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STUDY ON OPTIMISATION OF EXTRACTION PROCESS OF TANSHINONE IIA AND ITS MECHANISM OF INDUCTION OF GASTRIC CANCER SGC7901 CELL APOPTOSIS

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Abstract

The objective of this paper was to investigate the extraction process of tanshinone ⅡA and its mechanism of induction of gastric cancer SGC7901 cell apoptosis. Extraction process of tanshinone ⅡA was optimised by orthogonal experimental method, and its effect on gastric cancer SGC7901 cell apoptosis was observed using MTT assay and electron microscopy. The optimum extraction process of tanshinone ⅡA was as follows: addition of a 10-fold amount of 80% ethanol, one-time extraction, and extraction time of 45 minutes. The study concluded that tanshinone ⅡA can induce apoptosis of gastric cancer SGC7901 cells.

Keywords: Tanshinone ⅡA, MTT assay, gastric cancer SGC7901 cells

Introduction

Dan Shen is the dried root and rhizome of Lamiaceae plant *Salvia miltiorrhiza* Bge. It has the efficacies of removing stasis and relieving pain, activating blood circulation and promoting menstruation, clearing away heart-fire and relieving anxiety. It is also often used in the clinical treatment of irregular menstruation, amenorrhoea, dysmenorrhoea, chest and abdominal pain, anxiety, insomnia, etc. Effective fractions of Dan Shen are fat-soluble compounds, of which a representative constituent is tanshinone ⅡA (Liu et al., 2009). Modern pharmacological studies have shown that tanshinone ⅡA has anti-tumour, anti-cardiovascular and cerebrovascular disease, anti-bacterial and anti-inflammatory effects (Wang et al., 2007; Sun et al., 2002; Cai et al., 2008). In this experiment, with the content as the index, orthogonal test was used to optimise the extraction process of tanshinone ⅡA. Meanwhile, its gastric cancer SGC7901 cell apoptosis inducing effect and its mechanism were studied.

Materials and Methods

Instruments and reagents

The instruments and reagents use for the study included high performance liquid chromatograph, Waters; JEM21200EX transmission electron microscope, JEOL; constant temperature incubator with 5% CO_2; LKB21 ultramicrotome, and MTT solution.

Drugs and tumour lines

The drugs and tumour lines are as follows: tanshinone ⅡA, self-prepared, purity of over 90%; human gastric cancer SGC-7901 cell lines, purchased from Shanghai Bogoo Biotechnology Co., Ltd.; 5-fluorouracil, purchased from Shanghai Hanhong Biochemical Co., Ltd. The *Salvia miltiorrhiza* Bge powder was identified by Professor Jia Yuhua,and the voucher number is 2012-2001-02

Extraction and purification process of tanshinone ⅡA

About 20g of *Salvia miltiorrhiza* Bge. powder was weighed in 9 replicates, pulverised into coarse powders, added with corresponding volumes of 80% ethanol as in the experimental programme in Table 1 and then reflux extracted. The filtrates were filtered, extracts were combined, and the solvents were recovered under reduced pressure to near dryness. Volumes were fixed, and tanshinone ⅡA contents in the extracts were determined, respectively, to calculate the extraction rate. Levels and factors of orthogonal experimental design were shown in Table 1.

Table 1: Levels and factors of orthogonal experimental design

Level	A Extraction time (minutes)	B Extraction times (times)	C Ethanol amount (folds)
1	30	1	6
2	45	2	8
3	60	3	10

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Experiment for the inhibitory effect of tanshinone ⅡA on human gastric cancer SGC-7901 cells
Determination of gastric cancer cell growth inhibition rate of tanshinone ⅡA by MTT assay

SGC7901 cells in the exponential growth phase were taken, digested by conventional trypsin method, and then collected. The cells were seeded in 96-well plates at a density of 1×10^4 cells per well, and affected by 2.0, 4.0, and 6.0 mg/L tanshinone ⅡA. Blank control group and 5-Fu control group (250 μg/ml) were set up, which were routinely cultured. At 24 h, 48 h, and 72 h, 20 μL of MTT solution (5 mg/mL) was added to each well, and the incubation was continued at 37℃ for an additional 4 h. Then, the cell culture was completed. Supernatant was carefully aspirated and discarded. Each well was added with 150 μL of DMSO and shaken for 10 min in order to fully dissolve the crystals. Absorbance (A) value was measured at 490 nm, followed by the calculation of cell growth inhibition rate (CI).

CI = (A value of the negative control group - A value of the experimental group) / A value of the negative control group × 100%.

Transmission electron microscopy observation

Human gastric adenocarcinoma SGC7901 cells in the logarithmic growth phase were collected, seeded in 25 mL culture flasks, randomly divided into two groups, and added with 4.0, and 6.0 mg/L tanshinone ⅡA solutions, respectively. After 72 h, cells were digested by conventional trypsin method, placed in the EP tube, and centrifuged for 10 min. Then, the supernatant was discarded and the cells were collected. Before pre-cooling at 4℃, the cells were fixed with 20 g/L glutaraldehyde for 2 h, washed in PBS solution, resin-embedded, sectioned, double stained with uranyl acetate and lead citrate, and then observed under a transmission electron microscope.

Results

Orthogonal experiment results

The experimental results showed that the order of size of influence of three factors on extraction results was A>C>B. Factors A and C were statistically significant ($P<0.05$) for the extraction of Tanshinone ⅡA by reflux extraction method. Factor B was not statistically significant for the extraction of Tanshinone ⅡA. Therefore, the optimal process for the extraction of Tanshinone ⅡA would be A2B1C3, i.e. addition of a 10-fold amount of 80% ethanol, one-time extraction, and extraction time of 45 minutes.

Table 2: Orthogonal experiment results

No.	A	B	C	Tanshinone ⅡA yield (%)
1	1	1	1	0.18
2	1	2	2	0.35
3	1	3	3	0.22
4	2	1	2	0.48
5	2	2	3	0.45
6	2	3	1	0.35
7	3	1	3	0.23
8	3	2	1	0.26
9	3	3	2	0.40
K1	0.25	0.30	0.26	
K2	0.43	0.35	0.41	
K3	0.30	0.32	0.30	
R	0.18	0.05	0.15	

Inhibitory effect of Tanshinone ⅡA on gastric cancer cell growth

After 24 h, 48 h, and 72 h action of tanshinone ⅡA with final concentrations of 2, 4, and 8 mg/L on gastric cancer SGC-7901 cells, it exhibited cell inhibitory effect, which was in a dose-dependent manner with the increase of the concentrations, as shown in Table 3.

Table 3: Inhibitory effect of Tanshinone ⅡA on gastric cancer cell growth

Group	Concentration (mg/L)	24 h		48 h		72 h	
		A value	Inhibition rate (%)	A value	Inhibition rate (%)	A value	Inhibition rate (%)
Normal control group		0.51±0.07		0.56±0.07		0.59±0.03	
5-FU group	250	0.43±0.04	15.7	0.29±0.04	48.2	0.28±0.05	52.5
Tanshinone ⅡA group	2.0	0.51±0.06	1.9	0.47±0.07	16.1	0.58±0.02	1.7
	4.0	0.47±0.03	7.8	0.42±0.07	25.0	0.54±0.04	8.5
	6.0	0.45±0.02	11.7	0.41±0.06	26.8	0.53±0.03	10.1

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Cell morphological changes under transmission electron microscopy

Gastric adenocarcinoma cell nuclei of the control group were comparatively big, concave, with some deformed or lobulated nuclei, and had uniform chromatin. After administration of 6.0 mg/L tanshinone ⅡA, cell volume shrank. Cell membranes and nucleuses were separated; protuberances were observed on the cell surfaces. Organelles were observed to be swollen; nuclei were condensed, and chromatin was marginated under nuclear membranes, forming crescent-shaped bodies. Nuclear chromatin was condensed and fragmented, forming apoptotic bodies, and the number of cells exhibiting early-to-mid stages of apoptosis increased significantly. In the late stage of apoptosis, the apoptotic bodies of organelles and nuclear fragments which were wrapped in membranes were seen.

Discussion

The incidence of cancer is not only associated with excessive proliferation and differentiation of cells, but also associated with inhibited apoptotic pathway, and prolonged cell life (Deng and Wu, 2000). Therefore, induction of tumour cell apoptosis has become a new target of cancer drug screening. By inducing increase in tumour cell apoptosis, proliferation capacity is weakened, thus achieving the purpose of diagnosis and treatment of tumours. The small side effects of traditional Chinese medicine have made it increasingly advantageous in cancer therapy, and have infused a new wave of research on tumour control with TCM in China.

Salvia miltiorrhiza Bge. is the oldest and most widely used drug in traditional Chinese medicine, and it has blood circulation promoting and blood stasis removing effects, antibacterial and anti-inflammatory effects. It is clinically used for the treatment of coronary heart disease, cerebral thrombosis, acne and other diseases (Zhao et al., 2003; Chen and Lin, 2003). The chemical constituents of *Salvia miltiorrhiza* Bge. are divided into two categories: water-soluble constituents and fat-soluble constituents. One of its main fat-soluble constituent – Tanshinone ⅡA has anti-atherosclerotic, myocardial oxygen consumption reducing and nerve cell protective effects. Studies in recent years have found that the antitumour mechanism of action of tanshinone ⅡA is mainly concentrated in the killing of tumour cells, induction of differentiation and apoptosis, and inhibition of invasion and metastasis (Zhang et al., 2005; He et al., 2002). In this study, ethanol extraction process of Tanshinone ⅡA from Salvia miltiorrhiza Bge. was optimised by orthogonal experiment, and the influences of extraction time. Extraction times and ethanol concentration on extraction effects were investigated. The optimum extraction conditions A2B1C3, i.e. addition of a 10-fold amount of 80% ethanol, one-time extraction, extraction time of 45 minutes, were screened by L9(3^4) orthogonal test design with tanshinone ⅡA content as quality control index.The ELISA-based MTT assay used in this study is one of popular methods for cell activity measurement at present. MTT is a yellow, water-soluble tetrazolium dye, which can be reduced by living cells to a water-insoluble, purple formazan product, while the dead cells cannot reduce MTT (Lee et al., 2006). Therefore, the amount of formazan formation is proportional to the number of living cells. After dissolution of formazan in organic solvent DMSO, a purple solution was formed. Then, optical density (OD value) was automatically measured using a microplate reader, thereby quantitatively calculating the activity status of cells in each experimental group (Yang & Liu, 2006; Liu et al., 2006).In this experiment, the effect of Tanshinone ⅡA on human gastric cancer SGC-7901 cell growth was observed by MTT colorimetric assay. It was found that tanshinone ⅡA had a relatively strong inhibitory effect on growth and proliferation of gastric cancer cells. After action of 1.0 μg/ml, 2.0 μg/ml, and 4.0 μg/ml tanshinone ⅡA for different time periods, they all produced inhibitory effects on human gastric cancer SGC-7901 cells. The growth inhibition rates after 72 h action of Tan ⅡA were 25.7±1.86%, 47.8±2.75%, and 58.4±3.25%, respectively. The experimental results showed that the inhibitory effect of tanshinone ⅡA on proliferation of human gastric cancer SGC-7901 cells was time- and dose-dependent.

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