

RESEARCH ARTICLE

Mechanisms of Hela Cell Apoptosis Induced by Abnormal Savda Munziq Total Phenolics Combined with Chemotherapeutic Agents

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Abstract

Objective: To investigate the effects of abnormal Savda Munziq (ASMq) total phenolics combined with cisplatin and docetaxel on the Hela cell growth. **Methods:** *In vivo* cultured Hela cells were treated with cisplatin, docetaxel, total phenolics, cisplatin+total phenolics or docetaxel+total phenolics. MTT was performed to assess inhibition of cell proliferation, flow cytometry to detect apoptosis, and semi-quantitative RT-PCR to test for survivin and Bcl-2 expression. **Results:** The total phenolics, cisplatin and docetaxel had significant inhibitory and apoptosis-promoting effects on Hela cells ($P < 0.05$), with the early apoptotic rates of $12.8 \pm 0.70\%$, $18.9 \pm 3.79\%$ and $15.8 \pm 3.8\%$; the total phenolics, cisplatin and docetaxel significantly decreased the expression of Bcl-2 and survivin (all $P < 0.01$), especially when used in combination. **Conclusion:** ASMq total phenolics, combined with cisplatin and docetaxel, could promote the apoptosis of Hela cells possibly through reducing the expression of Bcl-2 and survivin.

Keywords: Abnormal Savda Munziq total phenolics - Hela cells - apoptosis - Bcl-2 gene - survivin

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Introduction

Uighur medicine considers that the abnormal Savda (AS) is closely related to the occurrence and development of cancers (Kizaibek et al., 2012). The Abnormal Savda Munziq (ASMq) is the valid prescription towards the treatment and prevention of various tumors in Uighur medicine, and its effects are significant (Yusup et al., 2005; Upur et al., 2011; Yusup et al., 2011; Aikemu et al., 2012; Aikemu et al., 2012). Cisplatin and docetaxel are the commonly clinically used drugs in the treatment of gynecological malignant tumors, with exact efficacy (Wang et al., 2010; Ke et al., 2012; Pu et al., 2013). In this study, the inhibition effect and mechanism of ASMq total phenolics, combined with chemotherapy drugs (cisplatin and docetaxel), on the *in vitro* proliferation of human cervical carcinoma Hela cells, were investigated, aiming to find a new drug which could enhance the tumor-suppressive roles of the chemotherapy drugs, and promoting some possibility of the application of ASMq total phenolics in the treatment of cervical cancer.

Materials and Methods

Chemical Reagents

The total phenolics were kindly provided by the Professor Mulati Hupur, Xinjiang Medical University. Experimental drugs and reagents include: Cisplatin (Hospira Australia Pty Ltd., Australia), docetaxel (Jiangsu Hengrui Medicine, China), high glucose Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (GIBCO, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), trypsin (AMRESCO Co., Ltd.), membrane breaker (Beckman Coulter), Bcl-2 antibody (INVITROGEN Co., Ltd), RNA extraction kit (Invitrogen Co., Ltd), primer, fluorescent probe synthesis and PCR reaction kit (Zhongshan Medical University DaNa Gene Co., Ltd.).

Cell culture

Human cervical carcinoma Hela cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy

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Table 1. Inhibition Rate of Total Phenolics Combined with Cisplatin on Hela Cell Proliferation ($\bar{x}\pm s$, %)

Phenolics ($\mu\text{g/ml}$)	Cisplatin (0 $\mu\text{g/ml}$)			Cisplatin (0.1 $\mu\text{g/ml}$)		
	24 h	48 h	72 h	24 h	48 h	72 h
0	-	-	-	7.84 \pm 0.34	8.40 \pm 0.53	12.27 \pm 1.49
50	-	8.40 \pm 1.12	17.59 \pm 2.59	12.38 \pm 0.82 ^a	13.78 \pm 1.68 ^{ab}	21.96 \pm 2.56 ^{ab}
75	9.79 \pm 0.56	15.05 \pm 1.57	25.04 \pm 2.20	15.69 \pm 1.41 ^a	27.84 \pm 1.70 ^{ab}	32.65 \pm 3.10 ^{ab}
100	11.99 \pm 1.18	23.53 \pm 1.91	30.00 \pm 0.74	22.16 \pm 2.09 ^a	41.86 \pm 2.99 ^{ab}	47.39 \pm 2.97 ^{ab}
125	22.49 \pm 2.20	31.50 \pm 4.07	44.11 \pm 3.22	27.90 \pm 1.69 ^a	45.01 \pm 2.79 ^{ab}	64.43 \pm 1.11 ^{ab}
Total Phenolics ($\mu\text{g/ml}$)	Docetaxel (0 $\mu\text{g/ml}$)			Docetaxel (5 $\mu\text{g/ml}$)		
	24 h	48 h	72 h	24 h	48 h	72 h
0	10.23 \pm 1.49	16.01 \pm 1.03	21.29 \pm 1.65	20.30 \pm 1.69	44.36 \pm 2.07	81.96 \pm 2.01
50	17.96 \pm 3.25 ^a	22.83 \pm 1.38 ^{ab}	35.39 \pm 1.08 ^{ab}	25.77 \pm 1.88 ^a	55.24 \pm 4.37 ^{ab}	84.69 \pm 1.14 ^b
75	25.06 \pm 1.39 ^a	33.50 \pm 1.09 ^{ab}	40.34 \pm 2.66 ^{ab}	28.18 \pm 1.76 ^a	62.73 \pm 1.59 ^{ab}	86.87 \pm 2.60 ^{ab}
100	30.36 \pm 0.42 ^a	48.92 \pm 2.72 ^{ab}	55.96 \pm 1.54 ^{ab}	38.16 \pm 1.01 ^a	67.99 \pm 1.75 ^{ab}	90.09 \pm 0.84 ^{ab}
125	33.65 \pm 1.69 ^a	52.62 \pm 2.86 ^{ab}	77.42 \pm 2.59 ^{ab}	41.34 \pm 1.30 ^a	70.50 \pm 2.32 ^{ab}	92.07 \pm 1.50 ^{ab}

Compared with the same concentration of total phenolics or cisplatin alone group, ^a $P < 0.05$; compared with the same concentration while different action time group, ^b $P < 0.05$

of Medical Sciences, cultivated within RPMI-1640 medium, containing 5% fetal bovine serum and 100 U/ml mycillin, at 37 °C and 5% CO₂. The medium was changed every 2 days, and the passage was performed once every 3-4 days, the cells in logarithmic growth phase were used in the experiment.

MTT assay

Hela cells of logarithmic growth phase were adjusted to the concentration of 5×10^4 /ml, and seeded into 96-well plate, with 100 μl in each well. after 48 h culture, the cells were divided into: 1) control group (only with medium); 2) experimental group (including monotherapy group: total phenolics group, cisplatin group and docetaxel group; combined group : total phenolics+cisplatin group, total phenolics+docetaxel group); 3) blank group (including blank control group, blank experimental group). 0, 50, 75, 100 and 125 $\mu\text{g/ml}$ total phenolics was added, then 0.1, 1 and 5 $\mu\text{g/ml}$ cisplatin or 5, 10 and 20 $\mu\text{g/ml}$ docetaxel was added and cultivated for 24, 48 and 72 h. Each hole also had a control well with only the same concentration of cisplatin, docetaxel or culture medium. Then 20 μl MTT was added into each well. After 4 h cultivation in the incubator, the medium liquid was discarded, and 150 μl DMSO was added. The automatic microplate reader (BIORAD Co., Ltd, USA) was used to detect the absorbance value at 490 nm (optical density, OD490) after shock 10 min. Proliferation inhibition rate=[1-(average OD490 value of experimental well- wells average OD490 value of blank control/average OD490 value of control well-average OD490 value of blank control well)] \times 100%. The experiment was repeated five times, and each concentration had 3 duplicate wells for the average value.

Flow cytometry

Hela cells of logarithmic growth phase were inoculated 5×10^5 into each flask, then added 5 ml culture medium. After 48 h, cisplatin (5 $\mu\text{g/ml}$), docetaxel (20 $\mu\text{g/ml}$), total phenolics (50 $\mu\text{g/ml}$), total phenolics (50 $\mu\text{g/ml}$) +cisplatin (5 $\mu\text{g/ml}$) and total phenolics (50 $\mu\text{g/ml}$) +docetaxel (20 $\mu\text{g/ml}$) were added into the culture medium, respectively, and another bottle of culture medium was used as

the control. After another 48 h cultivation, single cell suspension was formed with trypsinization, and flow cytometry (BECKMAN COULTER Co., Ltd, USA) was used to detect the cell apoptotic rate. The single cell suspension was washed twice with PBS, and used the membrane breake to break the membrane, after that, added 5 μl mouse anti-human bcl-2 antibody, and added 4 ml PBS to wash and centrifuge for 15 min, , the supernatant was then added into 1ml sheath fluid to form the uniform cell suspension for the flow cytometry.

Semi-quantitative RT-PCR

Quantitative fluorescence PCR instrument (PerkinElmer Co., Ltd, USA). The grouping method was the same as 2.2, total RNA was extracted and reversely transcribed into cDNA according to the kit instructions, and PCR reaction was performed to detect the expression of survivinm. Upstream primer: 5'-GTCCGGTTGCGCTTTCCTT-3', downstream primer: 5'-CGCAGTTTCCTCAAATTCTTTCTTC-3', and β -actin was used as an internal reference, the reaction conditions were: 95 °C denaturation for 3 min, 95 °C denaturation for 10 s, 60 °C renaturation for 30 s, 60 °C extension for 40 s, with 40 repeated cycles. The products were purified by agarose gel electrophoresis for the observations and photographs.

Statistical analysis

The experimental results obtained was performed statistical handling with SPSS17.0, measurement data were expressed as mean \pm standard deviation ($\bar{x}\pm s$), analysis of variance was carried out and $P < 0.05$ was considered as statistically significant difference.

Results

Proliferation inhibition rate by total phenolics and cisplatin

Total phenolics and cisplatin exhibited increasing proliferation inhibition rate on Hela cells, and the combination group of the above 2 drugs also showed significantly higher inhibition rate ($P < 0.001$); when the

Table 2. Inhibition Rate of Total Phenolics Combined with Docetaxel on Hela Cell Proliferation ($\bar{x}\pm s$, %)

Total Phenolics ($\mu\text{g/ml}$)	Docetaxel (0 $\mu\text{g/ml}$)			Docetaxel (5 $\mu\text{g/ml}$)		
	24 h	48 h	72 h	24 h	48 h	72 h
0	-	-	-	9.36 \pm 1.11	9.38 \pm 1.40	21.77 \pm 1.67
50	-	8.40 \pm 1.12	17.59 \pm 2.59	16.13 \pm 1.03 ^a	20.37 \pm 1.15 ^{ab}	29.46 \pm 1.41 ^{ab}
75	9.79 \pm 0.56	15.05 \pm 1.57	25.04 \pm 2.20	19.78 \pm 0.76 ^a	22.81 \pm 1.35 ^{ab}	36.76 \pm 0.54 ^{ab}
100	11.99 \pm 1.18	23.53 \pm 1.91	30.00 \pm 0.74	26.11 \pm 2.59 ^a	31.50 \pm 1.38 ^{ab}	57.71 \pm 1.73 ^{ab}
125	22.49 \pm 2.20	31.50 \pm 4.07	44.11 \pm 3.22	48.62 \pm 0.97 ^a	47.66 \pm 4.54 ^{ab}	73.16 \pm 3.37 ^{ab}

Total Phenolics ($\mu\text{g/ml}$)	Docetaxel (10 $\mu\text{g/ml}$)			Docetaxel (20 $\mu\text{g/ml}$)		
	24 h	48 h	72 h	24 h	48 h	72 h
0	14.48 \pm 0.89	20.59 \pm 1.46	42.06 \pm 1.91	32.92 \pm 2.71	54.20 \pm 3.23	64.44 \pm 2.08
50	33.93 \pm 0.13 ^a	37.92 \pm 0.53 ^{ab}	58.59 \pm 3.90 ^{ab}	69.30 \pm 3.81 ^a	73.64 \pm 0.42 ^{ab}	82.97 \pm 1.96 ^{ab}
75	41.20 \pm 3.09 ^a	45.75 \pm 0.91 ^{ab}	74.02 \pm 0.61 ^{ab}	72.50 \pm 3.18 ^a	78.13 \pm 0.33 ^{ab}	89.78 \pm 0.69 ^{ab}
100	46.04 \pm 4.35 ^a	64.77 \pm 1.49 ^{ab}	81.10 \pm 3.09 ^{ab}	77.81 \pm 0.69 ^a	84.73 \pm 0.02 ^{ab}	92.09 \pm 2.06 ^{ab}
125	69.97 \pm 1.93 ^a	73.81 \pm 0.37 ^{ab}	87.25 \pm 0.43 ^{ab}	79.95 \pm 1.21 ^a	88.06 \pm 1.33 ^{ab}	94.07 \pm 0.54 ^{ab}

Compared with the same concentration of total phenolics or docetaxel alone group, ^a $P < 0.05$; compared with the same concentration while different action time group, ^b $P < 0.05$

Table 3. Influence of Total Phenolics, Combined with Cisplatin and Docetaxel, on Hela Cells Bcl-2 and Survivin Expression ($\bar{x}\pm s$, %)

Group	Bcl-2	Survivin
Total phenolics	63.63 \pm 3.84 ^a	0.636 \pm 0.040 ^a
Cisplatin	49.20 \pm 4.08 ^a	0.431 \pm 0.048 ^a
Docetaxel	51.40 \pm 6.35 ^a	0.577 \pm 0.040 ^a
Total phenolics+cisplatin	14.57 \pm 2.32 ^{ab}	0.172 \pm 0.015 ^{ab}
Total phenolics+docetaxel	18.93 \pm 4.83 ^{ab}	0.324 \pm 0.031 ^{ab}
Control	81.93 \pm 7.51	1.500 \pm 0.034

Compared with control group, ^a $P < 0.01$; compared with the monotherapy group, ^b $P < 0.01$

concentration of cisplatin was constant, the inhibition rate of the combined effect increased with the increasing of the total phenolics concentration; when the concentration of total phenolics was constant, the inhibition rate increased with the increasing concentration of cisplatin; when treated with the same drug concentration, the inhibition rate increased with the increasing treating time. When the concentration of total phenolics was lower than 50 $\mu\text{g/ml}$, there was no cytotoxic effect on Hela cells within 24 h, while it was able to significantly increase the cell proliferation inhibition rate of cisplatin (Table 1).

Proliferation inhibition rate by total phenolics and cisplatin docetaxel

Total phenolics and docetaxel exhibited increasing proliferation inhibition rate on Hela cells, and the combination group of the above 2 drugs also showed significantly higher inhibition rate ($P < 0.001$); when the concentration of docetaxel was constant, the inhibition rate of the combined effect increased with the increasing of the total phenolics concentration; when the concentration of total phenolics was constant, the inhibition rate increased with the increasing concentration of docetaxel; when treated with the same drug concentration, the inhibition rate increased with the increasing treating time. When the concentration of total phenolics was lower than 50 $\mu\text{g/ml}$, there was no cytotoxic effect on Hela cells within 24 h, while it was able to significantly increase the cell proliferation inhibition rate of docetaxel (Table 2).

Changes of early apoptosis rate

After 48 h cultivation, the apoptosis rate of total phenolics+cisplatin group was (31.87 \pm 3.82)%, significantly higher than those of total phenolic group (12.80 \pm 0.70)% and cisplatin group (18.87 \pm 3.79)%, and the P were all < 0.05 ; the apoptosis rate of total phenolics+docetaxel group was (36.47 \pm 3.98)%, significantly higher than total phenolic group (12.80 \pm 0.70%) and docetaxel group (15.87 \pm 3.81)%, $P < 0.05$. The apoptosis rates of total phenolics+cisplatin, total phenolics+docetaxel, total phenolics, cisplatin and docetaxel group were all higher than the control group (4.87 \pm 0.65)%, $P < 0.05$.

Bcl-2 and survivin expression

As it could be seen from Table 3, the bcl-2 and survivin expression levels of total phenolics, cisplatin, docetaxel, total phenolics+cisplatin and total phenolics+docetaxel groups were lower than the control group ($P < 0.01$), and bcl-2 and survivin expression of total phenolics+cisplatin and total phenolics+docetaxel group were lower than the monotherapy group ($P < 0.01$).

Discussion

AS is closely related to the occurrence and development of tumors. Modern researches have confirmed that ASMq could scavenge free radicals, preventing hydroxyl radical induced oxidative DNA damage and mitochondrial oxidative damage. ASMq might possess the active ingredients which could act on the apoptosis signaling pathways of cell proliferation and regulate the related gene expression, playing the roles of inducing cells apoptosis, increasing the expression of tumor suppressor gene and apoptosis inhibition gene. Total phenolics is the aqueous extract from ASMq, studies have reported that among the aqueous extracts of ASMq, total phenolics accounts for the highest content, as 38.28 mg/g, significantly higher than the total flavones, which accounted for the 2nd content of ASMq as 16.21 mg/g (Chen et al., 2011). Docetaxel is a newly-developed Taxol-category anticancer drug, exhibiting cytotoxicity towards a variety of human tumors, such as cervical cancer, colon cancer, ovarian cancer, lung

cancer and breast cancer, and could induce tumor cell apoptosis (Bergqvist et al., 2003).

MTT results of this study indicated that cisplatin and docetaxel alone could significantly inhibit the growth of HeLa cells, similar to the previous studies (O'Connor et al., 2013; Pu et al., 2013), and there was a synergistic effect when ASMq total phenolics was applied with the above drugs, which could significantly increase the inhibition rate of tumor cell proliferation, providing a new choice towards the treatment of the cervical cancer patients in the middle and late, recurrence and uncontrolled stages. When kept the concentrations of cisplatin and docetaxel constant, the inhibition rate of ASMq total phenolics towards HeLa cells exhibited a dose-time dependent manner. The study also showed that low concentration of total phenolics (50 $\mu\text{g/ml}$) had no inhibitory effect on HeLa cells, while it could increase the inhibitory effects of the chemotherapy drugs, such as cisplatin and docetaxel, towards the proliferation of HeLa cells, indicating that low concentration of total phenolics might be used as a chemosensitizer for the treatment of cervical cancer in future.

Bcl-2 gene is the suppressor gene of cell apoptosis, playing its anti-apoptotic effects through closing the nucleus transportation, inhibiting the increasing of intracellular calcium and antioxidanting (Vidya Priyadarsini et al., 2010; Gan et al., 2011; Chen et al., 2012; Gupta et al., 2013). It is one of the anti-apoptotic genes which has been studied much more deeply currently. Report revealed that (Yusup et al., 2012) ASMq could induce the expression of wild-type p53 gene and reduce the expression of bcl-2 gene in tumor cells. This study showed that after acted on HeLa cells for 48 h, total phenolics, combined with cisplatin and docetaxel, significantly reduced the Bcl-2 expression levels than those of the control, cisplatin, docetaxel and total phenolics groups, suggesting that the mechanism might be related with the regulation of the Bcl-2 gene expression, the decreased expression of Bcl-2 might release the inhibition effects of Bcl-2 on the tumor cell apoptosis, therefore promoting the apoptosis of cancer cells. The exact mechanism still remained to be elucidated.

Survivin is the most active apoptosis-inhibiting protein, mainly through indirectly or directly inhibiting the activities of caspase-3 and caspase-caspase-7 to play the role of apoptosis. Studies have shown that the expression of survivin gene is closely related to the occurrence and development of cervical cancer, (Chen et al., 2010; Vidya Priyadarsini et al., 2010; Gan et al., 2011; Wu et al., 2012; Gupta et al., 2013; Chen et al., 2013). In this study, it showed that the survivin expression level in total phenolics+cisplatin and total phenolics+docetaxel groups were significantly lower than the control, cisplatin, docetaxel and total phenolic groups, after acted on HeLa cells for 48 h, suggesting that the mechanism might be related to the regulation of survivin expression, through inhibiting the expression of survivin gene to induce the apoptosis of cancer cells, and the specific mechanism still needed deeper study.

In short, ASMq total phenolics had significant inhibition towards HeLa cells, and could improve the sensitivity of HeLa cells to cisplatin and docetaxel. When

the concentrations of cisplatin and docetaxel were kept constant, the inhibition rate of ASMq total phenolics towards HeLa cells exhibited a dose-time dependent manner. ASMq could be used in the combination of cisplatin and docetaxel, which might promote the apoptosis of HeLa cells, and the possible mechanism might be associated with the decreasing expressions of Bcl-2 and survivin. However, it still remained to be further studied that through what means the total phenolics regulated the expressions of Bcl-2 and survivin.

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