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HSP70 inhibitor combined with cisplatin suppresses the cervical cancer proliferation *in vitro* and transplanted tumor growth: An experimental study

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ABSTRACT

Objective: To study the regulating effect of HSP70 inhibitor (PES) combined with cisplatin on cervical cancer proliferation *in vitro* and transplanted tumor growth.
Methods: Cervical cancer Hela cell lines were cultured and divided into control group, cisplatin group, PES group and cisplatin + PES group that were treated with serum-free DMEM, cisplatin with final concentration of 10 μmol/L, PES 20 μmol/L and cisplatin 10 μmol/L combined with PES with 20 μmol/L, respectively; animal models with cervical cancer xenografts were established and divided into control group, cisplatin group, PES group and cisplatin + PES group who received intra-tumor injection of normal saline, 10 μmol/L cisplatin, 20 μmol/L PES as well as 10 μmol/L cisplatin + 20 μmol/L PES, respectively. Cell proliferation activity, transplanted tumor volume and mitochondria apoptosis molecule expression were detected.
Results: Cell viability value and Bcl-2 mRNA expression in cells of cisplatin group, PES group and cisplatin + PES group were significantly lower than those of control group while Bax, Caspase-3 and Caspase-9 mRNA expression in cells were significantly higher than those of control group; transplanted tumor volume and the Bcl-2 mRNA expression in transplanted tumor tissue of cisplatin group, PES group and cisplatin + PES group were significantly lower than those of control group while Bax, Caspase-3 and Caspase-9 mRNA expression in transplanted tumor tissue were significantly higher than those of control group.
Conclusions: HSP70 inhibitor combined with cisplatin can inhibit cervical cancer cell proliferation *in vitro* and transplanted tumor growth through mitochondrial apoptosis pathway.

1. Introduction

Cervical cancer is the most common malignant tumor of the female reproductive system, and an important cause of cancer-related death in women. At present, the pathogenesis of cervical cancer is still not clear, and whether regulatory molecules play a key role in the development and change of cervical cancer also need further research. Heat shock protein (HSP) family is highly conserved protein family in evolution that can act as molecular chaperones to be involved in intracellular peptide chain folding, modification, maturation and transport, and have regulatory effect on intracellular protein structure and function. As an important member of heat shock protein family, heat shock protein 70 (HSP70) can adjust the structure and function of a variety of proteins to promote cell survival and inhibit cell apoptosis [1–3]. Studies have shown that the HSP70 expression significantly increased in cervical cancer tissue [4,5], but there is no report on the effect of HSP70 on cervical cancer cell proliferation *in vitro* and cervical cancer xenograft. In the following study, the regulating effect of HSP70 inhibitor (PES) combined with cisplatin on cervical cancer proliferation *in vitro* and transplanted tumor growth was analyzed.

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2. Materials and methods

2.1. Experimental materials

Cervical cancer Hela cell lines were bought from ATCC Cell Company, DMEM medium, fetal bovine serum and trypsin were purchased from Hyclone Company, MTS cell viability detection kits were bought from Promega Company, and the animal tissue/cell RNA extraction kits (article No: CW0560S), SuperRT cDNA Synthesis kits (article No: CW0741M) and UltraSYBR One Step RT-qPCR kits (article No: CW2624S) were bought from Beijing ComWin Biotechnology Co., Ltd.

2.2. Experimental methods

2.2.1. Cell culture and drug treatment methods

Hela cell lines were recovered and then cultured with DMEM containing 10% fetal bovine serum, the medium was replaced every 2 days, the cells were digested and sub-cultured with 0.25% trypsin after the cell density was higher than 90%, the sub-cultured cells were inoculated in culture plate and treated with drugs, control group were treated with DEME not containing drugs or serum, cisplatin group were treated with cisplatin with final concentration of 10 μmol/L, PES group were treated with PES with final concentration of 20 μmol/L, and cisplatin + PES group were treated with cisplatin with final concentration of 10 μmol/L and PES with final concentration of 20 μmol/L.

2.2.2. Cervical cancer xenograft model establishment and drug intervention methods

Cervical cancer cells with log-phase growth were collected, cell density was adjusted to 1×10^7/mL, 0.2 mL cell suspension was subcutaneously inoculated in the right nape of nude mice. A total of 28 transplanted tumor model mice with tumor diameter more than 5 mm were selected 2 weeks after inoculation, and were divided into control group, cisplatin group, PES group, cisplatin + PES group, with 7 in each group. Control group received intraperitoneal and intra-tumor injection of normal saline, cisplatin group received 0.2 mL intraperitoneal injection of 10 μmol/L cisplatin, PES group received 0.1 mL intra-tumor injection of 20 μmol/L PES, cisplatin + PES group received 0.2 mL intraperitoneal injection of 10 μmol/L cisplatin, and 0.1 mL intra-tumor injection of 20 μmol/L PES.

2.2.3. Cell viability detection methods

The Hela cells for cell viability test were inoculated in the 96-well cell plate. Cell number was 1×10^4 per well, medium volume was 200 μL. MTS cell viability detection liquid 20 μL was added in the medium before treatment and 12 h, 24 h, 36 h and 48 h after treatment with different conditions. Cells continued to be incubated for 4 h in the incubator, and the culture plate was taken out and fully shaken. Then the absorbance value at 450 nm wavelength was determined on the microplate reader, and the absorbance value was used as the cell viability value.

2.2.4. mRNA expression detection methods

Cells for mRNA expression detection were inoculated in 12-well cell plate. The medium was abandoned after treatment with different conditions for 48 h, and the cells were kept and stored in at −80 °C; 30 d after transplanted tumor animal model intervention, the animals were executed and anatomized to get transplanted tumors, rinse them with saline for 2–3 times and then stored at −80 °C. Animal tissue/cell RNA extraction kits were used to extract total RNA from Hela cells and cervical cancer xenograft tissue, and SuperRT cDNA Synthesis kits were used to reverse-transcribe the RNA into cDNA; the cDNA samples were taken, UltraSYBR One Step RT-qPCR kits were used for fluorescence quantitative PCR amplification, the amplified genes included Bcl-2, Bax, Caspase-3, Caspase-9 and GAPDH, the primer sequences and annealing temperature were shown in Table 1, and after amplification curves were obtained, GAPDH was used as reference to calculate Bcl-2, Bax, Caspase-3 and Caspase-9 mRNA expression according to $2^{-\Delta\Delta Ct}$.

2.3. Statistical analysis

SPSS20.0 software was used for to input and analyze data, measurement data analysis among groups was by variance analysis and $P < 0.05$ indicated statistical significance in differences.

3. Results

3.1. Regulating effect of PES combined with cisplatin on cervical cancer cell proliferation activity

At 12 h, 24 h, 36 h and 48 h after treatment with different conditions, analysis of cervical cancer cell proliferation activity was as follows: (1) cell viability was different under the same treatment condition and at different time points after treatment, and cell viability value of cisplatin group, PES group and cisplatin + PES group at 12 h, 24 h, 36 h and 48 h were significantly lower than those at 0 h; cell viability value of

Table 1
PCR primer sequences and annealing temperature.

Gene	Primers	Sequences (5′ → 3′)	Temperature (°C)
Bcl-2	Upstream	AGCTATGCATGCTAGTAGC	58.0
	Downstream	ACGGTTAGCATCAAGTCAGC	
Bax	Upstream	TCGACCAGTGCATAGCTAG	60.0
	Downstream	AATGCTAGCAGCTAGCTAG	
Caspase-3	Upstream	TGCGCATAGCTAGCAGCGC	56.0
	Downstream	TTCATCGTAGCTCGATGCAT	
Caspase-9	Upstream	GCTAAGCTAGCTGAGGCTAG	58.0
	Downstream	AAGGTATGCGATCGTAGCTA	
GAPDH	Upstream	GTCCCGTAGCTAGCCGGTTA	60.0
	Downstream	GTCAGTACGTAGCTAGTTTC	

Table 2
Effect of PES combined with cisplatin on cervical cancer cell proliferation activity and effect of PES on cervical cancer xenograft volume (cm^3).

Groups	PES + cisplatin cell proliferation activity					PES xenograft volume			
	0 h	12 h	24 h	36 h	48 h	0 d	10 d	20 d	30 d
Control	0.84 ± 0.13	1.02 ± 0.13^{a}	1.15 ± 0.15^{ab}	1.22 ± 0.20^{abc}	1.35 ± 0.20^{abcd}	0.15 ± 0.04	0.58 ± 0.09^{e}	0.92 ± 0.17^{eg}	1.37 ± 0.18^{egf}
Cisplatin	0.87 ± 0.15	0.72 ± 0.17^{*a}	0.65 ± 0.16^{*ab}	0.51 ± 0.16^{*abc}	0.44 ± 0.12^{*abdd}	0.17 ± 0.03	0.41 ± 0.06^{*e}	0.68 ± 0.11^{*ef}	0.89 ± 0.14^{*efg}
PES	0.85 ± 0.16	0.76 ± 0.15^{*a}	0.69 ± 0.12^{*ab}	0.58 ± 0.17^{*abc}	0.49 ± 0.16^{*abcd}	0.16 ± 0.02	0.42 ± 0.05^{*e}	0.70 ± 0.12^{*ef}	0.81 ± 0.14^{*efg}
PES + cisplatin	0.88 ± 0.15	0.56 ± 0.16$^{*\&\hat{}a}$	0.47 ± 0.15$^{*\&\hat{}ab}$	0.34 ± 0.15$^{*\&\hat{}abc}$	0.24 ± 0.15$^{*\&\hat{}abcd}$	0.17 ± 0.03	0.25 ± 0.04$^{*\&\hat{}e}$	0.39 ± 0.08$^{*\&\hat{}ef}$	0.47 ± 0.09$^{*\&\hat{}efg}$

$^{*}P < 0.05$ compared with control group; $^{\&}P < 0.05$ compared with cisplatin group; $^{\hat{}}P < 0.05$ compared with PES group.
$^{a}P < 0.05$ compared with same group at 0 h; $^{b}P < 0.05$ compared with same group at 12 h; $^{c}P < 0.05$ compared with same group at 24 h; $^{d}P < 0.05$ compared with same group at 36 h; $^{e}P < 0.05$ compared with same group at 0 d; $^{f}P < 0.05$ compared with same group at 10 d; $^{g}P < 0.05$ compared with same group at 20 d.

cisplatin group, PES group and cisplatin + PES group at 24 h, 36 h and 48 h were significantly lower than those at 12 h; cell viability value of cisplatin group, PES group and cisplatin + PES group at 36 h and 48 h were significantly lower than those at 24 h; cell viability value of cisplatin group, PES group and cisplatin + PES group at 48 h were significantly lower than those at 36 h; (2) cell viability was different at same time point after treatment and under different treatment conditions, cell viability value of cisplatin group, PES group and cisplatin + PES group were significantly lower than those of control group, and cell viability value of cisplatin + PES group were significantly lower than those of cisplatin group and PES group (Table 2).

3.2. Regulating effect of PES combined with cisplatin on mitochondria apoptosis molecule expression in cervical cancer cells

At 48 h after treatment with different conditions, analysis of Bcl-2, Bax, Caspase-3 and Caspase-9 expression in cervical cancer cells was as follows: Bcl-2 mRNA expression in cells of cisplatin group, PES group and cisplatin + PES group were significantly lower than that of control group while Bax, Caspase-3 and Caspase-9 mRNA expression were significantly higher than those of control group; Bcl-2 mRNA expression in cells of cisplatin + PES group was significantly lower than that of cisplatin group and PES group while Bax, Caspase-3 and Caspase-9 mRNA expression were significantly higher than those of cisplatin group and PES group (Table 2).

3.3. Regulating effect of PES on cervical cancer xenograft volume

On 10 d, 20 d and 30 d after treatment with different conditions, analysis of cervical cancer xenograft volume was as follows: (1) cervical cancer xenograft volume was different under same treatment condition and at different time points after treatment, and cervical cancer xenograft volume of cisplatin group, PES group and cisplatin + PES group at 10 d, 20 d and 30 d were bigger than those at 0 d; cervical cancer xenograft volume of cisplatin group, PES group and cisplatin + PES group at 20 d and 30 d were bigger than those at 20 d; cervical cancer xenograft volume of cisplatin group, PES group and cisplatin + PES group at 30 d were bigger than those at 20 d; (2) cervical cancer xenograft volume was different at same time point after treatment and under different treatment conditions, cervical cancer xenograft volume of cisplatin group, PES group and cisplatin + PES group were significantly lower than those of control group, and cervical cancer xenograft volume of cisplatin + PES group were significantly lower than those of cisplatin group and PES group (Table 2).

3.4. Regulating effect of PES on mitochondria apoptosis molecule expression in cervical cancer xenograft tissue

On 30 d after treatment with different conditions, analysis of Bcl-2, Bax, Caspase-3 and Caspase-9 expression in cervical cancer xenograft tissue was as follows: Bcl-2 mRNA expression in cervical cancer xenograft tissue of cisplatin group, PES group and cisplatin + PES group were significantly lower than that of control group while Bax, Caspase-3 and Caspase-9 mRNA expression were significantly higher than those of control group;

Table 3
Effect of PES combined with cisplatin and effect of PES on mitochondria apoptosis molecule expression in cervical cancer cells and effect of PES on mitochondria apoptosis molecule expression in cervical cancer xenograft tissue.

Groups	PES + cisplatin				PES			
	Bcl-2	Bax	Caspase-3	Caspase-9	Bcl-2	Bax	Caspase-3	Caspase-9
Control	1.00 ± 0.22	1.00 ± 0.20	1.00 ± 0.17	1.00 ± 0.18	1.00 ± 0.23	1.00 ± 0.23	1.00 ± 0.16	1.00 ± 0.24
Cisplatin	0.65 ± 0.12*	1.78 ± 0.26*	1.84 ± 0.28*	1.89 ± 0.25*	0.77 ± 0.12*	0.72 ± 0.12*	0.68 ± 0.10*	0.66 ± 0.12*
PES	0.62 ± 0.14*	1.82 ± 0.29*	1.86 ± 0.25*	1.84 ± 0.27*	0.72 ± 0.14*	0.78 ± 0.13*	0.70 ± 0.11*	0.68 ± 0.11*
PES + cisplatin	0.39 ± 0.09*&ˆ	2.74 ± 0.52*&ˆ	2.88 ± 0.42*&ˆ	2.71 ± 0.41*&ˆ	0.40 ± 0.09*&ˆ	0.35 ± 0.08*&ˆ	0.32 ± 0.09*&ˆ	0.37 ± 0.09*&ˆ

*$P < 0.05$ compared with control group; &$P < 0.05$ compared with cisplatin group; ˆ$P < 0.05$ compared with PES group.

Bcl-2 mRNA expression in cervical cancer xenograft tissue of cisplatin + PES group was significantly lower than that of cisplatin group and PES group while Bax, Caspase-3 and Caspase-9 mRNA expression were significantly higher than those of cisplatin group and PES group (Table 3).

4. Discussion

The pathogenesis of cervical cancer and the key molecules that regulate cervical cancer cell proliferation are still not clear, and clinical targeted drugs for the treatment of cervical cancer are also short. Chemotherapy based on cisplatin is still the main mode for treatment of advanced cervical cancer, but the percentage of drug resistance during chemotherapy is high, and the effect of chemotherapy is not ideal. Therefore, exploring the key molecules that regulate cervical cancer cell proliferation can provide theoretical reference for targeted drug research and development. HSP is a family of proteins with the characteristics of molecular chaperones, and it plays an important role in the processes of new peptide chain folding, assembly, modification and transport as well as denatured protein degradation; it can also promote the cellular self-healing during the damage process caused by different external stimuli, and maintain the normal cellular biological function. HSP70 is an important member of the HSP family that can form complexes with various proliferation and apoptosis-related genes and then regulate cell proliferation and apoptosis process [6–9]. Studies have shown that the HSP70 expression significantly increases in cervical cancer tissue and is closely related to the clinical pathological process [4,5]. This means that the occurrence of cervical cancer tissue is associated with high expression of HSP70, and the highly expressed HSP70 may be able to promote the pathological process of cervical cancer by regulating cell proliferation and apoptosis process.

At present, the regulating effect of HSP70 on cervical cancer cell proliferation and apoptosis is still not clear. PES is the highly selective inhibitor of HSP70 that can interact with HSP70 and inhibit its biological function [10,11]. Studies have shown that PES can inhibit the activity of gastric cancer, bladder cancer, thyroid cancer and other malignant tumor cells [12–14], but there is no report about the effect of PES on cervical cancer cell proliferation activity. In the study, in order to define the role of HSP70 in the development and change of cervical cancer as well as the effect of HSP70 inhibitor PES on cervical cancer cell proliferation activity, PES combined with cisplatin was adopted to treat cervical cancer cells and the cell proliferation activity was analyzed. Results showed that cisplatin treatment alone, PES treatment alone and cisplatin + PES treatment could all reduce cervical cancer cell viability in time-dependent manner. Cisplatin + PES treatment has more significant effect on reducing the cell proliferation activity than cisplatin treatment alone and PES treatment alone. On one hand, it can explain that HSP70 inhibition by PES can reduce cervical cancer cell proliferation activity; on the other hand, it also shows that HSP70 inhibition by PES and cisplatin have synergetic effect, and can enhance the killing effect of cisplatin on cervical cancer cells.

In recent years, studies on HSP70 have confirmed that the molecule has regulating effect on the expression of a variety of molecules in mitochondrial apoptosis pathway, can inhibit mitochondrial cell apoptosis, and is conducive to cell proliferation [6,15]. Cytochrome C enters into cytoplasm from mitochondria is the first step of the mitochondrial apoptosis pathway. Cytochrome C that enters into the cytoplasm can form apoptotic bodies with Apaf-1 and Caspase-9, activate Caspase-9, then make Caspase-3 activate through the cascade activation reaction of Caspase-9 and mediate apoptosis [16,17]. Bcl-2/Bax are the key molecules that adjust the cytochrome C release within mitochondria; Bax can increase the permeability of the mitochondrial outer membrane and promote cytochrome C release into the cytoplasm; Bcl-2 can be combined with Bax and then inhibit the cytochrome C release [18,19]. In order to define the effect of HSP70 on mitochondria apoptosis of cervical cancer cells, the expression levels of above mitochondrial apoptosis molecules in cervical cancer cells were analyzed. Result showed that the cisplatin, PES and cisplatin + PES could all increase the Bax, Caspase-3 and Caspase-9 expression and decrease the Bcl-2 expression; and cisplatin + PES had more significant regulating effect on the Bcl-2, Bax, Caspase-3 and Caspase-9 expression than cisplatin and PES treatment alone. This means that HSP70 has regulating effect on the expression of mitochondrial apoptosis-related molecules in cervical cancer cells, HSP70 inhibition by PES can collaborate with cisplatin to play the role of promoting cervical cancer cell apoptosis.

Above cell experiment study shows that HSP70 can regulate cervical cancer cell proliferation and apoptosis through the mitochondrial apoptosis pathway; and targeted inhibition of HSP70 by PES can activate the mitochondrial apoptosis pathway and inhibit cervical cancer cell proliferation. In order to further clarify the effect of targeted inhibition of HSP70 by PES on cervical cancer occurrence and the lesion growth, cervical cancer xenograft animal models were established in the study and received intra-tumor injection of PES and intraperitoneal injection of cisplatin. In order to define the transplanted tumor growth, the transplanted tumor volume was analyzed, and the results showed that cisplatin, PES and cisplatin + PES could all inhibit the cervical cancer xenograft growth; and cisplatin + PES

could more significantly inhibit the transplanted tumor growth than cisplatin and PES treatment alone. This means that PES can not only enhance the killing effect of cisplatin on cervical cancer, but can also collaborate with cisplatin to inhibit the growth of cervical cancer tissue. Based on this, the expression levels of mitochondrial apoptosis molecules in transplanted tumor tissue were also analyzed in the study, the changing trend of mitochondrial apoptosis molecules Bcl-2, Bax, Caspase-3 and Caspase-9 in the transplanted tumors was consistent with that in cervical cancer cells; and HSP70 inhibition by PES has significant promoting effect on the mitochondrial apoptosis of cancer cells during cervical cancer growth.

In conclusion, it is believed that HSP70 is involved in the regulation of cervical cancer cell proliferation and apoptosis, HSP70 inhibitor PES can enhance the killing effect of cisplatin on cervical cancer cells, and PES combined with cisplatin can inhibit cervical cancer cell proliferation *in vitro* and transplanted tumor growth through mitochondrial apoptosis pathway.

Conflict of interest statement

We declare that we have no conflict of interest.

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