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ORIGINAL ARTICLE

Comparison between sonodynamic effect with protoporphyrin IX and hematoporphyrin on sarcoma 180

QuanHong Liu · XiaoBing Wang · Pan Wang · LiNa Xiao · Qiao Hao

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Abstract
Purpose The comparison between sonodynamic anti-tumor effect with protoporphyrin IX (PPIX) and hematoporphyrin (Hp) at a concentration of 5 mg/kg on Sarcoma 180 (S180) cells was studied in vivo, and the potential cell damage mechanism was also investigated.
Methods The sonodynamically induced anti-tumor effect of PPIX was studied in mice bearing S180 solid tumors. In order to determine the optimum timing of ultrasound exposure after administration of PPIX, the PPIX concentrations in plasma, skin, muscle and tumor were determined by the fluorescence intensity of tissue extractions with a fluorescence spectrophotometer based on the standard curve. Anti-tumor effects were estimated by measuring the tumor size and the tumor weight. Additionally, the morphological changes of S180 cells were evaluated by transmission electron microscope (TEM) observation immediately after sonodynamic therapy (SDT) treatment.
Results A time of 24 h after the intravenous administration of PPIX was chosen as the best time for ultrasound exposure. The antitumor effect induced by PPIX mediated sonodynamic therapy (PPIX-SDT) was in a dose dependent manner when ultrasound intensity was at or above the inertial cavitation threshold (5 W/cm^2). A significant tumor growth delay was observed both in PPIX mediated sonodynamic therapy and in Hp mediated sonodynamic therapy treatments (Hp-SDT), and the tumor weight inhibition ratios after the synergistic treatments were 42.82 ± 0.03 and $35.22 \pm 0.03\%$, respectively, this difference was significant at $P < 0.05$. While ultrasound alone (5 W/cm^2) showed a slight tumor growth inhibitory effect compared with the control group, and PPIX or Hp alone showed almost no significant effect. Furthermore, TEM observation indicated cell damage was more serious in PPIX-SDT treatment group than in Hp-SDT treatment group. After sonication, the cell ultra-structure such as cell membrane destruction, mitochondria swelling, chromatin condensation might be important factors that inhibited the tumor growth and even induced cell death.
Conclusions The comparative results suggested that PPIX as a sonosensitizer might have more potential cytotoxicity than Hp when irradiated with ultrasound, and the ultra-structural changes may account for cell destruction induced by sonodynamic therapy in our experiment mode.

Keywords Sonodynamic therapy · Anti-tumor effect · Protoporphyrin IX · Hematoporphyrin · Sarcoma 180

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Introduction

Sonodynamic therapy (SDT) is a relatively new approach for cancer treatment, which involves the systemic administration of a sonosensitizer, then followed by local activation by ultrasound exposure to induce tissue or cell destruction [1]. A series of in vivo and in vitro experiments have demonstrated that ultrasound can activate certain porphyrins and thereby induce significant anti-tumor effects, while in which either the ultrasound or the porphyrins alone had no or very low

cytotoxicity [2, 3]. Ultrasound, especially focused ultrasound, could be precisely focused on the target volume, which made it possible to effectively activate the cytotoxicity of sonosensitizers that preferentially accumulate in tumor sites while with minimal damage to peripheral healthy tissues, this indicates that SDT has potential value for cancer therapy [4, 5].

The sonosensitizers most commonly used are hematoporphyrin (Hp) and their derivatives. Protoporphyrin IX (PPIX) is as an effective compound of hematoporphyrin derivatives, which was found preferentially accumulated in rapid proliferating cancer cells, thus providing selective destruction of tumors exposed to ultrasound. Concentration of PPIX in tissue or cell could be increased not only by administration of PPIX itself but also by administration its precursors such as δ-aminolevulinic acid (5-ALA) or 5-ALA derivatives [6–8]. The effect of exogenously providing ALA to induce PPIX accumulation in cell is based on the bypass of negative feedback controls in the heme biosynthetic pathway this phenomenon is exaggerated in malignant tumor cells, since ferrochelatase which is enzyme to convert ALA to PPIX is lower in tumor cells than in the tumor cells [9–11]. The mechanisms of 5-ALA mediated photodynamic therapy (PDT) at the cellular and tissue levels have been thoroughly investigated in the past decade [12–15], and found that endogenous PPIX is almost completely cleared from the body within 24 h, such rapid clearance reduced the risk of a general photosensitization for longer periods of time [16–18]. However, there are few reports about exogenous PPIX by administration protoporphyrin IX itself in PDT or SDT. Kinoshita [19] reported that the exogenous PPIX might have more advantages to cause cytotoxicity than endogenous PPIX in SDT, which can be considered as an effective sono-sensisizer in SDT.

Previously, the in vitro ultrasound induced cell killing effects of PPIX have been briefly reported in our experiment system [20], and our result was in agreement with the Umemura's [21] report. Hp mediated sensitization to ultrasound of S180 cells has been well studied in vivo and in vitro experiments, and the accumulation of Hp in the tumor has been reported in a variety of animals [1, 22, 30]. However, the sonodynamic effect of PPIX on solid tumors have not been studied in details so far, so it is very important to know whether PPIX shows significant in vivo anti-tumor effects in solid tumors in combination with ultrasound. In this study, we compared the antitumor effects between PPIX-SDT and Hp-SDT treatments on sarcoma 180 in vivo, with ultrasound at a frequency of 2.2 MHz in a standing wave mode. In order to determine the optimum time for ultrasonic irradiation of the tumor, the pharmacokinetics of PPIX in mice bearing sarcoma 180 tumor cells was firstly investigated, and the time courses of PPIX concentrations in plasma, tumor, muscle and skin by the fluorescence intensity of tissues extractions with a fluorescence spectrophotometer based on the standard curve. In order to destroy target malignant tissue and reduce the poisoning of normal cells, insonation was administrated at the specific time, when the ratio of concentration of the PPIX in the tumor to that in plasma reached a maximum. Then, the anti-tumor effects were estimated by measuring the tumor size and calculating the tumor weight inhibition ratios 10 days after PPIX-SDT and Hp-SDT treatments. Additionally, the morphological changes of S180 cells were evaluated by transmission electron microscope (TEM) observation immediately after different treatments. At last we evaluated the potential mechanism about the cell damage effect of sonodynamic therapy with PPIX and Hp in vivo in our experiment system.

Materials and methods

Chemicals

Protoporphyrin IX disodium salt, Hematoporphyrin, were purchased from Sigma chemical company (St. Louis, MO, USA) and had a purity of 94 and 60% (HPLC), respectively. Their chemical structures were shown in Fig. 1. All other reagents were commercial products of analytical grade.

Tumor cells and animals

Sarcoma 180 cells and ICR mice were supplied by the Experimental Animal Center of Shaanxi, Chinese Traditional Medicine Institution. The cell line was passaged weekly through ICR mice with a body weight 18–22 g in the form of ascites. Cells were harvested from the peritoned cavity of a tumor-bearing mouse 7–10 days after inoculation. S180 cells were suspended in an air-saturated physiological saline solution at a concentration of 1×10^7 cells/ml, and 0.1 ml aliquots were subcutaneously inoculated into the left oxter region of ICR mice (18–22 g). The experiment was begun when the tumor size reached an average diameter of 6–8 mm, at 7–10 days after inoculation. The experiment animals were treated according to the standards supported by the animal protection committee of China.

Estimate of PPIX concentrations in plasma and tissue

PPIX was dissolved in a sterilized saline solution and administered to tumor bearing mice at a dose of 5 mg/kg

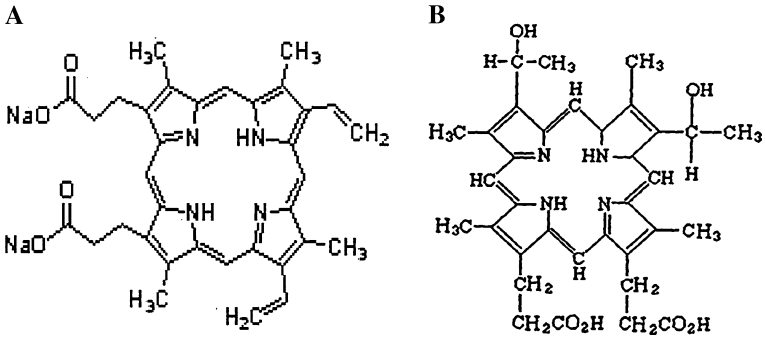


Fig. 1 Chemical structure of **a** protoporphyrin IX and **b** hematoporphyrin

by intravenous injection into the caudal vein. At each time point (0, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 48, 72 h after administration of PPIX), the blood samples were obtained by removing the eyeballs of the tumor-bearing mice. Immediately after sampling, the blood was placed into a 1 ml heparin-coated eppendorf tube, and centrifuged (3,000 rpm, 30 min) to separate the plasma. The animals were killed 2, 4, 6, 8, 10, 12, 24, 48, 72 h after injection by cervical dislocation. The tumor tissues and other tissues were excised immediately, rinsed in physiological saline solution, blotted dry, and weighed. Samples were stored at −20°C in the dark until needed. Plasma and tissue samples were taken from the same animals. Plasma (0.2 ml) was mixed with 2 ml 0.85% physiological saline solution, a portion of tissue (0.2 g) was homogenized in 2 ml of the same buffer in a tissue grinder, then the sample was extracted with 4 ml of ethylacetate: glacial acetic acid mixture (4:1, v/v). The suspension was centrifuged for 10 min (4,000 rpm), then, the supernatant was taken and mixed with 4 ml 0.5 M hydrochloric acid (HCL) for extraction, two layers with ethylacetate rich and HCL rich phases resulted. After a second centrifugation for 10 min (4,000 rpm), the upper ethylacetate layer could then be removed, leaving PPIX dissolved in the lower HCL phase. The PPIX concentration in the solution was determined by measuring the fluorescence intensity using a fluorescence spectrophotometer (F-2500, Hitachi, Japan) at an excitation wavelength of 405 nm and emission wavelength of 625 nm [23]. The background autofluorescence intensity in the test samples was subtracted from the PPIX fluorescence determined in the corresponding tissues digests in control group mice.

A standard curve was obtained by adding known gradient concentrations of PPIX to corresponding tissue digests prepared from untreated animals.

Ultrasonic exposure system

The experimental device for insonation was shown in Fig. 2. An ultrasound transducer with a diameter of 15 mm and a focal length of 22 mm was horizontally

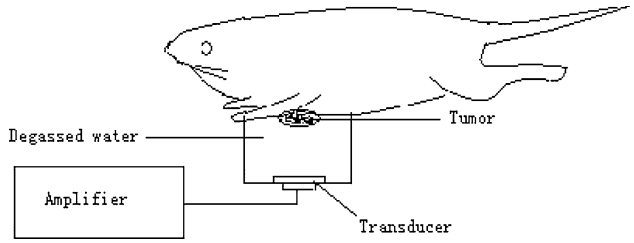


Fig. 2 Ultrasound exposure set-up

submerged in the bottom of a glass container filled with degassed water. Under anesthesia with pentobarbital, a mouse with its left oxter transplanted tumor was fixed in a rotating holder and placed into the focal spot, so that the focused ultrasound could penetrate through the tumor and the skin without any other acoustic interference. The purpose of this rotation was maintained the constant insonation conditions.

The ultrasound transducer was manufactured by the Institution of Applied Acoustics, Shaanxi Normal University. The same transducer was used for all the experiment, with a resonant frequency of 2.2 MHz in a standing wave mode, and it was used to convert the electrical power measured by the amplifier (T&C Power Conversion Inc., USA) into acoustic power. We used the reading output power from the amplifier representing our spatial average ultrasound intensity in our experiment system.

Treatmental protocol

Experimental tumor treatment was performed using PPIX or Hp in combination with focused ultrasound. The tumor bearing mice were divided into six groups: (1) the control group, and those treated with (2) Hp alone, (3) PPIX alone, (4) ultrasound alone, (5) ultrasound plus Hp, and (6) ultrasound plus PPIX. Each group had 8 mice. PPIX or Hp was injected to mice through the caudal vein. For ultrasound treatment, the mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Prior to ultrasound applications, to

minimize cavitation events and interference at the tumor surface, the hair over the tumor regions was shaved using an animal shaver, treated with depilatory cream, washed with liquid soap, and rinsed. Then, the naked skin was partially immersed into the water bath, and the tumor was exposed to the focused ultrasound for 3 min, at the frequency of 2.2 MHz in a standing wave mode. For the combined treatment groups, the tumor was exposed to ultrasound 24 h after PPIX or Hp administration at a concentration of 5 mg/kg. For all experiments, the cold degassed water was used as ultrasonic medium that reduced thermal effect caused by ultrasound irradiation, the temperature within the tumor was checked and found this level of temperature rise is not likely to induce thermal damage of tissues during such a short period of irradiation time.

Evaluation of anti-tumor effect

The therapeutic response was evaluated by observing tumor growth and comparing it to the control group after SDT treatment. The long (a) and short (b) diameters of the tumors were measured with a slide caliper every day after treatment. Tumor size was calculated as $(a + b)/2$. Ten days after the treatment, the mice were killed and the tumors were dissected out and weighted, the inhibition ratio was calculated as follows: (1−average tumor weight of treated group/average tumor weight of the control group) × 100. The mean and standard error (SE) were calculated for each group. The value were compared by Student's *t*-test with $P < 0.05$ as the minimum level of significance.

Transmission electron microscope observation

The tumor samples were collected immediately after PPIX-SDT and Hp-SDT treatments, cut the tumor tissues in pieces of about 1 mm^3, put the pieces in a small glass bottle, then the samples were washed with PBS and fixed in 2.5% glutaraldehyde for 1 h at 4°C, followed by post-fixation for 1 h at 4°C in 1% Osmium tetroxide (OsO_4). After washing by PBS, the samples were dehydrated by graded alcohol, embedded with Epon812 and ultrathin sectioned. The sections were stained with uranium acetate and lead citrate, and examined under a TEM (H-600, Hitachi, Japan).

Results

According to the fluorescence intensity of known gradient concentration of PPIX, the standard curve was plotted in Fig. 3, and the relative analysis equation was:

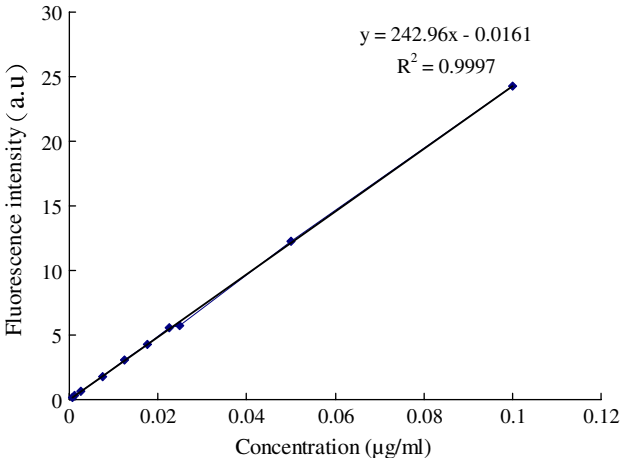


Fig. 3 Standard curve of PPIX

$y = 242.96x - 0.0161$ (R^2=0.9997, $P < 0.05$). Based on the standard curve of PPIX, the concentration changes of PPIX after injection in the tissues could be estimated by the fluorescence intensity. The concentration of PPIX in the plasma after intravenous administration was shown in Fig. 4. The data indicated that after administration, the concentration of PPIX in the plasma decreased rapidly reaching a minimum at 4 h that was maintained for up to 72 h. The time courses of PPIX concentrations in the tumor, in the skin, and in the muscle were shown in Fig. 5. The PPIX concentration in the tumor was significantly higher than in the skin and in the muscle through out the experiment. In the tumor, there was an obviously rising for the first few hours, which then peaked at 10 h, and was 2.6 times higher than in the muscle, but there were moderate PPIX concentrations in the skin for up to 10 h. Therefore, from a pharmacological respective, the

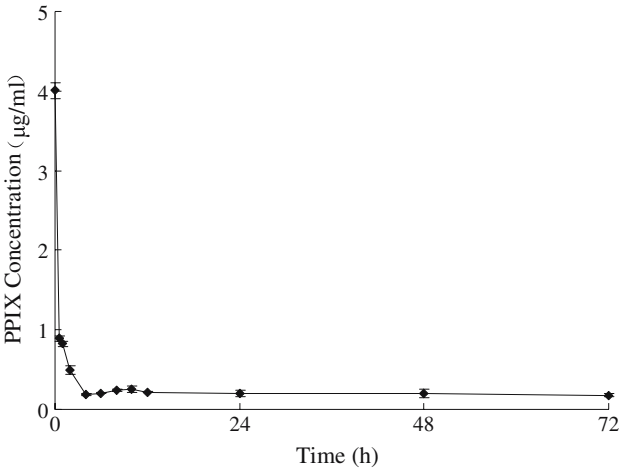


Fig. 4 Time course of PPIX concentration in plasma after intravenous injection

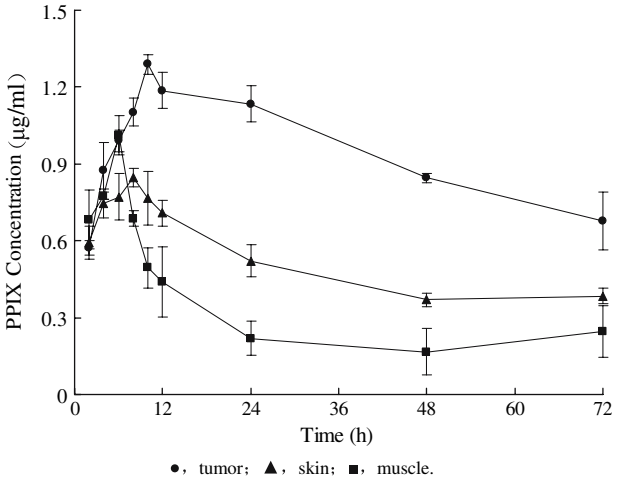


Fig. 5 Time courses of PPIX concentrations in tumor, skin and muscle after intravenous injection. *Filled circle* tumor, *filled triangle* skin, *filled square* muscle

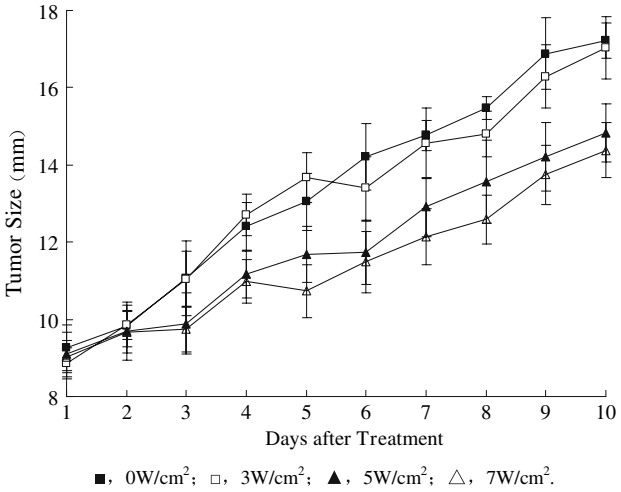


Fig. 7 Effect of ultrasound intensity on tumor. *Filled square* 0 W/cm^2, *open square* 3 W/cm^2, *filled triangle* 5 W/cm^2, *open triangle* 7 W/cm^2

ratios of PPIX concentration in tumor to other tissues were very important in order to maximize treatment effect while minimize side effect. The tumor/plasma, tumor/skin and tumor/muscle concentration ratios after administration were plotted in Fig. 6. The tumor/plasma ratio rose very rapidly during the first 4 h and peaked at 24 h, then declined very slowly. The tumor/muscle and tumor/skin concentration ratios also reached the relatively high and stable levels between 24 and 48 h, so a time of 24 h after administration was likely to be the best time for ultrasound exposure treatment.

The anti-tumor effect of ultrasound alone on S180 solid tumors was shown in Fig. 7, at an intensity of 0, 3, 5, and 7 W/cm^2, respectively, which indicated the tumor growth curve for 10 days after the day of treatment. The tumor growth inhibitory effect increased as the ultrasound intensity increased, and which became more and more obvious as the time prolonged. Ultrasound alone at the intensity of 3 W/cm^2 showed no obvious tumor growth inhibitory compared with the control group ($P > 0.05$), while there was significant tumor growth delay at 3 days after the treatment when ultrasound intensity was at 5 W/cm^2 or above ($P < 0.01$). So ultrasound alone at the intensity of 5 W/cm^2 showed a threshold for anti-tumor effect in our experiment.

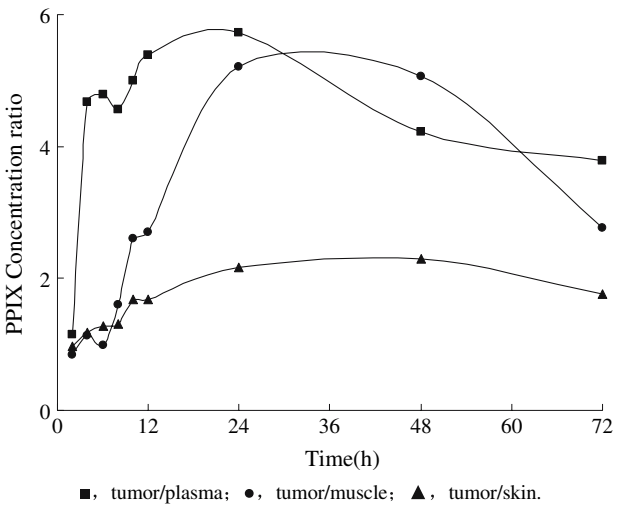
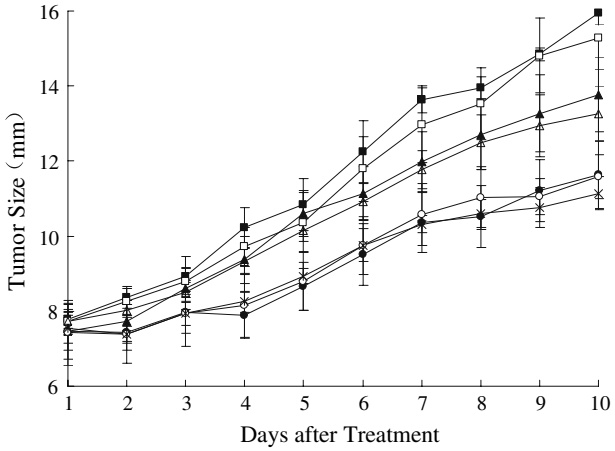


Fig. 6 Tumor/tissue concentration ratios. *Filled square* tumor/plasma, *filled circle* tumor/muscle, *filled triangle* tumor/skin

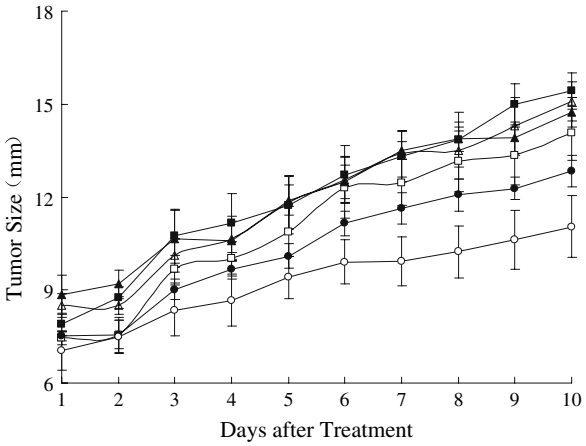
The effect of PPIX dose on anti-tumor effect of ultrasound was shown in Fig. 8, with PPIX dose of 0, 2.5, 5, 12.5, 25 mg/kg and ultrasound exposure at an intensity of 5 W/cm^2. PPIX alone at a dose of 25 mg/kg was basically the same as that of no treatment, showed no inhibitory effect. Ultrasound alone at an intensity of 5 W/cm^2 showed a slight anti-tumor effect, and which was enhanced by PPIX in a does dependent manner, the synergistic inhibitory effect was significant when PPIX dose was about 5 mg/kg or higher, but there were no obvious differences among the ultrasound combined with 5 mg/kg, 12.5 mg/kg, and 25 mg/kg PPIX groups.

The growth curve of S180 solid tumor with Hp-SDT and PPIX-SDT treatments at a PPIX or Hp concentration of 5 mg/kg was compared in Fig. 9, by plotting the tumor size for 10 days after inoculation. PPIX or Hp alone had almost no inhibitory effect, while ultrasound alone showed a slight anti-tumor effect. A relatively obvious suppression of tumor growth over 10 days after sonication was observed both in PPIX-SDT and Hp-SDT combined treatment groups. The synergistic effect of PPIX and ultrasound on inducing anti-tumor



■，Control group；□，25 mg/kg PPIX alone；▲，5 W/cm^2 ultrasound alone；△，2.5 mg/kg PPIX ＋ 5 W/cm^2 ultrasound；●，5 mg/kg PPIX ＋5 W/cm^2 ultrasound；○，12.5 mg/kg PPIX ＋ 5 W/cm^2 ultrasound；*，25 mg/kg PPIX ＋ 5 W/cm^2 ultrasound.

Fig. 8 Effect of PPIX dose on tumor growth: *filled square* Control group, *open square* 25 mg/kg PPIX alone, *filled triangle* 5 W/cm^2 ultrasound alone *open triangle* 2.5 mg/kg PPIX + 5 W/cm^2 ultrasound *filled circle* 5 mg/kg PPIX + 5 W/cm^2 ultrasound *open circle* 12.5 mg/kg PPIX + 5 W/cm^2 ultrasound, * 25 mg/kg PPIX + 5 W/cm^2 ultrasound



■，Control；□，5 W/cm^2 ultrasound alone；▲，5 mg/kg Hp；△，5 mg/kg PPIX ；●，5 mg/kg Hp ＋ 5 W/cm^2 ultrasound；○，5 mg/kg PPIX ＋ 5 W/cm^2 ultrasound；

Fig. 9 The tumor growth curve with different treatments. *Filled square* Control, *open square* 5 W/cm^2 ultrasound alone, *filled triangle* 5 mg/kg Hp, *open triangle* 5 mg/kg PPIX, *filled circle* 5 mg/kg Hp + 5 W/cm^2 ultrasound, *open circle* 5 mg/kg PPIX + 5 W/cm^2 ultrasound

effect was more and more remarkable as time delayed, and the tumor size was constantly about a half of the untreated group 3 days after treatment, which showed more marked synergistic antitumor effect than Hp-SDT treatment. The tumor weight 10 days after each treatment was shown in Table 1, there were no significant difference among the control, Hp, PPIX, and ultrasound groups. The tumor weight after the combination of PPIX or Hp with ultrasound was significantly smaller than the control group, the inhibition ratios were 42.82 ± 0.03 and 35.22 ± 0.03%, respectively, and there was significant difference between PPIX-SDT and Hp-SDT treatment groups ($P < 0.05$).

The morphological changes of S180 solid tumors immediately after PPIX-SDT and Hp-SDT treatments were shown in Fig. 10. The cells in control group were intact with rich cytoplasm, cell membranes were integrated, mitochondria were clear, nuclei were dense (Fig. 10a). The morphology of cells in Hp and PPIX groups was similar to that in control group, cell membranes were still intact, the cytoplasm of these cells was relatively homogeneous with rich organelles (Fig. 10b, c). Cells were injured to some extent in ultra-

Table 1 Antitumor effect of Hp or PPIX with ultrasound on sarcoma 180

Treatment group	Tumor weight[a]	Inhibition ratio (100%)[b]
Control	1.58 ± 0.05	0
Hp	1.50 ± 0.03	4.67
PPIX	1.52 ± 0.03	3.93
Ultrasound	1.39 ± 0.07	12.09
Hp + ultrasound	1.02 ± 0.03*	35.22
PPIX + ultrasound	0.90 ± 0.03*	42.82

[a] The mean ± SE of eight mice are list

[b] The inhibition ratio was calculated as (1 – average tumor weight of treatment group/average tumor weight of control group) × 100

*Significantly different ($P < 0.01$) from control group

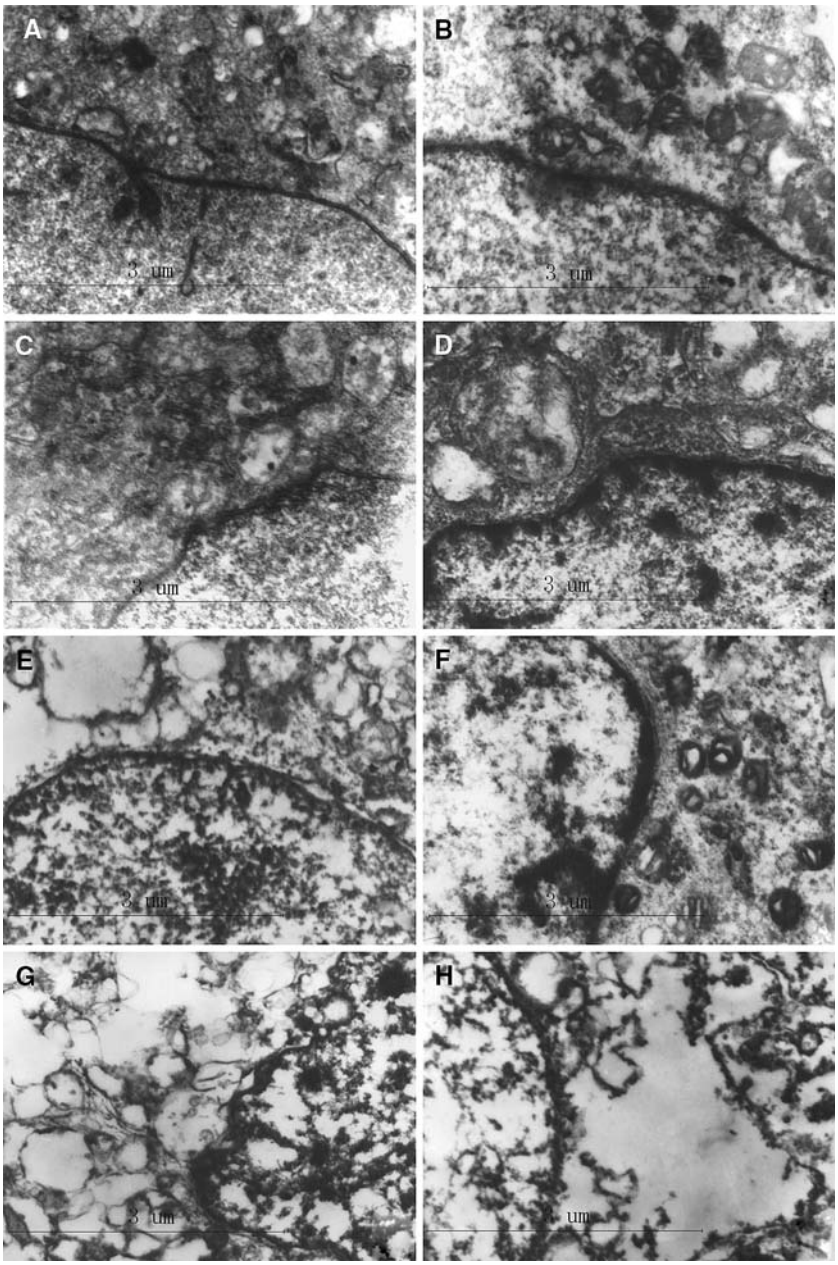


Fig. 10 Transmission electron microscopic images of S180 cells in vivo (×10 000). **a** Control group cell without any treatment, **b** tumor cell treated with 5 mg/kg Hp alone, **c** tumor cell treated with 5 mg/kg PPIX alone, **d** tumor cell treated with 5 W/cm^2 ultrasound alone, **e**, **f** Tumor cells treated by 5 W/cm^2 ultrasound plus 5 mg/kg Hp, **g**, **h** Tumor cells treated by 5 W/cm^2 ultrasound plus 5 mg/kg PPIX

sound group, cytoplasm became less dense, part of the membrane of mitochondria was damaged slightly, some chromatin slightly condensed in nuclear (Fig. 10d). The cell morphology was changed greatly in the synergistic treatment groups, in the Hp-SDT treatment group, some mitochondria were swelling with cristae decreased, there were sparse organelles in the cytoplasm in most cells (Fig. 10e), and there were many bubbles appeared in the less dense cytoplasm in some cells (Fig. 10f). In the PPIX-SDT treatment group, the surface membranes of most cells have been disrupted, the number and volume of bubbles in cytoplasm increased, the nuclear membrane was also slightly injured with condensed chromatin (Fig. 10g), some materials in cytoplasm were partly lost due to the destroyed cell membrane, and there were many spaces appeared in cytoplasm and nucleoplasm in some cells (Fig. 10h).

Discussion

It is known that the effects of SDT are dependent mainly on the concentration of porphyrins accumulated in the tumor and adequate intensity of ultrasound applied, there is usually a tissue- and tumor-specific sonodynamic does threshold for cell or tissue damage, which means that tissue concentration of porphyrins

and the activating ultrasound intensity does must reach a threshold level which allow tissue damage to happen [3]. The accumulations of porphyrin componds have been reported in a variety of tumors, this is important because these time courses of distribution can be different depending not only on the sensitizers but also on animal species, and the tumor cell lines [24–26]. And many studies in vitro suggested the involvement of specific tumor tissues properties, including an elevated number of low-density lipoprotein receptors, poor lymphatic drainage, leaky vasculature, and poor stroma structure [27, 28]. Hp, the most widely used sonosensitizer, it mediated sonodynamic therapy has been well investigated in vivo and in vitro [1, 22, 29]. PPIX, as one component of the hematorphyrin derivatives, may have more potential sonosensitization than Hp but less well studied. We have previously studied the metabolic distribution of Hp in S180 solid tumors in vivo and the cell killing effect of Hp or PPIX combined with ultrasound on isolate 180 cells in vitro [20, 22, 29], and found that the cell damage rate induced by PPIX-SDT was significantly higher than that treated by Hp-SDT at the same concentration. That is to say, PPIX can be induced more potential cytotoxicity than Hp when irradiated with ultrasound at the same experiment conditions, so it is very important to know whether it is true for sonodynamically induced antitumor effect in vivo. The aim of the present study was to compare the antitumor effect caused by PPIX-SDT treatment and Hp-SDT treatment, and to evaluate the possible mechanism accounting for the cell damage in sonodymic therapy.

Because the pharmacology of PPIX has not been carefully investigated, we firstly measured the concentrations of PPIX in plasma and other tissues in tumor bearing mice. For quantitative evaluation of PPIX accumulation both in the tumor and in normal tissues, we used porphyrin extraction methods to investigate the kinetics of PPIX concentration in mice bearing sarcoma 180 solid tumors using a fluorescence spectrophotometer. The results of fluorescence studies show the time-dependent kinetics of PPIX in the tumor and normal tissues (Figs. 4, 5). SDT would be most effective if the tumor is exposed to ultrasound when PPIX concentration in the tumor is at its maximum, furthermore, the adverse effects of SDT treatment can be minimized by exposure to ultrasound when the sensitizer concentration in the tumor is significantly higher than in normal tissues. So the ratios of PPIX accumulation in the tumor to that in other tissues were important in order to maximize the treatment effect while minimize side effect. From our result, the concentration of PPIX in the tumor gradually increased and reached a peak about 10 h after administration, however, PPIX also retained in the skin and muscle at a moderate concentration at this time point. Therefore, 10 h after administration was unlikely to be best time for ultrasound exposure. Figure 6 showed that about 24 h after administration, the tumor/plasma concentration ratio peaked and relatively high tumor/skin and tumor/muscle concentration ratios were seen, pointing to the best treatment time for preventing side effect and maintaining anti-tumor effect.

When used alone, ultrasound displayed a relative threshold for anti-tumor effect on sarcoma 180 as estimated by measuring the tumor size. The tumor growth inhibitory effect increased as the ultrasound intensity increased, and which became more and more obvious as the time after treatment prolonged. There was significant tumor growth delay 3 days after the treatment when ultrasound intensity was not less than 5 W/cm^2 compared with the control group ($P < 0.01$). So ultrasound alone at the intensity of 5 W/cm^2 showed a threshold for inducing anti-tumor effect in our experiment. And the ultrasonically induced antitumor effect can be enhanced by PPIX in a PPIX-dose dependent manner, at a PPIX dose not less than 5 mg/kg and the ultrasound intensity of 5 W/cm^2, the synergistic effect between PPIX and ultrasound exposure on the tumor growth inhibition was marked. A similar tendency has been observed in an in vitro experiment of ultrasound induced cell damage with and without PPIX, and PPIX alone inactivated S180 cells by the trypan blue exclusion test [20]. Therefore, based on the presented results and the previous report in vitro experimental, we inferred that certain PPIX could enhance tissue or cell damage induced by ultrasound.

The tumor growth inhibitory effect induced by ultrasonically activating PPIX and Hp was plotted in Fig. 9, the ultrasonically induced antitumor effect of sarcoma 180 was enhanced more significantly by PPIX than by Hp when they were compared at the same experiment conditions. Additionally, the tumor weight inhibition ratios 10 days after PPIX-SDT and Hp-SDT treatments were compared in Table 1. There were no significant inhibitory effects among the Hp, PPIX, and ultrasound groups compared with control group. The tumor weight after the combination of PPIX or Hp with ultrasound was significantly smaller than the control group, the inhibition ratios were 42.82 ± 0.03 and $35.22 \pm 0.03\%$, respectively, there was significant difference between the two combined treatment groups ($P < 0.05$). Furthermore, the morphologic changes of S180 solid tumors immediately after different treatments showed that cell damages induced by PPIX-SDT were more serious than those treated by Hp-SDT, and the results indicated that cell membrane and mitochondria were the mainly destruction targets in the synergistic effects.

The above comparative results implied that PPIX was an effective sensitizer for sonodynamic tumor treatment, it may have ever better potential than Hp. The enhancement of synergistic inhibitory effect by PPIX demonstrated was significantly higher than Hp when they were compared with the same ultrasound exposure on sarcoma 180 solid tumors, and the ultra-structure changes might play important roles in inhibiting tumor growth and even inducing cell death. Therefore, our results can be analysed from the perspective of tissue structure and function. The exposure of biological tissues to sonodynamic treatment can result in structure of functional alterations [30, 31]. On most occasions, structural changes in tissues brought about functional alterations, and vice versa. The ultra-structure changes reflected inertial morphological destruction. TEM observation immediately after sonodynamic treatment showed altered cell membrane, swollen mitochondria, lose of cytoplasm and nucleoplasm, injured nuclear membrane with condensed chromatin, and so on, which might suggested that ultrasound irradiation with PPIX and Hp played an important role in damaging cell membrane system and the inheritance information expression system. The damages of membranous organelles greatly influenced the stabilization of micro-surrounding and energy exchange from in and out; Condensation of nuclear chromatin would result in interruption of gene expression and regulation, these changes could destroyed metabolism, affected functions and lead to cell death eventually [32]. Additionally, many cytoplasmic vacuoles were detected in both PPIX-SDT and Hp-SDT treatment group cells, and the number and volume in the former group cells were more and larger than in the latter group cells, which might account for their different tumor inhibitory effects. The cytoplasmic vacuoles are usually regarded as direct evidence of cavitation [33, 34]. The efficiency of SDT, which resulted from cavitation, in cancerous cells both in vitro and in vivo had been manifested in recent years [35]. Cavitation is considered the mechanism responsible for sonochemically generated radical production and a quantitative phenomenon, which could lead to transient membrane ruptures, resulting in promotion of membrane permeability [36]. Products of ultrasonic cavitation were free radicals, such as hydroxyl, singlet oxygen and hydroperoxyl et al, where researchers believed that the singlet oxygen played the determining role, free radicals scanvengers reduced its damage to cells and tissue [37, 38].

The enhancement of sonodynamic anti-tumor effect caused by PPIX was more significantly higher than Hp when it was compared at the same molarity in suspension of isolated 180 cells in vitro and at the same administration concentration on sarcoma 180 in vivo [21]. The characteristic difference between PPIX and Hp may cause their different impact on SDT: on the one hand, they are porphrins with different structures and potential sensititization. There was report about that the photosensitive damage of PPIX to the main-chain protein was stronger than the damages of Hp, which mainly depended on the structures of PPIX and Hp [39]. On the other hand, they have different tumor preferential abilities and distinct subcellular localization patterns. The sonosensitizer and ultrasound are known as indispensable determinant for SDT, however, an increasing evidence has emerged the effectiveness of SDT may also depend on subcellular and intracellular localization patterns of a sonosensitizer. This may be related to the fact that some organelles of cell and structural elements of tissues are more sensitive then other for cell survival and tissue repair [12]. In the experiment, exogenous PPIX disodium salt was hydrophilic, and mainly accumulated in the cell membrane and diffused in the cytoplasm, while Hp was lipophilic and mainly distributed on the mitochondria in the cytoplasm [12]. Generally, the most sensitive structures in the cell are biomembranes, the permeability of which can be influenced by ultrasound [27], and mitochondria may be another important target for SDT, this was in accordance with previous reports [31, 32, 40].

In summary, we estimated the distributions of PPIX in the tumor tissue in comparison to normal tissues in order to predict the treatment effects, and a significant in vivo anti-tumor effect was observed with low energy focused ultrasound, in combination with intravenous administration of PPIX or Hp. Based on the presented results and the previously reported in vitro, we think that PPIX has more potential sonosensitization than Hp, the enhancement of sonodynamic antitumor effect by PPIX might be attribute to the sonochemical activation.

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