

Original Article

Impacts of eicosapentaenoic acid on proliferation of colon cancer SW480 cells under normoxic and hypoxic conditions

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Abstract: Objective: The study aim was to investigate the impacts of eicosapentaenoic acid (EPA) on the proliferation of SW480 cells under normoxic and hypoxic conditions. Methods: Methyl thiazolyl tetrazolium (MTT) assay was used to detect the proliferation inhibition rate and half maximal inhibitory concentration (IC₅₀) of EPA towards SW480 cells; trypan blue staining was used to draw cell growth curve; flow cytometry was performed to detect the cell cycle, thus observing the impacts of EPA on the proliferation of SW480. Under normoxic and hypoxic conditions, acridine orange fluorescence staining was performed to observe the morphological changes of apoptotic SW480 cells, AnnexinV/PI dual-staining was used to detect the apoptosis rate; western blot assay was performed to detect the expression changes of caspase-9 and caspase-3 proteins. Real time-polymerase chain reaction (RT-PCR) was performed to detect the expression changes of hypoxia inducible factor-1 α (HIF-1 α) mRNA inside the cells, and laser confocal assay was performed to detect the changes of HIF-1 α protein inside the cells. Results: After EPA treatment, proliferation of the SW480 cells was significantly inhibited, with IC₅₀ as 84.2 μ g/ml. Cell cycle was arrested in G₀/G₁ phase; acridine orange staining exhibited significant yellow-green staining inside the cells. EPA could induce the apoptosis of SW480 in a dose-dependent manner. With the increasing concentration of EPA, the levels of HIF-1 α mRNA and protein exhibited the increasing trend. Compared with normoxic condition, the above phenomena were more significant under hypoxic condition. Conclusions: EPA could induce the apoptosis of SW480 cells in vitro, which would be more significant under hypoxic condition.

Keywords: Eicosapentaenoic acid EPA, human colon cancer SW480 cells, hypoxic condition, HIF-1 α

Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal cancers, and would seriously threat people's health. The incidence rate of colon cancer in China has exhibited continuously increasing trend, and its mortality is only after lung cancer and liver cancer, and it's one of Chinese three major cancers [1]. Looking for the treatment methods of CRC is still the key issue urgently needed to be solved in current clinical work. With the improvements of surgery, chemotherapy and radiation levels, the survival rate of CRC had been greatly improved, but it still remains to be a serious threat to patients' life and health. Currently, due to such

advantages as convenient and non-toxic, food therapy, especially the chemopreventive materials inside diet has become a hotspot of research [2]. Modern epidemiological studies had found that intaking a large quantity of unsaturated fatty acids could reduce the incidence rates of breast cancer, colon cancer and prostate cancer to some extent [3-6]. In addition, a large number of in vivo and in vitro experiments had shown that unsaturated fatty acids could slow the growth of cancer cells, induce their apoptosis, and increase the efficacies of chemotherapies [7-9]. Although the specific mechanisms are not very clear, the intake of unsaturated fatty acids could kill tumor cells through regulating cells' behavior as well as

other signaling pathways such as proliferation [10].

ω -3 polyunsaturated fatty acids (ω -3PUFA) are one kind of nutrients necessary for human body, and also one kind of important structural fatty acids involved in constructing membrane phospholipids and regulating intracellular metabolism. In vivo and in vitro pharmacological experiments had confirmed that ω -3PUFA could inhibit the growth, invasion and metastasis of tumors, and enhance the efficacies of certain anticancer drugs, prolong the survival time of tumor-bearing hosts. As a key member of ω -3PUFA, eicosapentaenoic acid (EPA) had exhibited its inhibitory effects on a variety of tumors in many in vivo and in vitro experiments [11-14]. Our previous studies had shown that EPA could effectively inhibit the proliferation of human bile duct cancer cell line FRH-0201, and promote its apoptosis. This study used in vitro experiment to observe the impacts of EPA on the proliferation of human colon cancer SW480 cells and its related mechanisms.

Materials and methods

Tumor cell lines

Human colon cancer SW480 cells were cultured in 10% Dulbecco minimum essential medium (DMEM) (Gibco Life Technologies, Carlsbad, CA, USA) containing fetal bovine serum (FBS) (Gibco Life Technologies, Carlsbad, CA, USA) at 37°C and 5% CO₂. The cells in logarithmic growth phase were then taken for trypsin-EDTA (Gibco Life Technologies, Carlsbad, CA, USA) digestion, and prepared the single cell suspension with 10% FBS-containing DMEM medium, after counted the cells with trypan blue staining method, the cell suspension was diluted to the desired test concentration.

Proliferation inhibition rate and half maximal inhibitory concentration (IC₅₀) towards SW480 cells

SW480 cells in logarithmic growth phase were firstly prepared to 1×10⁵ cells/mL with DMEM culture medium; seeded the cells into 96-well plate, with 100 μ L of medium in each well, added EPA when the cells adhered to the wall, with the drug concentrations as: 0 μ g/mL, 10 μ g/mL, 20 μ g/mL, 40 μ g/mL, 80 μ g/mL, 160 μ g/mL, 320 μ g/mL, 640 μ g/mL and 1280 μ g/mL; cultured the cells at 37°C, 5% CO₂ and saturated humidity for 72 h; each group was set

three repeated wells; 72 h later, absorbed excessive medium, added 80 μ L of fresh medium and 20 μ L of MTT for 4 h culture at 37°C, 5% CO₂ and saturated humidity; absorbed the medium, added 150 μ L of dimethylsulphoxide (DMSO) and oscillated in darkness with low speed for 10 min to fully dissolve the crystals, then measured the OD value of each well with one ELISA instrument at 490 nm.

Impacts on cell growth curve

The subcultured SW480 cells were treated with different concentrations of EPA (0 μ g/mL, 42.1 μ g/mL, 84.2 μ g/mL, 168.4 μ g/mL) for 24, 48, 72 and 96 h, after performed trypan blue staining, counted the cells resisted Trypan blue staining to calculate the concentrations of live cells and drew the cell growth curve after 96 h EPA treatment.

Impacts on cell cycle

After treated with different concentrations of EPA (0 μ g/mL, 42.1 μ g/mL, 84.2 μ g/mL, 168.4 μ g/mL) for 72 h, SW480 cells were harvested, washed twice with ice-cold PBS, adjusted the concentration to 1×10⁶ cells/mL, and fixed with pre-cooled 70% ethanol, after stored at 4°C in darkness for 24 h, added 100 μ L of Rnase A solution, heated in 37°C water bath for 30 min, then added 400 μ L of propidium iodide (PI) staining solution, mixed and stood at 4°C in darkness for 30 min; BECKMAN COULTER Epics Altra flow cytometer was then used to detect the cell cycle, recording the red fluorescence excitation with wavelength at 488 nm.

Establishment of hypoxic condition

The following experiments were all performed under normoxic and hypoxic conditions; in the normoxia group, the cells were cultured at 37°C, saturated humidity and 5% CO₂ for 72 h; while the cells in the hypoxia group were cultured in sealed conditions with 95% N₂ and 5% CO₂ for 72 h.

Morphological observation of fluorescence staining

After the SW480 cells in logarithmic growth phase were treated with different concentrations of EPA (0 μ g/mL, 42.1 μ g/mL, 84.2 μ g/mL, 168.4 μ g/mL) for 72 h, acridine orange dye (5 μ g/mL) was added for staining, observed and photographed with microscope.

Eicosapentaenoic acid on proliferation of SW480 cells

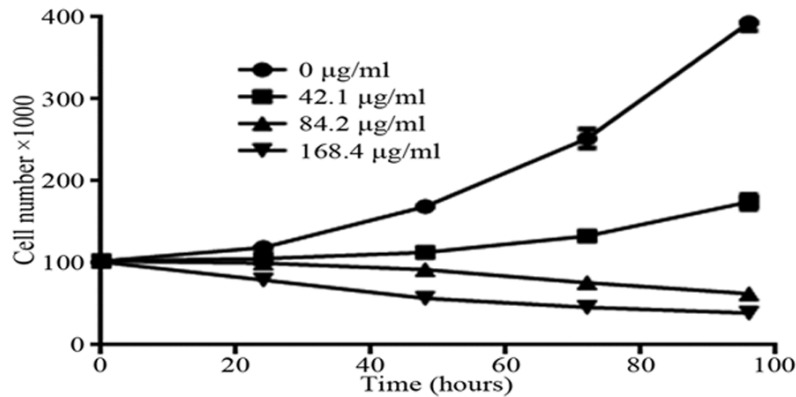


Figure 1. The cellular growth curve drawn by trypan blue staining.

Measurement of apoptosis rate by flow cytometry

After treated with different concentrations of EPA solutions (0 µg/mL, 42.1 µg/mL, 84.2 µg/mL, 168.4 µg/mL) for 72 h, about $1\sim 5 \times 10^5$ SW480 cells were collected, washed with PBS twice, added 500 µL of Binding Buffer to suspend the cells; added 5 µL of Annexin V-FITC, mixed, added 5 µL of PI and mixed, stained in darkness at room temperature for 5~15 min; used flow cytometer to analyze DNA with excitation wavelength at 488 nm, the data obtained were then analyzed with the software of flow cytometry.

Expression detection of activated Caspase-9, Caspase-3 and HIF-1α proteins by Western blot

Collected the SW480 cells in logarithmic growth phase, treated with EPA (0 µg/mL, 42.1 µg/mL, 84.2 µg/mL, 168.4 µg/mL) for 72 h, then lysed the cells with RIPA lysis buffer, extracted the supernatant, and detected the protein concentrations; took the equal amount of protein sample (40 µg/well) for 12% SDS-PAGE electrophoresis, after transferred with wet method, used 5% nonfat milk to block and incubated with the specific primary antibody, as well as with horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody; developed by ECL and X-exposure.

Expression detection of HIF-1α mRNA by quantitative real time-polymerase chain reaction (qRT-PCR)

Trizol method was performed to extract the total RNA of SW480 cells after treated with dif-

ferent concentrations of EPA under normoxic and hypoxic conditions, which were then reversely transcribed into total cDNA, and the expression of HIF-1α mRNA was then detected by RT-PCR.

Intracellular distribution of HIF-1α protein by laser confocal

SW480 cells treated by different concentrations of EPA were performed immunohistochemical staining, then performed laser confocal to observe the intracellular distribution changes of HIF-1α.

Results

Proliferation inhibition and IC₅₀ of EPA on SW480 cells

MTT assay revealed that after treated with EPA for 72 h, the proliferation of SW480 cells was significantly inhibited, with IC₅₀ as 84.2 ± 1.2 µg/mL.

Impacts on cell growth curve

Trypan blue staining method was performed to determine the impacts of EPA on the cell growth curve of SW480. The results showed that, various concentrations of EPA exhibited proliferation inhibition on SW480 cells between 0~96 h in a time-dependent manner (**Figure 1**).

Impacts on cell cycle

Flow cytometry assay revealed that, after treated with EPA (0 µg/mL, 42.1 µg/mL, 84.2 µg/mL, 168.4 µg/mL) for 72 h, the percentage of SW480 cells in G₀/G₁ phase was increased with the increasing of EPA concentrations, while those in S and G₂/M phase were significantly decreased (**Figure 2**). Cell cycle was arrested in G₀/G₁ phase.

Morphological observation by fluorescence staining

After acridine orange staining, observation under fluorescence microscope revealed that the nuclear DNA of SW480 cells in the control

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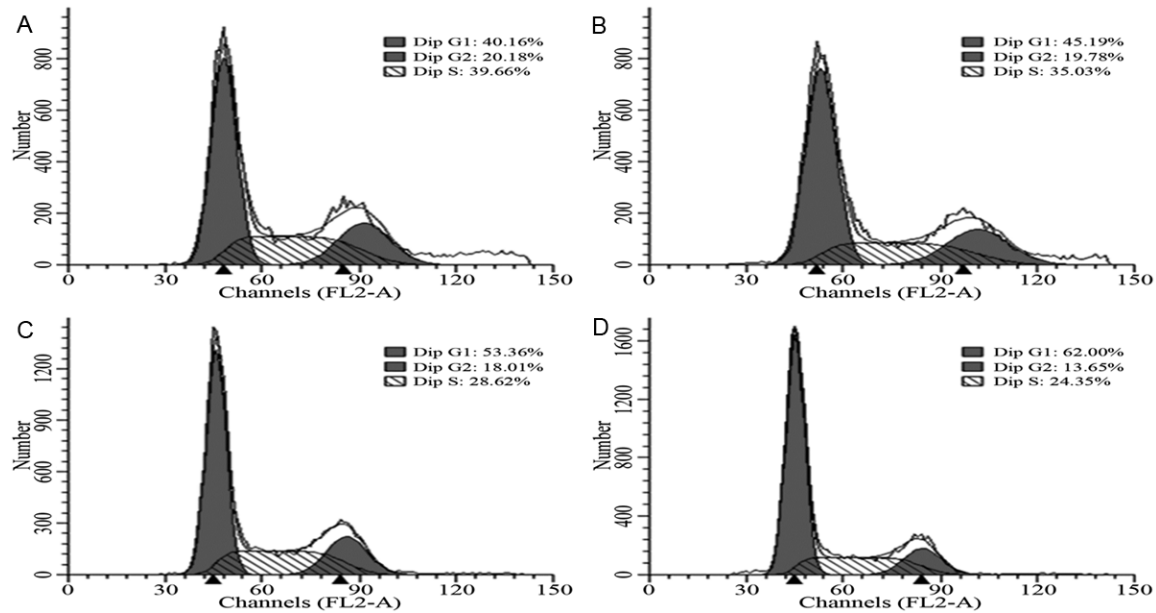


Figure 2. The cell cycle was analyzed using Flow Cytometry.

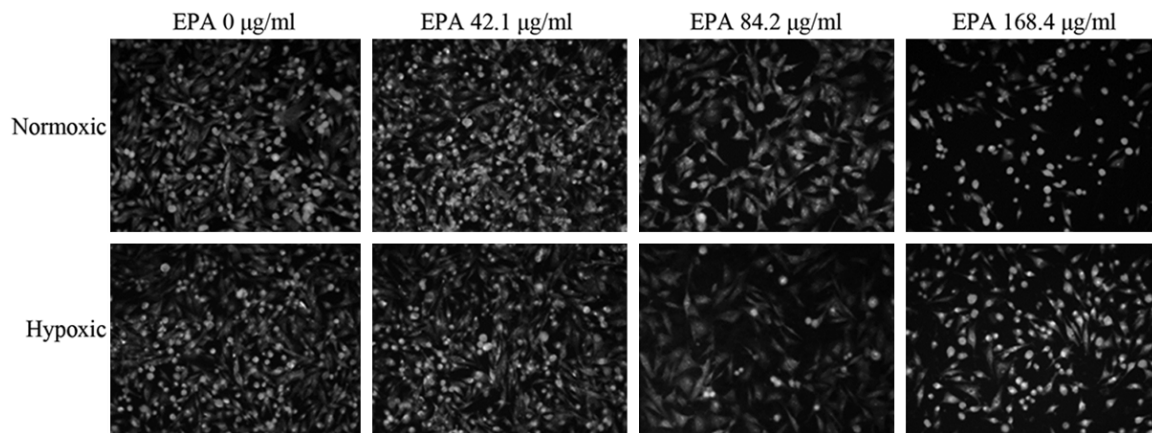


Figure 3. The apoptosis was determined by acridine orange staining.

group exhibited uniform yellow-green fluorescence, while that of each EPA treatment group exhibited dense yellow-green fluorescence in nuclei or cytoplasm, even heavily stained yellow-green fragments, showing typical apoptotic morphological changes (**Figure 3**).

Analysis of apoptosis rate by flow cytometry

AnnexinV/PI dual-staining method is a sensitive method to detect early apoptosis, early apoptotic cells would mainly exhibit AnnexinV-FITC staining, while late apoptotic or necrotic cells would mainly exhibit AnnexinV-FITC/PI dual-staining. The apoptosis rates after treated by

different concentrations of EPA for 72 h were shown in **Figure 4**, there were statistical differences when compared with the control group. Compared with normoxic condition, the proapoptotic effects of EPA towards SW480 under hypoxic condition were more significant.

Impacts of EPA on the expressions of Caspase-9 and Caspase-3 proteins in SW480

Western blot analysis was performed to detect the impacts of EPA on the protein expressions of Caspase-9 and Caspase-3 in SW480. The results showed that compared with the control group, after treated with different concentra-

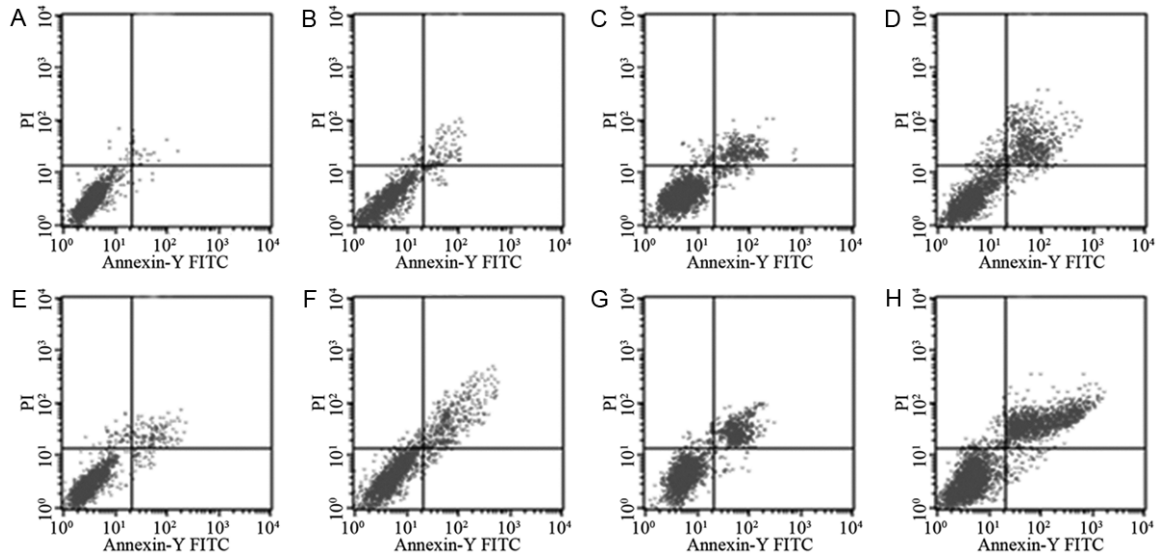


Figure 4. The apoptosis was determined by Flow Cytometry.

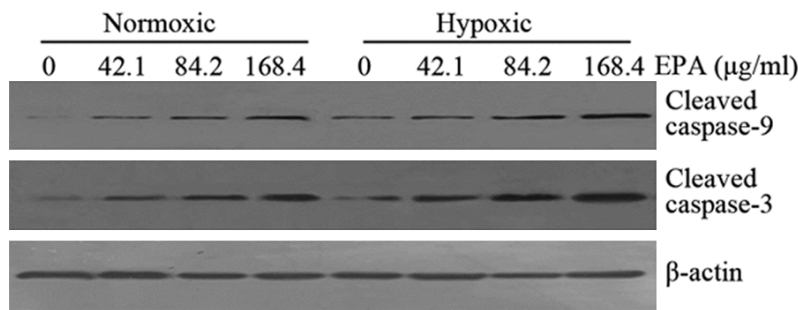


Figure 5. Western blot analysis the expression of cleaved Caspase-3 and Caspase-9.

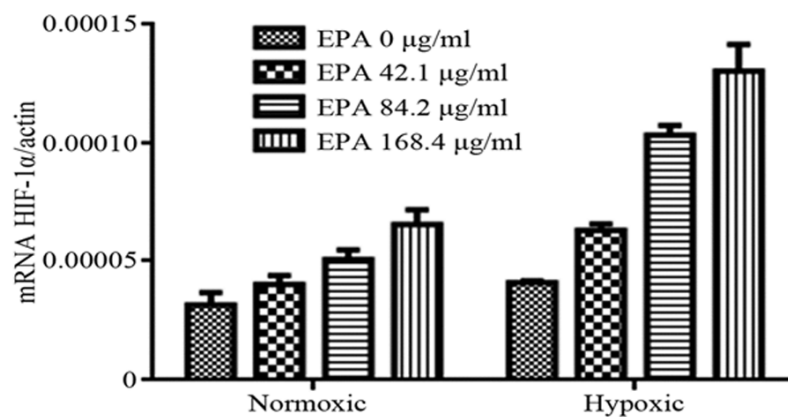


Figure 6. qRT-PCR analysis of the expression of HIF-1α mRNA.

tions of EPA, the activated Caspase-9 and Caspase-3 proteins were significantly expressed in SW480 (**Figure 5**).

was also increased; and compared with normoxic condition, the expression was much more increased under hypoxia.

Expression detection of HIF-1α mRNA by qRT-PCR

The cells were cultured with EPA at different concentrations (0 μg/mL, at 42.1 μg/mL, 84.2 μg/mL and 168.4 μg/mL) under normoxic and hypoxic conditions for 72 h, qRT-PCR was performed to detect the expressions of HIF-1α gene in each group (**Figure 6**).

Intracellular distribution of HIF-1α protein by laser confocal

Laser confocal was performed to observe the distribution changes of HIF-1α in SW-480 cells after treated with different concentrations of EPA and immunohistochemically stained. The results were shown in **Figure 7**, with the increasing of EPA concentrations, the expression of HIF-1α

Eicosapentaenoic acid on proliferation of SW480 cells

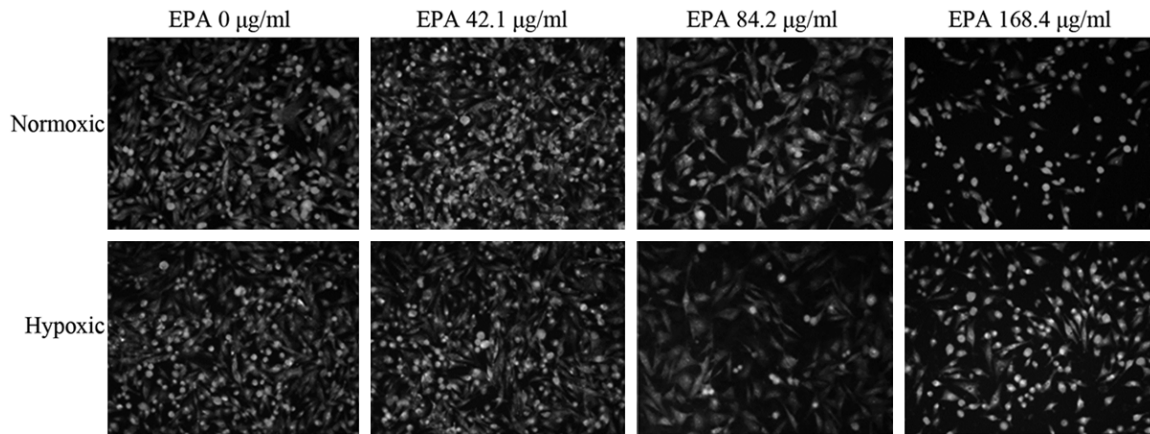


Figure 7. Confocal laser scanning microscope analysis of the expression of HIF-1 α .

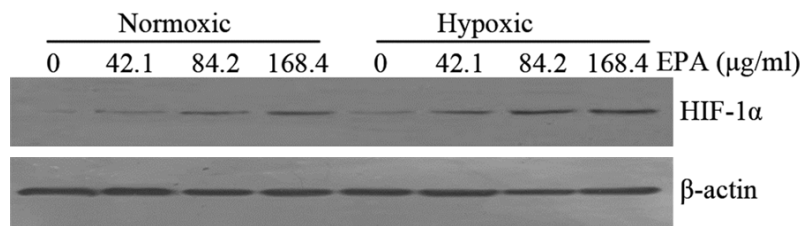


Figure 8. Western blot analysis the expression of HIF-1 α .

Impacts of EPA on the expression of HIF-1 α protein in SW480

Western blot assay was performed to detect the impact of EPA on the expression of HIF-1 α protein in SW480. The results showed that compared with the control group, after treated with different concentrations of EPA, HIF-1 α protein was significantly expressed in SW480 (**Figure 8**). Compared with normoxic condition, the expression was much more increased under hypoxia.

Discussion

It would be very attractive to use natural foods, such as EPA and DHA, etc., as prevention and treatment towards cancers, because these chemicals are usually low-cost, and would be easily metabolized in human body, and they usually would not exhibit significant cytotoxicities on normal cells. A large number of studies have shown that, EPA and DHA could inhibit the proliferation of tumor cells in vitro through a variety of ways, and exhibited significant inhibitory effects on HepG2 cells, leukemia HL-60 cells, human pancreatic cancer SW1990, and AsPC-1 cells, etc. [11-14].

The results of this study showed that EPA could inhibit the proliferation of SW480, with IC₅₀ as 84.2 \pm 1.2 μ g/mL. The growth curve of SW480 cells after treated with EPA for 96 h showed that there was a good linear relationship between inhibitory effects

and action time. Because cell proliferation is achieved through cell cycle [15], certain studies had reported that affecting cell cycle was one of the main ways of ω -3 polyunsaturated fatty acid-induced apoptosis of tumor cells [16]. This study found that after treated with EPA, the percentage of SW480 cells in G₀/G₁ phase was increased with the increasing of EPA concentrations, while those in S and G₂/M phase were significantly decreased; cell cycle was arrested in G₀/G₁ phase.

Hypoxia is one of the inherent characteristic of most solid tumors, because the proliferation rate of tumor cells was faster than the speed of the formation of new blood vessels, a series of pathological changes were thus caused. In order to better understand the anti-tumor mechanisms of EPA, we conducted a preliminary study about the proliferation inhibition effects of EPA under normoxic and hypoxic conditions towards SW480. After acridine orange staining, fluorescence microscopy revealed that after EPA treated, SW480 cells showed typical features of apoptosis, namely heavy yellow-green staining [17], thus it could prove that EPA could induce the apoptosis in SW480.

Annexin V/PI kit and flow cytometry were then used to detect the apoptosis rate of EPA on SW480, the results further demonstrated that EPA could induce the apoptosis of SW480 in a dose-dependent manner. When compared with normoxic condition, it was found that under hypoxic condition, the pro-apoptotic effects of EPA on SW480 were much more significant.

Cytochrome C is a key enzyme in caspase pathway, the activation of Caspase is the central element to induce the apoptosis signaling pathway [18-21], Caspase-9 and Caspase-3 are the two most critical proteins, Caspase-9 is the precursor of Caspase cascade, and Caspase-3 is considered to be the execution Caspase in most cells' apoptosis [22, 23]. As the decreased downstream of mitochondrial transmembrane potential ($\Delta\psi_m$), we detected the impacts of EPA on the protein expressions of Caspase-9 and Caspase-3 in SW480 using Western blot. The results showed that compared with the control group, after treated with different concentrations of EPA for 72 h, the expressions of Caspase-9 and Caspase-3 proteins were significantly increased. Compared with normoxic condition, under hypoxic condition, the amounts of activated caspase proteins were increased, and it also explained the mechanism of proliferation inhibition of EPA on SW480 from another point of view.

HIF-1 protein is important to cells' growth under hypoxic environment. HIF-1 contains two subunits, α and β , HIF-1 α is a functional subunit, regulating the activity and function of HIF-1 protein [24]. We also studied the impacts of EPA on the expressions of HIF-1 α protein under normoxic and hypoxic conditions, the experimental results showed that no matter from gene level, or from protein, after treated with EPA, the expression of HIF-1 α was increased in a dose-dependent manner, though matched with some reports that EPA could reduce the expression of HIF-1 α protein, of course, it was also reported that similar DHA could increase the expression of HIF-1 α , and the reason might be related with different cells. On SW480 cells, we could apply EPA, and at the same time, use HIF-1 α inhibitor for synergic effects, thus further strengthening the proliferation inhibitory effects of EPA on tumor cells.

Disclosure of conflict of interest

None.

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