In recent years, Many efforts have focused on the anticancer mechanism of Traditional Chinese Medicine (TCM) worldwide [22-24]. Researchers expect to find new and more effective anticancer drugs through TCM investigation with both low side effect and high toleration. More knowledge has been obtained about how TCM induce cell death of human cancers. It was found, in our previous study, Emodin isolated from the roots and rhizomes of numerous plants can inhibit the proliferation of EC-9706 cells in a dose- and time-dependent manner obviously. In this study, we sought to reach the anti-proliferative and apoptotic effects of Emodin, DDP against EC-9706 human ESCC and its underlying molecular mechanisms in vitro.

To determine the growth inhibitory activity and the induction of apoptosis by Emodin and DDP on morphological, we used the AO/EB staining for fluorescence microscopy. The result showed, in the drug group and control group, the intra-cellar jacinth fluorescence arised in different levels but no difference on green fluorescence, with the combined group was the highest, which indicated the EB stain can easily transit the cell membrane. The result cued that Emodin and DDP can anti-proliferative of EC-9706 and promote the death. In order to explore the inhibition degree of EC-9706 by emodin, DDP and Emodin combined DDP, MTT assay was used to check the inhibition level in 12 h, 24 h and 48 h. The survival rate were Emodin 58.35±5.03%, 45.18±1.33%, 40.24±3.71%; DDP 72.06±2.92%, 56.60±6.41%, 23.06±4.52%; combined group 47.35±5.78%, 37.29±4.85%, 15.45±1.46%. The combination index analysis was 0.94±0.03, 0.91±0.04, 0.90±0.03 in each time point. It was not difficult to conclude that the survival rate go down with the time went by in each group. The combined group showed more anti-proliferation compare to the other two groups ($P<0.05$). The average CDI in each group were less than one, which indicated there exited synergism between the two drugs that had more power induced cell to death.

In the experiment, the EC-9706 cells grow depression in different degree in each group and the cell apoptosis emerge with the time went by. The combined group showed the biggest inhibition and apoptosis, and the apoptosis was obvious after 24 h. The FCM results showed each group emerge in equal nonage apoptosis in each group. The nonage apoptosis rate were 15.39±3.40%, 12.80±2.41% and 23.09±3.97% with the control group was just 1.34±0.24%, which had statistical significance ($P<0.05$) compare to single drug group. The FCM results indicated the two combined drugs can enhance apoptosis with a time-dependent manner.

In order to investigate the molecule mechanisms in the progress of EC-9706 Apoptosis, we choose to check the ROS levels in the carcinoma cell. According our early results and many literature reports [25-27], we checked the intracelluar ROS levels on several time points. The specimens were marked by fluorescence probe [28] and detected by FCM, we found the ROS level in each group went up obviously, especially in the combined group whose ROS level was highest comparing to other three groups. From the results we concluded that the more created intracelluar ROS levels the more apoptosis cells it was. So we presume that ROS plays an important part in the process of emodin and DDP induce EC-89706 cells to death. We also found that the appearance of cell inhibition was correlated with the intracelluar ROS level. The two drugs combined group exist more power to induce EC-9706 cells to death compared to the other solo-drug group ($P<0.05$). Our experiment revealed that the drugs emodin and DDP induce EC-9706 cells to death is dependent on increasing the intracelluar ROS levels. The above mechanism will be the effective method on treating caner, which expected to find a new way to cure caner.

The results of this experiment indicate that both Emodin and DDP can inhibit proliferation and induced apoptosis in human EC-9706 cells and there exist synergistic effect between the two drugs. The intracelluar ROS arises sharply in the induced way to apoptosis and evoke intracelluar ROS off-balance to further apoptosis. Now the most investigation just stay on the cell level with only a little on the molecule and body level, so there still exist large work to do about Emodin on the anti-carcinoma research.

Disclosure of conflict of interest

None.

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