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Article

Toxicity Evaluation of Geniposide on MCF-7 Cancer Cells

Kena Lv[1], Shuangshuang Zheng[2], Xiangqin Li[1], Yi Nie[2,3,*], Tianqing Liu[1] and Kedong Song[1,*]

[1]State Key Laboratory of Fine Chemicals, Dalian R&D Center for Stem Cell and Tissue Engineering, Dalian University of Technology, Dalian, 116024, China
[2]Zhengzhou Institute of Emerging Industrial Technology, Zhengzhou, 450000, China
[3]Key Laboratory of Green Process and Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, 100190, China
[*]Corresponding Authors: Kedong Song. Email: Kedongsong@dlut.edu.cn; Yi Nie. Email: ynie@ipe.ac.cn
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Abstract: As one of the adjuvant treatments for cancer treatment, traditional Chinese medicine treatment has a wide range of cancer treatments, such as preventing metastasis and relapse, improving the efficacy of radiotherapy and chemotherapy, reducing the side effects of chemotherapy, improving body function, extending life and improving the life quality. Geniposide (GEN) is a bioactive substance extracted from the fruit of gardenia. In recent years, it has attracted attention due to its anti-tumor effect. In this study, we aimed to investigate whether GEN could inhibit the proliferation of human breast cancer cells (MCF-7) and promote its apoptosis. The half-inhibitory concentration (IC50) values of GEN were firstly determined as 16.06, 14.85 and 13.14 mg/mL by the CCK-8 experiment after cells treated for 24 h, 48 h, and 72 h, respectively. The inhibitory effect of GEN on MCF-7 cells was in concentration- and time-dependent manners from the results of CCK-8 experiment and Live/Dead staining. AO/EB staining result has shown that GEN has induced MCF-7 cell apoptosis.

Keywords: MCF-7; geniposide; anti-cancer

1 Introduction

Breast cancer is the most common cancer among women worldwide, and it has also become a global health problem. According to the global cancer statistics in 2018, the incidence of cancer in a total of 185 countries has been analyzed, of which 154 countries had the highest incidence of breast cancer [1]. In China, the incidence of breast cancer has been increasing more than twice the global incidence of breast cancer rate since the 1990s, and it has increased significantly from 31.90/100,000 in 2000 to 63.30/100,000 in 2014 [2]. At present, the traditional breast cancer treatment method mainly includes surgery, radiotherapy, chemotherapy, hormone therapy and immunotherapy [3]. In recent years, studies have found that some traditional Chinese medicines have anti-tumor effects and attracted more and more attention.

Traditional Chinese medicine is an adjuvant therapy in the treatment of cancer, especially in the treatment of postoperative complications, reducing the resistance to chemotherapy and side effects of radiotherapy, improving life quality and prolonging survival rate [4]. Gubenyiliu II, a traditional Chinese medicine formula has shown anti-tumor growth and anti-metastatic effects on a murine breast cancer model [5]. Astragalus polysaccharide (APS)

plays its adjunct anticancer role by improving immune function, counteracting the side effects of chemotherapeutic drugs and increasing the sensitivity of chemo-therapeutics, for example, it has synergistic anti-tumor effect with doxorubicin [4]. Paclitaxel is considered to be one of the first-line drugs to treat breast cancer and one of the effective methods for clinical treatment of breast cancer [6].

Geniposide (GEN) is a bioactive substance extracted from the fruit of *Gardenia jasminoides* Ellis. GEN has a variety of pharmacological effects, including anti-inflammatory, anti-diabetic, anti-oxidant, neuroprotective, anti-viral, liver protection and anti-tumor [7]. In recent years, a number of studies have reported that GEN has shown anti-tumor effect. Yu et al. [8] found that GEN can not only inhibit the proliferation of HepG2 and Huh7, promote their apoptosis, but also reduce their migration and invasion ability. Zhang et al. [9] have found that the suppression of HCC induced by GEN was related to a decrease of VEGF expression and HCC angiogenesis. Huang et al. [10] have found that GEN and doxorubicin used together significantly increased the accumulation of doxorubicin in resistant cells.

Although studies have shown that GEN can inhibit the proliferation of some cancer cells, it is not clear whether GEN can inhibit the growth of human breast cancer (MCF-7) cells. In this study, we aimed to investigate the effect of GEN on the growth of MCF-7 cells. To explore the effect of GEN, CCK-8 experiment, Live/Dead staining and AO/EB staining were conducted. The results have shown that GEN can inhibit MCF-7 cells growth in a concentration- and time-dependent manner and induce cell apoptosis.

2 Experimental

2.1 Preparation of GEN Solution

A total of 0.2 g GEN solid powder was weighed with an electronic analytical balance and poured into a small beaker. Then 10 mL of complete medium was transferred by a pipette to dissolve GEN powder in a sterile environment and stirred with a magnetic stirrer while heated with 37°C water bath until completely dissolved. Under sterile conditions, GEN solution was collected with a 0.22 μm microporous filter membrane into a centrifuge tube. The concentration of the prepared GEN solution was 20 mg/mL and stored in a refrigerator at 4°C.

2.2 CCK-8 Test Detection of IC50 of GEN

MCF-7 cells (3000 cells/well) were seeded into three 96-well plates and cultured in an incubator. After cultured for 1d, 100 μL GEN solution at a gradient concentrations (1, 3, 5, 8, 10, 12, 15, 18 and 20 mg/mL) was added into 96-well plates. Then after cultured for 24 h, 48 h and 72 h, CCK-8 assay was conducted to assess the cell OD value and cell survival rate, respectively. Before the experiment, CCK-8 stock solution was diluted (V_{CCK}:V_{medium} = 1:10). Take out the 96-well plate and remove GEN solution, then wash with PBS. Add 110 μL of diluted CCK-8 solution to each well and incubate cells in a 5% CO_2, 37°C incubator for 3 h. The IC50 value of GEN at 24 h, 48 h and 72 h were calculated by simulating the cell survival rate-concentration curve equation, respectively.

2.3 Cell Viability Assay after Exposed to GEN

CCK-8 assay was used to estimate the effect of GEN on the proliferation of MCF-7 cells. 3000 MCF-7 cells were seeded into each well of 96-well plates and exposed to GEN at different concentrations of 2, 10, 13, 15, and 16 mg/mL for 24, 48 and 72 h after cells were seeded 24 h, respectively. Then after cultured for 24 h, 48 h and 72 h, CCK-8 assay was conducted. The specific steps are as described above.

2.4 Live/Dead Staining to Observe Cell Life and Death

Each well was seeded 2×10^4 MCF-7 cells and cultured with 400 μL complete medium in 24-well plates. After cultured for 1 d, GEN solution at different concentrations of 2, 10, 13, 15 and 16 mg/mL was added. When exposed for 24 h, 48 h and 72 h, cells were stained by Calcein-AM, Hoechst33324 and PI composite fluorescent dye and incubated for 20 min, respectively. Then cells were observed under an inverted fluorescence microscope.

2.5 AO/EB Staining to Observe Cell Apoptosis

2×10^4 MCF-7 cells were seeded into 24-well plates and each well was added 400 μL complete medium. After cultured for 1 d, cells were exposed to GEN at different concentrations of 2, 13, 15 and 16 mg/mL for 48 h. Then cells were stained with AO and EB composite dye (V_{AO}:V_{EB} = 1:1). After incubation for 20 min, cell were washed twice with PBS and observed under an inverted fluorescence microscope.

3 Results and Discussions

3.1 IC50 Value of GEN

The results of the CCK-8 experiment are shown in Fig. 1. The OD value was read by the microplate reader, and cell survival rates of MCF-7 cells were calculated. The goodness of fit (R2) of the fitting curve at 24 h, 48 h and 72 h was 0.98025, 0.98972 and 0.97438, respectively. The goodness of fit that closer to 1 indicates the better fitting effect. The IC50 values of GEN at 24 h, 48 h and 72 h were calculated according to the fitted curve equation. The result was 16.06 mg/mL at 24 h, 14.85 mg/mL at 48 h and 13.14 mg/mL at 72 h. According to the IC50 value, the concentration of GEN used in subsequent experiments was determined from 2 mg/mL to 16 mg/mL.

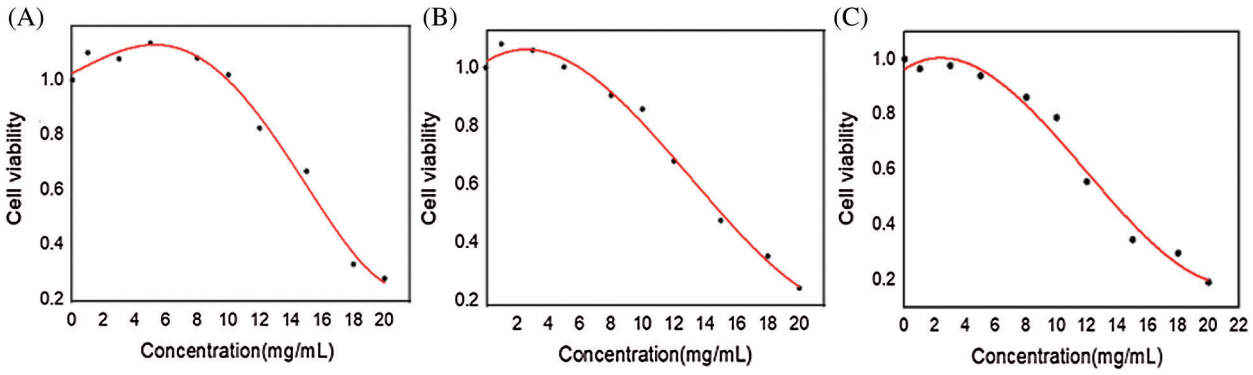

Figure 1: The cell viability-concentration fitting curves after MCF-7 cells were treated with GEN for 24 h, 48 h and 72 h (n = 3)

3.2 The Inhibition of GEN on MCF-7 Cell Proliferation

The results of CCK-8 experiment to detect the inhibition of GEN on MCF-7 cell proliferation were shown in Fig. 2. The OD value of MC-7 cells was shown in Fig. 2A. The OD value was directly proportional to the number of living cells, so comparing the OD value of the experimental group with the control group can estimate the inhibitory effect of GEN on MCF-7 cells. From Fig. 2A, it can be found that when GEN was at a low concentration of 2 mg/mL, the OD value of the experimental group was not significantly different from that of the control group, indicating that GEN of low concentrations had no significant inhibitory effect on the growth of MCF-7 cells. As the concentration of GEN increased, the OD value of the experimental group decreased significantly. When the concentration was over 13 mg/mL, the decline rate of OD value has become faster. The results have indicated that at 24 h, 48 h and 72 h, the inhibitory effect of GEN on MCF-7 cells was concentration-dependent. It was seen from the increasing OD value of the control group that MCF-7 cells had been continuously proliferating. However, when the concentration was 10 mg/mL, GEN had a significant inhibitory effect on MCF-7 cells. It was caused by the prolonged duration of drug action. When the concentration was 16 mg/mL, the OD value of the experimental group decreased continuously, because the inhibitory effect of GEN on MCF-7 cells exceeded the proliferation rate of cells.

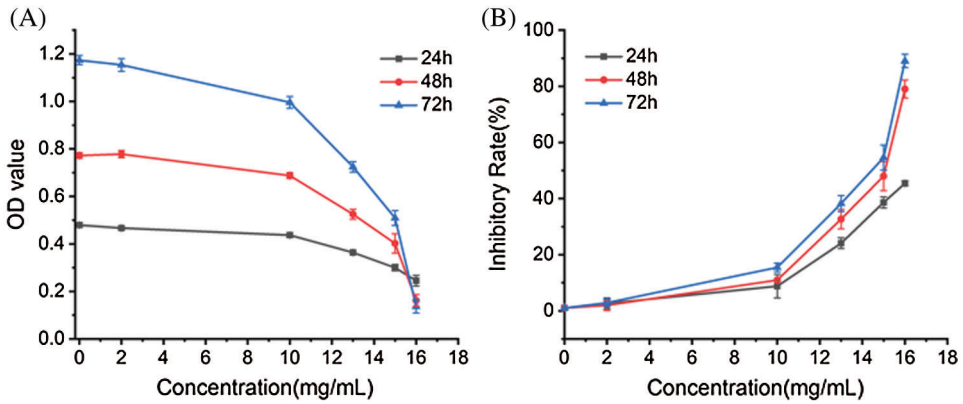

Figure 2: Effect of GEN on the proliferation of MCF-7 cells. (A) The OD value of MCF-7 cells cultured with different dosages of GEN at 24 h, 48 h and 72 h. (B) The survival rate of MCF-7 cells cultured with different dosages of GEN at 24 h, 48 h and 72 h (n = 3). The bar was shown as SD

The cell survival rate calculated from the OD value was shown in Fig. 2B. The inhibitory rate was proportional to the inhibitory effect of GEN. In summary, considering the two factors of time and concentration, GEN had an inhibitory effect on MCF-7 cells of a time- and concentration-dependent manner.

3.3 Live/Dead Staining to Observe the Life and Death of MCF-7 Cells

The results of composite fluorescence staining were shown in Fig. 3. Calcein-AM staining dye can make living cells glow green fluorescence, so as to locate the distribution of living cells and qualitatively observe the number of living cells. PI staining dye can combine with the nucleus of dead cells and glow red fluorescence, so it can be used to observe the status of cell death. After Hoechst33342 staining, the nucleus of live cells appear dark blue, and dead cells show bright blue fluorescence, which can locate the distribution of live and dead cells.

When the concentration of GEN was 2 mg/mL and 10 mg/mL at 24 h, there was no significant changes in the number of live cells, but when the concentration increases to 13, 15 and 16 mg/mL, the number of live cells significantly reduced. At the same time, from the Hoechst33342 staining results, it can be seen that the overall cell number has decreased and most cells were dissolved. As the drug concentration increases, the nucleus has appeared to glow bright blue, which was a manifestation of cell apoptosis. After cells exposed to GEN for 48 h, it was obvious that the number of cells has increased, which was the result of cell proliferation. In addition, it was obvious that PI staining gradually brightens, indicating that the number of apoptotic cells has increased. It can be seen from Fig. 3 that the inhibitory effect of GEN on MCF-7 was closely related to time and concentration. Live/Dead staining results were consistent with CCK-8 quantitative experiment results.

3.4 AO/EB Staining to Observe MCF-7 Cells Apoptosis

The AO/EB staining result was shown in Fig. 4. It was known from Live/Dead staining experiment that MCF-7 has undergone apoptosis under the action of GEN. AO/EB staining experiment further explored the effect of GEN on MCF-7 cell apoptosis by distinguish MCF-7 cells into living cells, early and late apoptotic cells and necrotic cells. The staining results of the control group and the 2 mg/mL experimental group showed uniform green fluorescence, indicating that there was almost no apoptosis. When the concentration increased to 13 mg/mL, the green fluorescence became brighter due to the cell nucleus shrankage under the action of the drug, showing early apoptosis characteristics. As the drug concentration increased, the fluorescence appeared orange-yellow bright spots. When the concentration was at 16 mg/mL, bright red spots started to appear, indicating that most cells were in the stage of late apoptosis or necrosis.

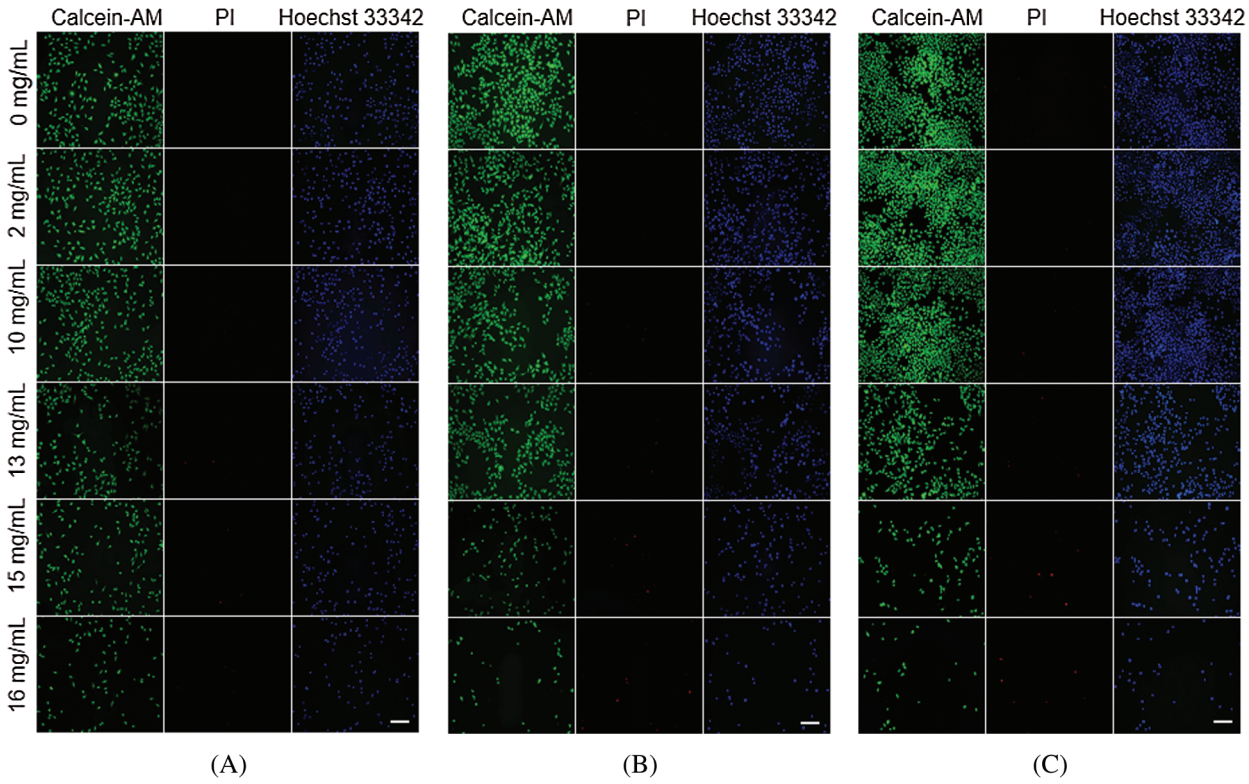

Figure 3: Live/Dead staining to observe the live and dead condition of MCF-7 cells after treated with GEN for 24 h, 48 h and 72 h. (A) Cells treated for 24 h. (B) Cells treated for 48 h. (C) Cells treated for 72 h, scale bar = 200 μm (n = 3)

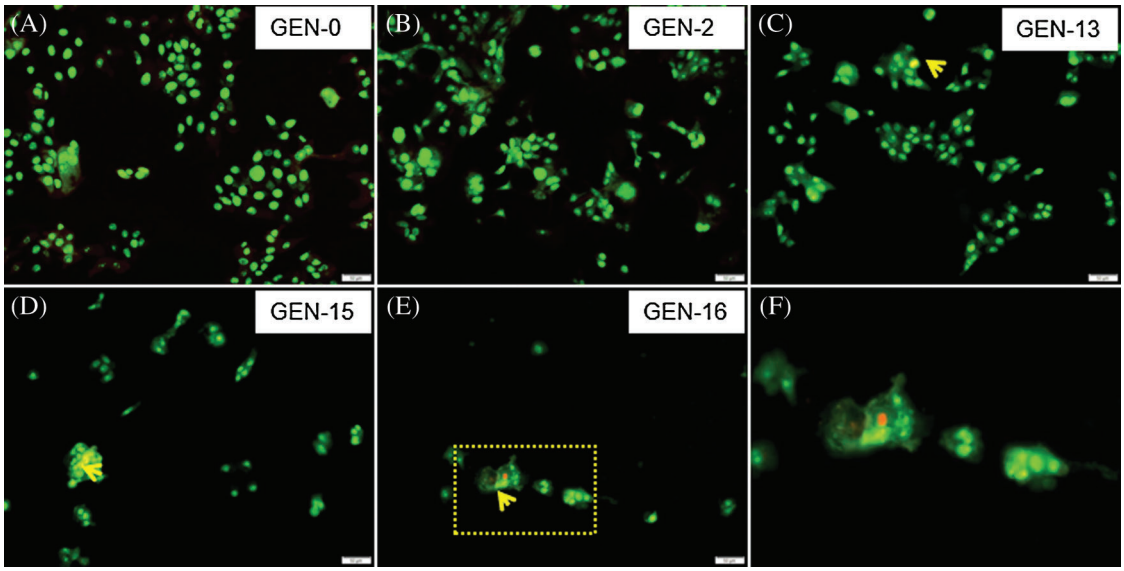

Figure 4: AO/EB staining to observe the apoptosis of MCF-7 cells after cells exposed to GEN at different concentrations for 48 h (n = 3, scale bar = 50 μm)

4 Conclusion

In this research, the inhibition of GEN on MCF-7 cells was estimated by CCK-8 staining, live/dead staining and AO/EB staining. The CCK-8 experiment and live/dead staining results have shown that GEN plays an inhibitory effect on MCF-7 cells proliferation in a time- and concentration- dependent manner. At a concentration of 13 mg/mL when exposed for 24 h, GEN could significantly inhibit cells growth and the inhibitory rate could reach 50% as the concentration increased. AO/EB staining result has shown that under the action of GEN, MCF-7 cells has undergone apoptosis. In a conclusion, GEN can not only inhibit MCF-7 cells proliferation but also promote cell apoptosis.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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