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ORIGINAL ARTICLE

Paclitaxel nanoparticle inhibits growth of ovarian cancer xenografts and enhances lymphatic targeting

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Abstract *Objectives*: Ovarian cancer has the highest mortality of all the gynecologic cancers. The antitumor agent paclitaxel has been proved to be efficient in the treatment of ovarian cancer. Our study is to develop a polymeric drug delivery system for paclitaxel and determine whether paclitaxel nanoparticle can inhibit growth of ovarian carcinoma xenografts in Fisher344 (F344) rats by intraperitoneal administration. The mechanism of paclitaxel nanoparticles in rats bearing ovarian cancer has been investigated in this study. *Methods*: Synthesize paclitaxel loading nanoparticle (PLA) by ultrasonic emulsification; MTT analysis identified cytotoxic activity of paclitaxel nanoparticle in vitro; rat ovarian carcinoma cells were injected into the peritoneal cavity of F344 rats. The antitumor effect of paclitaxel nanoparticle in vivo has been evaluated by measuring tumor weight and ascite volume. At the end of the procedure rats were sacrificed; tumors were excised and processed for PCNA staining, tissue terminal deoxynucleotide transferase-mediated dUTP nick and labeling assay and RT-PCR to evaluate the proliferative and apoptotic changes and cancer transfer-related gene expression induced by PLA. Paclitaxel concentration in plasma, pelvic lymph nodes, liver, and heart were determined by high-performance liquid chromatography. *Results*: Paclitaxel nanoparticle and PTX (Cremophor) showed equivalent cytotoxic activity in vitro. In rats implanted carcinoma cells, paclitaxel nanoparticles significantly reduced tumor weight and ascites volume, and induced apoptosis of tumor cells. PLA also inhibited cell proliferation and matrix metalloproteinase 9 mRNA expression. The paclitaxel concentration of pelvic lymph nodes in PLA treated animals was 20-fold higher than that of free PTX treated animals at 48 h after intraperitoneal administration. *Conclusion*: The intraperitoneal administration of paclitaxel nanoparticle can significantly inhibit the progression of ovarian carcinoma in peritoneal cavity of female F344 rat. The paclitaxel nanoparticle is safe and lymphatic targeting.

Keywords Paclitaxel nanoparticle · Ovarian cancer · Intraperitoneal administration · Lymphatic targeting

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Introduction

Ovarian cancer remains the leading cause of death from gynecological malignancies. One of therapeutic approaches of ovarian cancer is surgical removal followed by systemic chemotherapy to kill malignant cells that may have survived the surgery, and to prevent metastasis and progression of tumor. Despite improved surgical technology and chemotherapy, the long-term prognosis of patients with advanced ovarian cancer has not been markedly improved during the past 10 years [1].

Paclitaxel is one of the most effective anticancer agents known for ovarian cancer. Currently, paclitaxel is marketed commercially in a formulation containing a solvent system of Cremophor and dehydrated ethanol.

However, the amount of Cremophor in paclitaxel per administration is relatively high and has been associated with serious toxicities, including severe hypersensitivity reactions [2, 3]. In order to develop safer formulations, many studies are directed to drug delivery system. The objective of drug delivery system is to maximize therapeutic effect of the drug while minimizing the adverse effect. Biodegradable nanoparticle formulations using poly (lactic-co-glycolic) acid have been first reported by Wang et al. in 1996 [4], and have shown comparable activity to traditional formulations and much faster administration. ABI007 is a Cremophor-free, albumin-bound, nanoparticle paclitaxel (mean diameter 130 nm), which was developed to retain the therapeutic benefits of paclitaxel but eliminate the toxicities associated with Cremophor in the taxol formulation. The maximum tolerated dose of ABI007 was 70–80% higher than that reported for Taxol and a more rapid CL (clearance) and greater V_z (the volume in the terminal state) were observed for ABI007 [5].

While ovarian cancer is confined to the peritoneal cavity, intraperitoneal chemotherapy can achieve dose-intensification on a regional level and systemic toxic effect can be reduced [6]. The main problem with regional antineoplastic therapy is probably the limited depth of penetration of drugs directly into the tumor by free-surface diffusion. To optimize intraperitoneal chemotherapy, Philippe et al. studied the targeting of the lymphatic system with nanoparticles after intraperitoneal administration. The study has demonstrated that there was a dramatic difference between blood and lymph concentrations as well as organ distribution with regard to the administration route. It is possible to target the lymphatic system after intraperitoneal administration [7].

We have studied the therapeutic effect and lymphatic targeting of nanoparticle containing PTX paclitaxel loading nanoparticle (PLA) after intraperitoneal administration to rats bearing ovarian cancer.

Materials and methods

Grant support

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Chemicals and antibody

The transferase-mediated dUTP nick and labeling (TUNEL) kit was purchased from R&D Corporation (USA), the mouse anti-rat PCNA monoclonal antibody was purchased from Serotec Corporation (USA). The free PTX was purchased from Shanghai Hualian Pharmaceutics (China). Acetonitrile and phosphoric acid high-performance liquid chromatography (HPLC grade) were purchased from Merck (USA). The tetrazolium dye 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma (USA). Trizol reagent was purchased from Gibco (USA), and reverse transcriptional kit was from Takara (Japan). The PCR primers were designed and provided by Shanghai Shanggong Company.

PLA, a PTX-incorporating micellar nanoparticle formulation

Paclitaxel loading nanoparticle is a formulation of paclitaxel loaded in PLA polymeric system by liquid-drying procedure. Ten milligrams of paclitaxel powder and 50 mg polylactic acid were dissolved in dichloromethane. The mixture was subsequently emulsificated by ultrasound after surfactant of 5% poly (vinyl alcohol) was added. The mixture was diluted with distilled water and stirred to evaporate the organic solvent. Finally mannitol was added as freeze-dry structure. The size of nanoparticle was detected by photon-relating spectrum. The morphology and microstructure of the nanoparticles were visualized by scanning electron microscope. The encapsulation efficiency was measured by HPLC (Agillent 1100 series, Germany; C_{18} column, 4.6 mm × 250 mm, 5 μm).

Drug-free nanoparticles (vehicle) were prepared according to the same procedure.

Cell culture

NuTu19 cell line is a generous gift from professor He Wang, Sichuan University, China. It was provided by Comprehensive Cancer Center, Michigan University, USA. Cells were maintained in Dulbecco's Modified Eagle Media supplemented with 10% heat-inactivated fetal bovine serum, sodium bicarbonate (0.375%), glutamine (2 mM), non essential amino acids (1 ×), and penicillin-streptomycin (0.01 mg/ml) in an atmosphere of 5% CO_2 (95% humidity, 37°C).

In vitro cytotoxicity

The 3000 NuTu19 cells in 100 μl culture medium were plated in 96-well plates and were incubated for 24 h at 37°C. Then, the cells were treated with various concentrations of PTX or PLA diluted in 100 μl of culture medium (the final concentration of the drugs was 0.2, 2,

20, 200, 2,000 ng/ml). After incubation for 24, 48, or 72 h, 5% MTT 20 μl was added, the plates were further incubated at 37°C for 4 h, and 150 μl DMSO (dimethylsulfone) was added; the absorbance at 490 nm was measured with microplate instrument, the cell growth inhibition rate = $(1 - OD_{experiment}/OD_{control}) \times 100\%$.

Assessment of the antitumor activity of PTX and PLA in vivo

Six to eight-week-old pathogen-free female Fisher344 (F344) rats were purchased from the National Academy of Science in China and housed in a temperature-controlled, light-cycled room.

All animal procedures were performed in compliance with the guidelines for the care and use of experimental animals, which had been drawn up by the committee for Animal Experimentation of the National Cancer Center. All guidelines met the ethical standards required by law and also complied with the guidelines for the use of experimental animals in China.

The antitumor activity of PTX and PLA was evaluated using F344 rats implanted with rat ovarian cancer cell line, NuTu19. To F344 rats aged 6–8 weeks, 3×10^6 of tumor cells were administered intraperitoneally. Four weeks later, when tumor was visible in the abdominal cavity of F344 rats, rats were randomly allocated to the PTX group, PLA group, PLA vehicle group, and control group. Each group contained 15 rats. Each experiment was carried out as follows: free PTX group was administered a dose of 5 mg/kg; PLA group was with the same PTX-equivalent doses; PLA vehicle group was the same doses with PLA group without PTX; and control group was given saline. Rats were administered intraperitoneal injection of PTX or PLA dissolved by 10 ml saline weekly for 5 weeks. At the end of the experiment, the rats were sacrificed, the tumor weight and ascite volume were measured. Body weight changes were also monitored as toxicity effect.

Assessment of tumor apoptosis and proliferation

In situ terminal deoxynucleotide TUNEL was done using the Apoptag Peroxidase In situ Apoptosis Detection kit (R&D). TUNEL-positive cells were quantified in 40 randomly selected high-power fields (200 ×) of each tissue section.

Cyro-section tissue slides were analyzed for PCNA expression using a mouse anti-rat monoclonal antibody (Serotec) according to the manufactures' recommendations. Positive cells were quantified as described above.

Assessment of cancer-related gene expression by RT-PCR

The total RNA was extracted by using Trizol reagent according to the procedural instructions described on the kit. Isolated RNA was reverse-transcribed by MMLV and amplified with RT-PCR system (Takara, Japan). The primer sequences used were as follows:

Matrix metalloproteinase 9 (MMP-9) forward (5′-AGCGAGACACTAAAGGCCAT-3′),

MMP-9 reverse (5′-TGCAGGACAAATAGGAGCGT-3′);

β-actin forward (5′-GATGACGATATCGCTGCGCT-3′),

β-actin reverse (5′-ATAGCTCTTCTCCAGGGAGG-3′);

TIMP-1 forward (5′-CCACCTTATACCAGCGTTATG-3′),

TIMP-1 reverse (5′-GGCAAAGTGATCGCTCTG-3′).

Amplification was conducted in 25-μl reactions, each containing cDNA 1 μl, primer forward and reverse 1 μl each, ddH_2O 9.5 μl, PCR mixture 12.5 μl. The cycling conditions included an initial incubation at 94°C for 5 min, followed by 36 cycles of 45 s at 94°C, 50 s at 56°C, and 1 min at 72°C. The final extension was for 10 min at 72°C. The PCR products were 632 bp for MMP-9, 443 bp for β-actin, and 383 bp for TIMP-1. RT-PCR products were separated on 1% agarose gels.

Assessment of tissue distribution of PTX and PLA by HPLC

Three animals were chosen from each group, 4, 8, 12, 24, 48 h after final administration (5 mg/kg). At least 500 μl blood was collected into a heparinized vacutainer tube from the tail vein. The blood was centrifuged to separate cells from plasma; samples were frozen at −20°C. The following tissues were harvested: heart, liver, tumor, pelvic lymph node.

A plasma sample was treated with 6 ml acetonitrile and drug was extracted on a vortex mixer. After centrifugation, the organic layer was transferred to another clean test tube and evaporated, and the residue was analyzed by HPLC following experimental conditions: Diamonsil™-C_{18} (4.6 mm × 250 mm, 5 μm), the column temperature was maintained at 35°C. The mobile phase was 75:25 (methanol:water) at flow rate of 1 ml/min. The effluent was detected at 233 nm and the area under the peak was used for quantification.

All tissue samples were accurately weighed and homogenized in 5 ml acetonitrile. The organic layer was treated as a plasma sample after centrifugation.

The residue was redissolved in 1 ml of mobile phase and filtered for injection into the HPLC system.

Statistical analysis

The data were expressed as mean ± SD, statistical analysis was performed with ANOVA using SPSS 11.5 software ($P < 0.05$ as significant difference).

Results

Characterization of paclitaxel-loaded nanoparticles

Particle size distribution was determined by photon-relating spectrum. As shown in Fig. 1, the mean diameter was about 200 nm, which was confirmed by scanning electron microscopy. The encapsulation efficiency was 70%, which was determined by HPLC.

In vitro cytotoxicity

The IC_{50} value of PLA was similar to that of PTX, indicating that both PLA and PTX showed equivalent cytotoxic activity in vitro (Fig. 2). The antitumor effect reached the peak at 24 h. There was no difference at 48 and 72 h.

In vivo antitumor effect

F344 rats bearing ovarian cancer showed decreased tumor weight and ascites after the administration of PTX and PLA (5 mg/kg, $q^w \times 5$). However, PLA exhibited superior antitumor activity as compared with PTX ($P < 0.01$). No obvious body weight loss was observed during the process (Table 1).

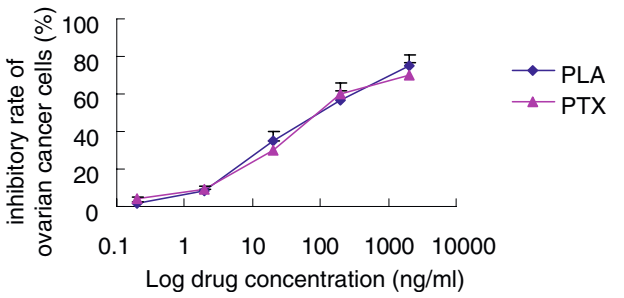

Fig. 2 The paclitaxel loading nanoparticle revealed similar in vitro antitumor effect with PTX

Table 1 Therapeutic effect for ovarian cancer in F344 rats xenografts (mean ± SD)

Experiment group	Tumor weight (g)	Ascite volume (ml)	*P*
Control	15.65 ± 0.80	85.30 ± 3.50	
Vehicle	15.85 ± 0.75	86.55 ± 2.50	
PLA	4.55 ± 0.11	3.55 ± 0.50	<0.001
PTX	10.13 ± 0.52	30.45 ± 1.55	<0.001

Control (saline), vehicle (drug-free nanoparticle), PLA (paclitaxel nanoparticle), PTX (free paclitaxel). The dose is 5 mg/kg, and the drug was given weekly for 5 weeks

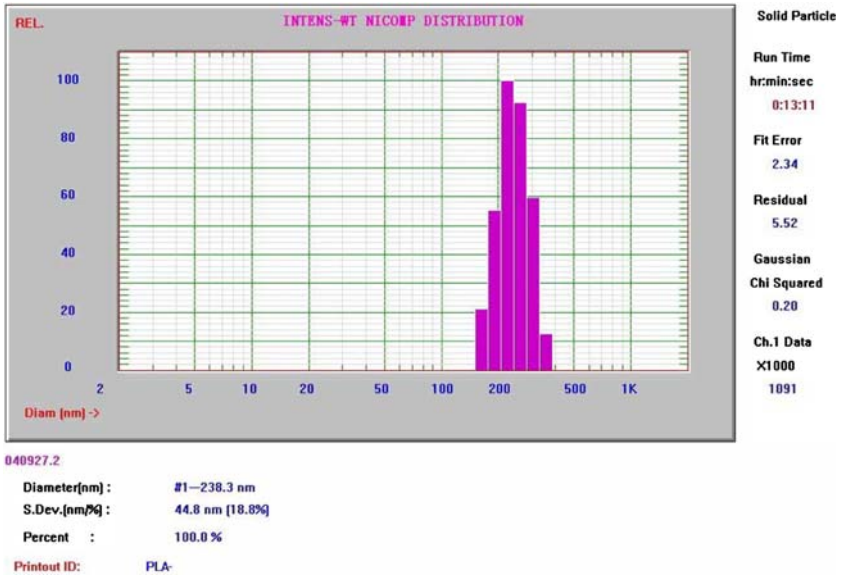

Fig. 1 The particle size distribution of paclitaxel loading nanoparticle determined by photon-relating spectrum

Table 2 PCNA expression and apoptotic analysis of tumors (mean ± SD)

Experiment group	PCNA+	TUNEL+
Control	110 ± 15	10 ± 3
Vehicle	107 ± 14	11 ± 5
PLA	20 ± 5	105 ± 15
PTX	85 ± 10	55 ± 10

Effect of tumor cell proliferation and apoptosis

Both PLA and PTX treatment increased apoptosis, however, treatment with PLA significantly increased the number of TUNEL positive tumor cells from 55 ± 10 in the PTX group to 105 ± 15 ($P < 0.01$). Similarly, PCNA was most significantly reduced in tumors received PLA vs PTX ($P < 0.01$). The results are shown in Table 2 and Fig. 3.

Analysis of cancer-related gene expression

Semi-quantitative RT-PCR analysis revealed that the mRNAs expression of MMP-9 in PTX group and PLA group were down-regulated when compared with the control. However, the PLA treated group significantly down-regulated MMP-9 levels ($P < 0.01$). In contrast, the TIMP-1 levels were significantly up-regulated in PLA treated group ($P < 0.01$). Table 3 and Fig. 4 showed the value of MMP-9 and TIMP-1 mRNA expression in tumors.

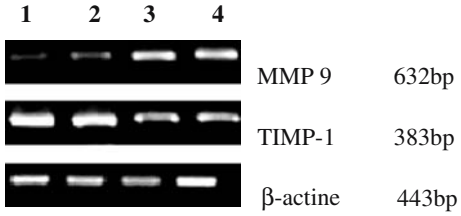

Fig. 4 The mRNA expression of matrix metalloproteinase 9 (MMP-9) and TIMP-1: *1* paclitaxel loading nanoparticle group, the mRNA expression of MMP-9 was significantly down-regulated while the expression of TIMP-1 was up-regulated; *2* PTX group; *3* control group; *4* vehicle group

Table 3 Matrix metalloproteinase 9 (MMP-9) and TIMP-1 mRNA expression in tumors (mean ± SD)

Treatment group	MMP-9	TIMP-1
Control	2.85 ± 0.25	0.57 ± 0.11
Vehicle	2.95 ± 0.27	0.55 ± 0.15
PLA	0.85 ± 0.15	1.55 ± 0.18
PTX	1.75 ± 0.20	0.88 ± 0.12

Analysis of tissue distribution

In PLA group, the mean drug concentration (paclitaxel) in the tumor and pelvic lymph nodes increased with the time after administration and reached the highest at 48 h after administration. However, in the

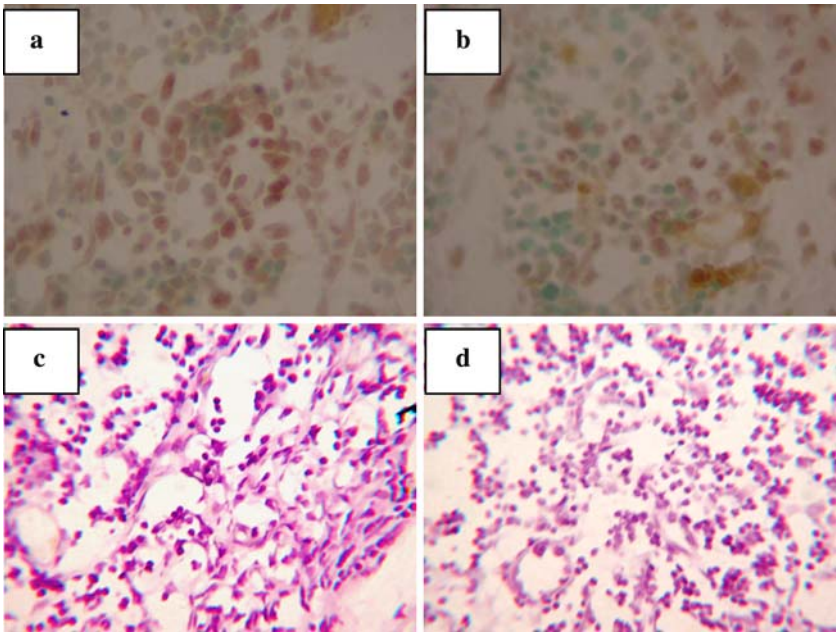

Fig. 3 Effect of paclitaxel loading nanoparticle (PLA) and PTX on tumor apoptosis and proliferation: **a** apoptosis of PLA group, positive cells were shown *brown* (200 ×); **b** apoptosis of PTX group, positive cells were shown *brown* (200 ×); **c** PCNA expression of PLA group, positive cells were shown *blue* (200 ×); **d** PCNA expression of PTX group, positive cells were shown *blue* (200 ×)

PTX group, the mean concentration reached the highest at 12 h in tumor while at 24 h in pelvic lymph nodes. As shown in Fig. 5, the differences in the plasma and tissue concentrations at 48 h after administration were statistically significant in the plasma ($P < 0.001$), tumor ($P < 0.005$), lymph nodes ($P < 0.001$), liver ($P < 0.01$), and heart ($P < 0.01$). Figures 6 and 7 revealed the paclitaxel concentration vs time after intraperitoneal administration in lymph nodes and tumor tissue, respectively. The paclitaxel concentration in plasma, as shown in Fig. 8, in the PLA group, it reached the peak at 48 h after administration, while it reached the peak at 24 h after administration in PTX group. The relative C_{max} in PLA and PTX was 0.457 ± 0.045 μg/g vs 0.348 ± 0.032 μg/g ($P < 0.05$).

Discussion

Ovarian cancer has poor prognosis, due to local distribution in the abdominal cavity and lymph system

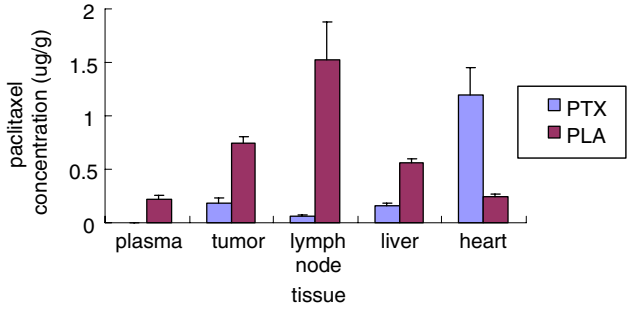

Fig. 5 Comparison of paclitaxel concentration in all tissues collected at 48 h after paclitaxel loading nanoparticle and PTX intraperitoneal administration at 5 mg/kg

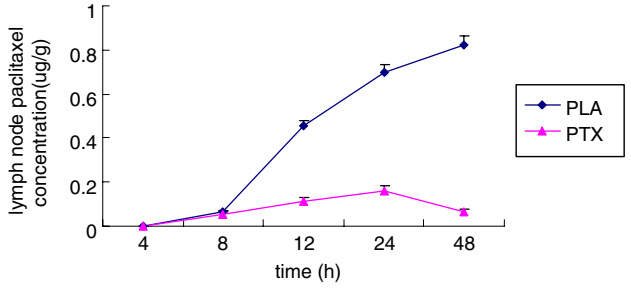

Fig. 6 Lymph nodes paclitaxel concentration vs time after intraperitoneal administration at 5 mg/kg paclitaxel loading nanoparticle (PLA) and PTX. The drug concentration of lymph nodes in PLA group increased with time after administration and reached the peak at 48 h. While the drug concentration in PTX group reached the peak at 24 h. The drug concentrations in lymph nodes have significant difference between the two groups at 48 h after intraperitoneal administration ($P < 0.01$)

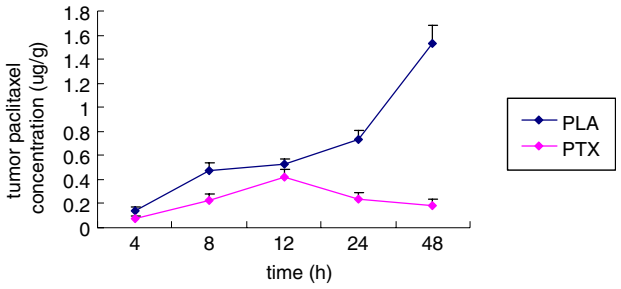

Fig. 7 Tumor paclitaxel concentration vs time after intraperitoneal administration of 5 mg/kg paclitaxel loading nanoparticle (PLA) and PTX. The drug concentration of tumor in PLA group improved with time and reached the peak at 48 h after administration. While the drug concentration in PTX group reached the peak at 12 h after administration. The drug concentrations in tumor have significant difference between the two groups at 48 h after intraperitoneal administration ($P < 0.01$)

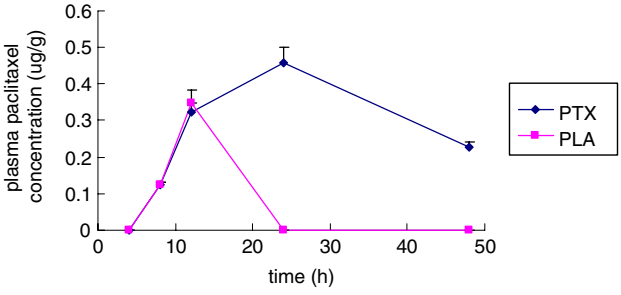

Fig. 8 Plasma paclitaxel concentration vs time after intraperitoneal administration of 5 mg/kg paclitaxel loading nanoparticle (PLA) and PTX. The drug concentration of plasma in PLA group reached the peak at 24 h after administration. While the drug concentration in PTX group reached the peak at 12 h after administration. The drug concentrations in tumor have significant difference between the two groups at 24 h after intraperitoneal administration ($P < 0.001$)

metastasis. To enhance the chemotherapy effect after surgery, intraperitoneal chemotherapy has been applied. Previous studies demonstrated that paclitaxel exhibited a profound pharmacokinetic advantage after regional delivery (1,000 times higher than systemic therapy) [8, 9]. Studies have revealed that paclitaxel nanoparticles could provide increased therapeutic benefit by delivering more drug to solid tumors in vivo and more efficient cellular uptake in vitro [10–13].

In the present study, we have shown for the first time that the synthetic paclitaxel nanoparticle PLA significantly inhibited the growth of ovarian cancer in vivo by intraperitoneal administration with less toxicity. We also showed that PLA and PTX had the equivalent antitumor effect in vitro, which was consistent with the result of Hamaguchi and Matsumura [14]. The significantly increased apoptosis and decreased proliferation of tumors in PLA treated group makes it reasonable to

conclude that both increased cell apoptosis and decreased cell proliferation contribute to the antitumor activity of PLA in vivo.

Matrix metalloproteinase 9 is an important collagenase contributing to the digestion of collagen type IV and has been shown to be related to tumor progression. TIMP-1 is the natural inhibitor of MMP. In this experiment, MMP-9 mRNA expression of tumors in PLA group is significantly weaker than that of PTX group, while the TIMP-1 mRNA expression is much higher. This result suggests that PLA suppress metastasis of ovarian cancer by regulating cancer metastasis-related gene expression.

In this study, drug concentration in PLA group varied in plasma and different tissues after intraperitoneal administration compared with PTX group. The drug concentration in plasma became detectable at 8 h after administration both in PLA and PTX treated group. In the PLA group, the drug concentration in plasma increased gradually with time and reached the peak at 24 h and was detectable at 48 h. While in the PTX group, the drug concentration in plasma reached the peak at 12 h and became undetectable at 24 h. The drug concentration in heart tissue was much lower in PLA group than in PTX group, which may result in less toxicity of PLA. The concentrations in tumors and lymph nodes were significantly higher in PLA group than in PTX group, and reached the peak at 48 h after administration. The liver targeting of PLA could be explained by the MPS (macrophage system) targeting feature of nanoparticles and could be related to lymphatic absorption by the right-side lymph duct [15]. The finding of lymph system targeting is not consistent with the results reported by Philippe et al. [7]. It may be due to the difference of lymph structure between healthy rats and rats bearing cancer.

There are several possible reasons why PLA exhibited stronger antitumor activity compared with PTX containing Cremophor EL and ethanol in present study: (1) The greater accumulation in pelvic lymph nodes, which may interrupt the lymphatic system metastasis of ovarian cancer more effectively; (2) The longer circulation in blood makes it accumulate more in tumor tissues due to the enhanced permeation and retention effect; (3) The polymeric carrier system of PLA has the potential to allow the effective sustained release of the drug inside the tumor and lymph nodes following the accumulation of drugs into the tissues. Regarding the toxicity profile, the repeated administration of PLA to rats at 7-day intervals produced no severe weight loss or death.

In conclusion, PLA could be an attractive candidate for further evaluation by intraperitoneal administration, due to the lack of cumulative toxicity and encouraging tumor responses observed in this study.

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