RESEARCH ARTICLE

Synergism of Cytotoxicity Effects of Triptolide and Artesunate Combination Treatment in Pancreatic Cancer Cell Lines

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Abstract

Background: Triptolide, extracted from the herb *Tripteryglum wilfordii* Hook.f that has long been used as a natural medicine in China, has attracted much interest for its anti-cancer effects against some kinds of tumours in recent years. Artesunate, extracted from the Chinese herb *Artemisia annua*, has proven to be effective and safe as an anti-malarial drug that possesses anticancer potential. The present study attempted to clarify if triptolide enhances artesunate-induced cytotoxicity in pancreatic cancer cell lines *in vitro* and *in vivo*. Methods: *In vitro*, to test synergic actions, cell viability and apoptosis were analyzed after treatment of pancreatic cancer cell lines with the two agents singly or in combination. The molecular mechanisms of apoptotic effects were also explored using qRT-PCR and Western blotting. *In vivo*, a tumor xenograft model was established in nude mice, for assessment of inhibitory effects of triptolide and artesunate. Results: We could show that the combination of triptolide and artesunate could inhibit pancreatic cancer cell line growth, and induce apoptosis, accompanied by expression of HSP 20 and HSP 27, indicating important roles in the synergic effects. Moreover, tumor growth was decreased with triptolide and artesunate synergy. Conclusion: Our result indicated that triptolide and artesunate in combination at low concentrations can exert synergistic anti-tumor effects in pancreatic cancer cells with potential clinical applications.

Keywords: Triptolide - artesunate - pancreatic cancer cell lines - cytotoxicity - synergism

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Introduction

Pancreatic cancer is a malignant neoplasm originating from transformed cells arising in pancreas, one of the highest fatality rates of all cancers, extremely poor prognosis and resistant to current chemotherapies, therefore, new strategies or reagents to tackle this disease are needed.

Triptolide (TPL) is a diterpenoid triepoxide and the principal active ingredient of Tripterygium wilfordii Hook. f. that has been used as a natural medicine in China for hundreds of years, which has pharmacological and biochemical properties in the treatment of autoimmune diseases such as nephritis and rheumatoid arthritis for centuries (Wang et al., 2012; Huang et al., 2013; Hung et al., 2013). Besides, triptolide is able to potently inhibit the growth of human cancer cells in vitro and prevents tumor growth in vivo via inhibiting cell proliferation and inducing apoptosis (Li et al., 2012; Tao et al., 2012; Chueh et al., 2013). However, triptolide possess both immunosuppressive and antifertility activities through its ability to inhibit the proliferation of both activated monocytes and spermatocytes (Huang et al., 2012; Xiaowen et al., 2012; Zhang et al., 2012).

Artesunate (ART), is a derivative of artemisinin isolated from the traditional Chinese herb Artemisia annua L, has been approved by the Chinese government for the treatment of malaria, especially against cerebral malaria, more recently, scholars had found it has a wide range of biological activities, such as hepatoprotective, antioxidative, anti-inflammatory, antidiabetic, antiallergic, and antibacterial effects (Ma et al., 2011; Jiang et al., 2012; Mao et al., 2012; Zhou et al., 2012). But now, a number of publications have shown that artesunate can inhibit the proliferation of cancer cells and inhibit angiogenesis in vitro and in vivo (Bachmeier et al., 2011; Berdelle et al., 2011; Thanaketpaisarn et al., 2011; Zhao et al., 2011).

In order to reduce the side effects and enhance the efficacy, traditional Chinese medicine (TCM) prescriptions from synergistic combinations of different TCM herbs is alternative strategy for cancer therapy. The present study was designed to determine combined efficacy of triptolide and artesunate on pancreatic cancer cell lines in vitro and in vivo.

Materials and Methods

Cell culture

Human pancreatic cancer cell lines PANC-1, CFPAC-1 and normal pancreatic cell line HPC-Y5, were obtained

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from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained as monolayer cultures in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 μg/ml of streptomycin and 100 U/ml of penicillin at 37 °C in a humidified atmosphere of 5% CO_2.

Drugs and reagents

Artesunate, provided by Guilin South Pharmaceutical Company Limited (purity > 99.0%, Guilin, Guangxi, China). Artesunate was dissolved in 1 ml of 5% sodium bicarbonate to obtain 1 mM stock solution, and then diluted in medium to different concentrations. Triptolide (purity > 99.0%, Institute of Dermatology, Chinese Academy of Medical Sciences, Nanjing, China) was prepared in dimethyl sulfoxide (DMSO) to obtain 1 μM stock solution and then was added in medium at required concentrations for a certain period of time. Methyl–thiazdyldiphenyl–tetrazoliumbromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma (NY, USA). RNase A and propidium iodide (PI) were obtained from Sigma (St. Louis). TransScript First-Strand cDNA Synthesis SuperMix was purchaseed from TransGen Biotech (Trans, Beijing, China). GeneRuler TM 100 bp DNA Ladder and DreamTaqTM Green PCR Master Mix were purchased from Fermentas Company (Fermentas, Shenzhen, China). Primers were synthetized by Sangon Biotech (Sangon, Shanghai, China). total protein extraction kit P1250 (Applygen Technologies Inc., Beijing, China); BCA protein assay kit (Biosynthesis Biotechnology Co., Ltd., Beijing, China); Anti-Hsp20, anti-Hsp27 and anti-Hsp60 antibodies, as well as horseradish peroxidase (HRP)-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit secondary antibodies, were from Santa Cruz Biotechnology (CA, USA). Caspase-3 Assay Kits were purchased from Molecular Probes (Eugene, OR, USA), and Caspase-9 Assay Kits were purchased from BioVision (Mountain View, CA, USA).

Proliferation assay

Exponentially growing cells (PANC-1, CFPAC-1 and HPC-Y5) were seeded into 96-well plates using 1×10^4 cells per well for viability measurements and incubated for 24 h. To examine the proliferation inhibitory effect of Artesunate and Triptolide, Artesunate (50 – 200 μM) and Triptolide (50 – 200 nM) was added to the wells, with a total volume of 200 μL in serum-free medium, and incubated for varying times at 37 °C. Cells treated with medium containing vehicle served as controls. On the day of collection, the cell number was measured at 24, 48 and 72 h using a standard methylthiazolyltetrazolium (MTT)-based assay. Briefly, MTT was added to each well at a working concentration of 1 mg/mL, and the plates were returned to the incubator for 4 h. After this time, the medium was removed by aspiration, 150 μL DMSO was added to each well and the plates were gently agitated for 10 min before measuring the optical density at 540 nm, and then determined using a microplate spectrophotometer (SpectraMax, Molecular Devices, CA, USA) to determine the cell viability. Treatment effects were determined as the percentage of viability compared with untreated cells.

Apoptosis analysis

Approximately 1×10^6 cells (PANC-1, CFPAC-1) were plated in 6-well plates with medium for 24 h. The cells were then treated for a further 48 h with Artesunate (50 μM and 100 μM) and Triptolide (50 nM and 100 nM). Both the foating and adherent cells were collected together for the analysis. Cells were washed with PBS and centrifuged, fixed with 70% (v/v) ice-cold methanol overnight at 4 °C. The fixed cells were collected by centrifugation, washed with PBS, and then resuspended in 100 μl of PBS containing 40 μg/ml RNase A, after being stained 5 μl Annexin V-FITC and 50 μg/ml propidium iodide, cells were placed in dark for 30 min at room temperature, then binding buffer (400 μl) was added to each tube. Analysis was performed on a FACSCalibur fow cytometer (Becton Dickinson, CA, USA) within 1 h of staining using Cellquest and ModFit software.

Caspase activity determination

The activity of caspase-3 and caspase-9 were measured with a colorimetric assay kit (BioBox, Nanjing, China) according to the manufacturer's instruction. The assay is based on the cleavage of the chromogenic substrates, DEVD-pNA and LEHD-pNA, by caspase-3 and caspase-9, respectively. PANC-1 and CFPAC-1 cells (1×10^4) were seeded into 96-well white opaque plates and a corresponding optically clear 96-well plate, and then allowed to adhere overnight. The next day, cells were treated with Artesunate and Triptolide. At the end of the incubation time, Cells were lysed in chilled lysis buffer on ice for 10 min and centrifuged for 5 min at 10,000 rpm. Caspase substrate solution containing the specific peptide substrate was then added to the supernatant and incubated for 2 h at 37 °C before measurement by microplate reader at a wavelength of 405 nm.

RNA isolation and real time RT-PCR analysis

PANC-1 and CFPAC-1 cells were seeded in six-well plates at a density of 3×10^5 cells and were then incubated overnight at 37 °C before treatment. After cells were treated with Artesunate and Triptolide for 48 h, total RNA was prepared using the TRIzol reagent (Tiangen, Beijing, China), according to the manufacturer's instructions. RNA was reverse transcribed into first-strand cDNA using a kit (Tiangen, Beijing, China) following the manufacturer's procedure. The synthesized cDNA was used as a template for polymerase chain reaction (PCR) amplification. Real-time PCR was performed using a Thermal Cycler Dice Real Time PCR System (Takara, Japan). The primers used for SYBR Green real-time RT-PCR were as follows: for Hsp20, sense primer (TCT TTG ACC AGC GCT TCG GC) and antisense primer (AGC AGC ACC GAA AAG TGG CC); for Hsp27, sense primer (CCA GAG CAG AGT CAG CCA GCA T) and antisense primer (CGA AGG TGA CTG GGA TGG TGA); for Hsp60, sense primer (AGT CAA GGC TCC AGG GTT TGG) and antisense primer (TGG CAT CGT CTT TGG TCA CA); and for GAPDH, sense primer (AGC CTC AAG ATC ATC AGC AAT G) and antisense primer (ATG GAC TGT GGT CAT GAG TCC TT). A dissociation curve analysis of Hsp20, Hsp27, Hsp60 and GAPDH showed a single peak. PCRs were

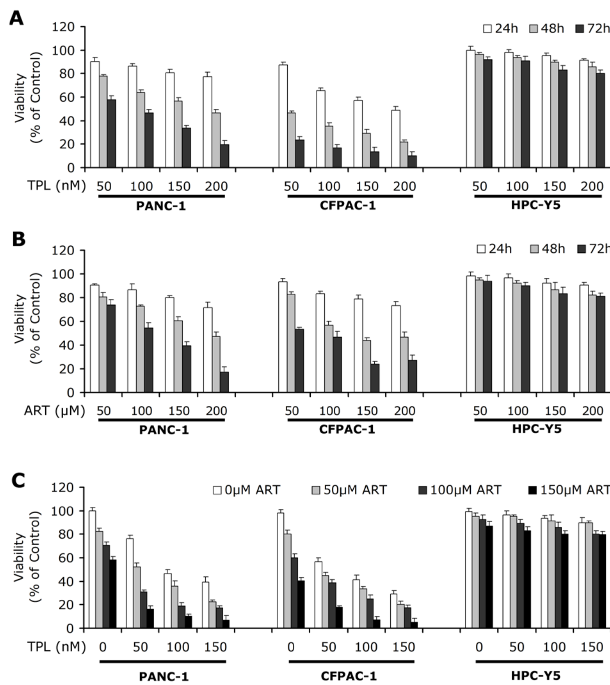

Figure 1. Viability of Triptolide (TPL) and Artesunate (ART) Combination Decreased on PANC-1, CFPAC-1 and HPC-Y5 Cells. The presence of 50 to 200 nM of triptolide. (A) and 50 to 200 μM of artesunate (B) in the culture medium significantly decreased the viability of both PANC-1 and CFPAC-1 cells with incubation time dependent manner. In contrast, little effect was observed on normal pancreatic cell (HPC-Y5) viability. Combined effect (C) of triptolide and artesunate on PANC-1, CFPAC-1 and HPC-Y5 cells for 24 h resulted in significant growth inhibition of pancreatic cancer cells, more than that by either compound alone; the degree of cytotoxicity in normal cells was not significant

carried out for 40 cycles using the following conditions: denaturation at 95 °C for 5 s, annealing at 57 °C for 10 s, and elongation at 72 °C for 20s.The mean Ct of the gene of interest was calculated from triplicate measurements and normalized with the mean Ct of a control gene, GAPDH.

Western blot analysis

PANC-1 and CFPAC-1 cells were seeded in six-well plates at a density of 2.5×10^5 cells and were then incubated overnight at 37 °C before treatment. After cells were treated with artesunate and triptolide for 48 h, the cells were harvested, washed with ice-cold PBS, suspended in 200 μl of ice-cold solubilizing buffer (300 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.6), 0.5% (v/v) Triton X-100, 2 mmol phenylmethanesulfonyl fluoride, 2 μl/ml aprotinin, and 2 μl/ml leupeptin), and incubated at 4 °C for 1 h. The extracts were cleared by centrifugation at 13 000 rpm for 20 min at 4 °C. The concentration of protein was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Protein samples of cell lysate were mixed with an equal volume of 5 × SDS sample buffer, boiled for 4 min, and then separated by 10–12% SDS-PAGE gels and transferred to 0.22 μm polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 0.05 g/ml non-fat dry milk for 1 h, incubated with primary antibody, including Hsp20 (1:200), Hsp27 (1:1000) and Hsp60 (1:1000) and GAPDH (1:1000) over night at 4 °C. After three washes, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h at room temperature, following three times of washing in TBST (Tris-buffered solution, pH 7.6, 0.05% Tween-20), and then developed in ECL-detection reagents, followed by exposure to X-ray film. Expression levels of the proteins were compared to the control based on the relative intensities of the bands.

Animal experiments

Five-week-old male BALB/c athymic nude mice were obtained from Shanghai Laboratory Animal Center of Chinese Academy of Sciences. They were maintained under specific pathogen-free conditions and supplied with sterilized food and water. All the animal experiments complied with guidelines of Animal Care and Use Committee. Tumors were established by the subcutaneous injection of 3 × 106 PANC-1 and CFPAC-1 cells separately into the right armpits of the mice. The mice were inspected for tumor formation twice per week, the length and width of the tumor along with the body weights of the mice were measured using a slide caliper. The tumor volume (V) was calculated according to the empirical equation $V = (\text{length} \times \text{width}^2)/2$. When the tumors reached a mean volume of approximately 100 mm3, the mice were randomly assigned to nine groups (n = 5) and administered the following treatments: mock group (injected intraperitoneally with 100 μl of saline), TPL group one (injected intraperitoneally with 50 μg/kg TPL), TPL group two (injected intraperitoneally with 100 μg/kg TPL), ART group one (injected intraperitoneally with 50 mg/kg ART), ART group two (injected intraperitoneally with 100 mg/kg ART), combination group one (injected intraperitoneally with 50 μg/kg TPL plus 50 mg/kg ART), combination group two (injected intraperitoneally with 50 μg/kg TPL plus 100 mg/kg ART), combination group three (injected intraperitoneally with 100 μg/kg TPL plus 50 mg/kg ART), combination group four (injected intraperitoneally with 100 μg/kg TPL plus 100 mg/kg ART). Each animal received the treatment through gavage once daily for two weeks. The mice were closely monitored, and after 18 d, all mice were anesthetized by ether and sacrificed by neck dissection. The tumors were removed and weighed.

Statistical analysis

All quantitative assays were performed in triplicate. The results are expressed as mean ± standard deviation (SD). Statistical analysis of the difference between treated and untreated groups was performed with Student's t-test. Values of $P < 0.05$ were considered as significant differences.

Results

Triptolide and artesunate enhanced inhibitory effects on pancreatic cancer cells in vitro

The viability of PANC-1 and CFPAC-1 cells treated with triptolide and artesunate was analyzed by MTT

100.0
75.0
50.0
25.0
0
6
56
31

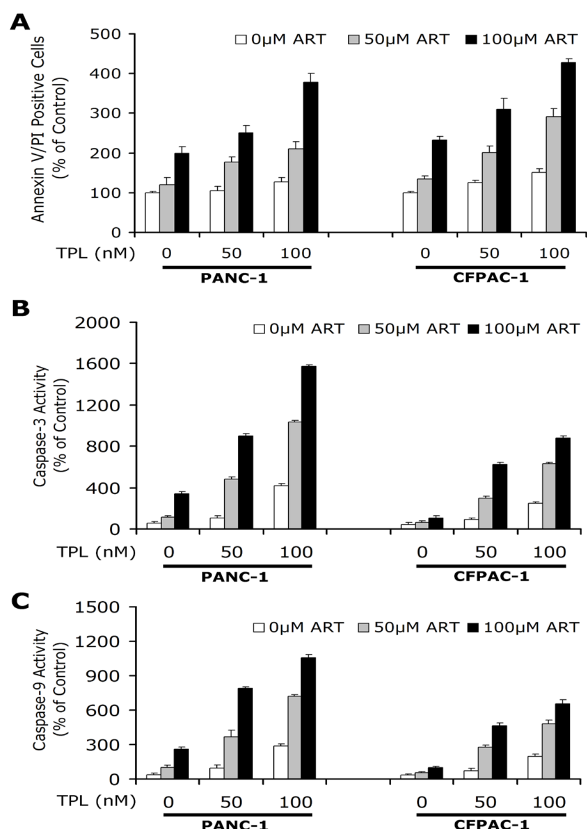

Figure 2. Triptolide and Artesunate Combination Induce PANC-1 and CFPAC-1 Cells Apoptosis and Caspase Activation. The presence of 50 and 100 nM of triptolide and 50 and 100 μM of artesunate in the culture medium for 24 h significantly induced both PANC-1 and CFPAC-1 cells apoptosis (A). Activity of caspase-3 (B) and caspase-9 (C) in PANC-1 and CFPAC-1 cells treated with triptolide and artesunate alone or in combination for 24 h

method. As seen in Figure 1, the growth of PANC-1 and CFPAC-1 cells was significantly inhibited by triptolide (Figure 1A) and artesunate (Figure 1B) in a dose dependent manner. To evaluate the synergistic effect of triptolide and artesunate in combination for subsequent studies, the viability of PANC-1 and CFPAC-1 cells decreased more in combination compared with drug used alone, and no synergistic cytotoxicity was observed in normal cells (Figure 1C).

Induction of apoptosis by triptolide and artesunate combination in pancreatic cancer cells

To further explore the mechanism by which triptolide and artesunate induced cell viability loss, Annexin V/PI staining and caspase-3 and caspase-9 activity assay were used. Apoptotic cells were observed in the combination as is shown in Figure 2A, the proportion of Annexin V/PI positive cells increased in triptolide and artesunate combination. In addition, the effect of triptolide and artesunate combination on the activation of the effector caspase (caspase-3 and caspase-9) was analyzed. A significant activity increase in caspase-3 (Figure 2B) and caspase-9 (Figure 2C) activation was observed after combination treatment. These findings indicate that activation of a caspase-involved apoptotic pathway is one of the major mechanisms via which triptolide

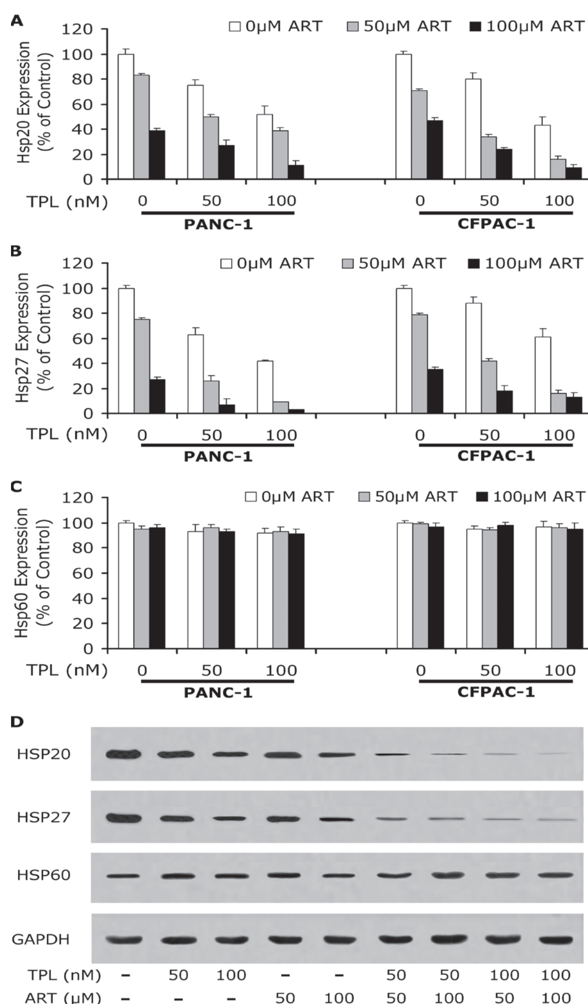

Figure 3. Triptolide and Artesunate Cooperate in Downregulating the Protein Expression of Hsp 20, HSP27 and Hsp60 in Pancreatic Cancer Cells. Hsp 20 (A) and HSP27 (B) mRNA expression (as assessed by real-time PCR) decreased in both PANC-1 and CFPAC-1 cells, however, triptolide and artesunate combination had no effect on Hsp60 (C) mRNA expression. PANC-1 cells were incubated with triptolide and artesunate alone or combination for 24 hours, effect of TPL combined with artesunate on the protein levels of HSP20 and HSP27 (D)

and artesunate combination exerts synergistic effect on pancreatic cancer cells.

Hsp20 and Hsp27 down regulated by tripolide and artesunate combination

Among the heat shock proteins, three chaperones, HSP20, HSP27 and HSP60 were reported as important target in anti-tumor therapy (Guo et al., 2008; Edwards et al., 2011; Hjerpe et al., 2013). We were interested to know how they keep their function in triptolide and artesunate combination. As indicated in Figure 3A and Figure 3B, the mRNA level of HSP20 and HSP27 dropped with triptolide and artesunate combination highly compared with drug used separately, whereas HSP60 mRNA level was unchanged in PANC-1 and CFPAC-1 cells (Figure 3C). From the Western blot analysis, HSP 20 and HSP27 expression decreased (Figure 3D) but HSP 60 and is consistent with their mRNA expression level analyzed by qRT-PCR. Therefore, our results indicate that triptolide

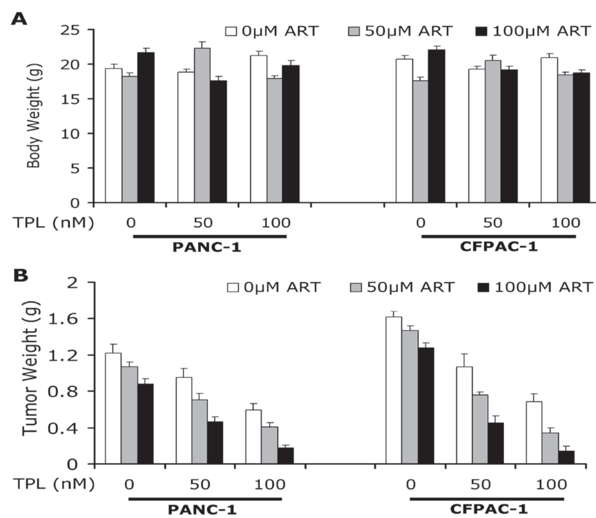

Figure 4. Triptolide and Artesunate Combination Significantly Suppressed the Growth of Pancreatic Tumor in vivo. To investigate the safety of this combination, body weight was assessed before and after treatment (A). Stronger antitumor effects were observed in the mice that were treated with triptolide and artesunate combination, and were indicated by markedly decreased tumor weight (B)

cooperates with artesunate to induce the decline of HSP20 and HSP27 expression via a pharmacological manner, which may be the crucial mechanism underlying the synergic apoptosis on pancreatic cancer cells.

Antitumor effects of triptolide in combination with artesunate: in vivo assay

The antitumor effect of triptolide and artesunate in combination was analyzed in a xenograft tumor model (pancreatic cancer cells bearing mice). After implantation for 7 days, the tumour xenografts reached size of 120 ± 12 mm3. We chose 45 mice with tumour xenografts of around 100 mm3 in size and randomly divided them into four groups (with 5 mice per group). There were no statistical differences among the sizes of all the groups. Thereafter, the mice were given different treatments. No significant change in body weight was observed in the mice treated with triptolide alone, artesunate alone, or combined triptolide and artesunate (Figure 4A). The tumor weight treated by drugs were decreased compared with the saline group, while degree of tumor decreased more in combination group compared with triptolide and artesunate used separately (Figure 4B). These results show that triptolide combined with artesunate have synergic antitumour effect.

Discussion

Pancreatic cancer is highly resistant to current chemotherapy agents. Recently, tumor therapy by traditional Chinese herb is becoming more and more attractive (He et al., 2008; Youns et al., 2009; Chen et al., 2010). We therefore attempted to examine the effects of triptolide and artesunate on pancreatic cancer.

In this study, we shown that triptolide combined with artesunate acts in synergy to inhibit pancreatic cancer cell line growth and induce its apoptosis. HSP20, HSP27 and HSP60 play an important role in carcinogenesis and tumor chemotherapy (Matsushima-Nishiwaki et al., 2007; Noda et al., 2007; Matsushima-Nishiwaki et al., 2008; Cappello et al., 2013). It's over expression is associated to antiapoptosis property and chemotherapy resistance in a variety of tumors including prostate, breast, gastric, and colon cancers. Newly reports have shown that these heat shock proteins may be act as a target for anti-tumor therapy (Li et al., 2008; Du et al., 2010). In the present study, we found that HSP20 and HSP27 declined during combination. That means that the synergetic inhibition induced by triptolide and artesunate combination was more likely due to the proliferation related pathway triggered by HSP20 and HSP27 but HSP60. Moreover, the synergic effect of triptolide and artesunate in vitro was verified in xenografts from PANC-1 and CFPAC-1 cells. In vivo, tumors with combination therapy grow slowly, and the tumor weights were much lighter than the drugs used separately.

In summary, the present study has demonstrated that growth inhibition by triptolide and artesunate combination on pancreatic cancer cells resulted from the induction of apoptosis in vitro, and this cooperation effectiveness is also exert in the pancreatic cancer cell bearing xenograft model in vivo. Our findings suggest that this approach effectively suppresses the growth of pancreatic cancer in vivo and in vitro.

Acknowledgements

The author(s) declare that they have no competing interests.

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