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Sonodynamically induced anti-tumor effect with protoporphyrin IX on hepatoma-22 solid tumor

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ABSTRACT

Objective: The purpose of this study was to evaluate sonodynamically induced anti-tumor effect of protoporphyrin IX (PPIX) in mice bearing hepatoma-22 (H-22) solid tumors, and the possible in vivo cell damage mechanism was also investigated.
Methods: The pharmacokinetics of PPIX was analyzed in plasma, skin, muscle and tumor of H-22 bearing mice. Tumors were irradiated with ultrasound (1.43 MHz, I_{SATA} 3 W/cm^2, 3 min) for three times at 8, 12 and 24 h after 5.0 mg/kg PPIX administration, respectively. The anti-tumor effects of sonodynamic therapy (SDT) were estimated by the tumor inhibition ratio (volume and weight). The bio-effects of SDT were evaluated by hematoxylin and eosin (H&E) staining, transmission electron microscope (TEM) observation, lipid peroxidation (LPO) measurement and anti-oxidative enzymes (glutathione peroxidase (GSH-PX), catalase (CAT) and superoxide dismutase (SOD)) assay.
Results: A significant anti-tumor effect was obtained by PPIX-mediated sonodynamic therapy (PPIX-SDT). At the fifteenth day after PPIX-SDT, the tumor growth and tumor weight inhibition ratios were 53.84% and 45.86%, respectively. In addition, the structure of tumor tissues and the anti-oxidative enzymes were obviously destroyed after SDT treatment.
Conclusions: A biochemical mechanism was involved in PPIX-SDT in vivo, and the free radicals produced by the synergistic treatment destroying the anti-oxidative system of tumor cells in vivo may play an important role in this action. Also, the thermal effect could not be excluded in inducing damage of cellular structures, like membrane disruption and chromatin condensation under current evaluation in this paper.

1. Introduction

Ultrasound is a kind of mechanical wave that has strong ability to penetrate deep biological tissue and activate a series of sono-sensitizers or anti-tumor drugs such as hematoporphyrin (Hp), photofrin II, ATX-70, adriamycin, and acridine orange [1–5], producing more remarkable inhibitory effect on tumors than ultrasound alone. This approach by combining ultrasound with sono-sensitizer to cure tumor is named sonodynamic therapy (SDT) [6,7]. Studies of SDT mainly focused on the mechanisms of killing effects by different ultrasound parameters and sono-sensitizers. Many mechanisms had been proposed by different researcher groups, including the singlet oxygen [8,9], alkoxyl radicals [10], lipid peroxidation [11], apoptosis [12–15] and so on. But until now, the exact mechanism of SDT is still unknown. It seems that many factors like the sono-sensitizer used, the acoustic parameters and the nature of biological samples being irradiated all affect the specific mechanism of SDT.

Protoporphyrin IX (PPIX), a kind of hematoporphyrin derivatives, has been found to be preferentially accumulated in rapid proliferating cancer cells, thus selectively destroy tumors when exposed to ultrasound or light [16–18]. Umemura S reported that PPIX-mediated sonodynamic therapy (PPIX-SDT) produced more serious cell killing effect than Hp mediated sonodynamic therapy (Hp-SDT) at the same experimental conditions [19]. Our studies also indicated that PPIX exhibited more potential cytotoxicity than did Hp when exposed to ultrasound in vivo and in vitro [16,20]. In addition, PPIX distributes reasonably well within tissues and metabolizes quickly in normal tissues, so it can be considered as a good sono-sensisizer in SDT.

PPIX mediated SDT therapy in our previous papers suggested that the generation of reactive oxygen species (ROS) during irradiation play some role in cell killing and apoptosis in isolate hepatma-22 (H-22) cells [13]. The in vivo PPIX-SDT also showed obvious tumor growth inhibition from the view of ultra-structure destruction effect on S180 tumors [16]. However, few reports concerning the in vivo inhibition mechanisms have been published. To

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further investigate the possible biological mechanism, in this current paper, we measured many parameters such as morphological observations, lipid peroxidation levels and key anti-oxidant enzymes activities, to reflect the anti-tumor effect of PPIX-SDT on H-22 solid tumors. The optimum time points for ultrasonic irradiation were determined by measuring the ratios of PPIX concentration in tumors to the surrounding normal tissues. Anti-tumor effects were estimated by the tumor inhibition ratios (volume, weight) and the weight change of mice after three times ultrasound exposure when PPIX concentrations in tumors were relatively higher. The morphological changes of H-22 tumor tissues were carefully examined by hematoxylin and eosin (H&E) staining and transmission electron microscope (TEM) observation. The biological responses of H-22 solid tumor cells to PPIX-SDT were analyzed by detecting levels of lipid peroxidation (LPO) and activities of anti-oxidative enzymes such as glutathione peroxidase (GSH-PX), catalase (CAT) and superoxide dismutase (SOD). Therefore, our findings may provide a fundamental understanding of SDT therapy from the clinical views.

2. Materials and methods

2.1. Chemicals

Protoporphyrin IX (PPIX) was purchased from Sigma Company (St. Louis, MO, USA). It was dissolved in phosphate buffer solution (PBS, pH 7.2–7.4), sterilized and stored in the dark at −20 °C. Malondialdehyde, glutathione peroxidase, catalase and superoxide dismutase detection kits were obtained from Nanjing Jiancheng Biotechnology Institute (Nanjing, China). All other reagents were commercial products of analytical grade.

2.2. Tumor cells and animals

H-22 cells were obtained from the Fourth Military Medical University (Xi'an, China). The Institute of Cancer Research (ICR) mice were supplied by the Experimental Animal Center of Xi'an Jiao Tong University (Xi'an, China). H-22 cells were passed through ICR mice in the form of ascites and harvested from the peritoneal cavity of mice at 5–7 days after inoculation. In the experiment, H-22 cells were suspended in physiological saline at a concentration of 5×10^6 cells/ml. The cell suspension of 0.1 ml was subcutaneously inoculated into the left oxter region of ICR mice (18–22 g). When the tumor size reached an average diameter of 5 mm (about 5 days later), the tumor-bearing mice were ready for analysis. The animal experiment was approved from the university's institutional animal care and use committee.

2.3. Detection of PPIX concentrations in plasma and tissues

PPIX was administered to tumor-bearing mice at a dose of 5.0 mg/kg by intravenous injection into the caudal vein. At each time point (0, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h after administration), the blood samples were obtained by removing the eyeballs of the mice. Immediately, the blood was placed into a 1.5 ml heparin-coated centrifuge tube, and centrifuged to separate the plasma. The mice were killed at 2, 4, 6, 8, 10, 12, 24, 36, 48, 72 h after administration by cervical dislocation. The tumor and normal tissues (skin and muscle) were excised immediately, rinsed in physiological saline solution, blotted dry and weighed. Plasma (0.2 ml) was mixed with 2 ml EDTA/PBS (0.02%), the tissues (0.2 g) were homogenized in 2 ml of the same buffer in a tissue grinder, then all the samples were extracted with 2 ml of methanol/perchloric acid (0.9 M) mixture (1/1, v/v). After blending and centrifugation, the supernatant was prepared for detection. The PPIX concentration was estimated by the fluorescence intensity of tissue extractions with a fluorescence photometer (F-2500, Hitachi, Japan) based on the standard curve. A standard curve was obtained by adding known gradient concentrations of PPIX to corresponding tissue digests prepared from untreated animals. The background auto-fluorescence in the samples was eliminated from the supernatant fluorescence in the corresponding tissue digests in control group mice.

2.4. SDT protocol

The tumor-bearing mice were divided into four groups: control group (CT), PPIX group (P), ultrasound group (U), and ultrasound plus PPIX group (UP). Each group had 7–8 mice. PPIX was administered to the mice of P and UP groups at a dose of 5.0 mg/kg by intravenous injection through the caudal vein. Prior to ultrasound applications, the hair over the tumors region was shaved using an animal shaver, treated with depilatory cream, and rinsed. The mice were anesthetized with 2.5% pentobarbital sodium and placed on a plexiglass plate with the axillary tumor immersed into the degassed water through an appropriate hole in the plate (Fig. 1). The exposed tumor was rightly in the focus center of the transducer. In U and UP groups, tumors were irradiated by ultrasound (I_{SATA} 3 W/cm^2, 3 min) for three times at 8, 12 and 24 h after PPIX administration, respectively, according to the result of pharmacokinetics. All of the experiments were performed in the dark to avoid PPIX excitation.

2.5. Ultrasonic exposure setup

As shown in Fig. 1, the focused ultrasound transducer with a circular ceramic plate of 38 mm in diameter, manufactured by the Institution of Applied Acoustics, Shaanxi Normal University (Xi'an, China), was horizontally submerged in the bottom of a glass container filled with cold degassed water. The focal length of the transducer is about 52 mm. The same transducer was used for all experiments, and was driven at its resonant frequency of 1.43 MHz in a continuous wave mode. The electrical signal was generated and amplified by a multi-functional generator (Model AG1020, T&C Power Conversion, Inc., Rochester, NY, USA) before being applied to the transducer. This generator/amplifier utilizes conservatively rated solid components and automatic power control circuitry to ensure reliable and continuous performance. The power supply switches automatically to the line voltage applied to the apparatus, and it also includes a simultaneous display system. In the study, the acoustic parameters were simultaneously detected by other instruments.

The efficiency of the electro-acoustic conversation of the transducer was measured by an Agilent 4294A Impedance Analyzer (Agilent Technologies, Santa Clara, California). The ultrasound

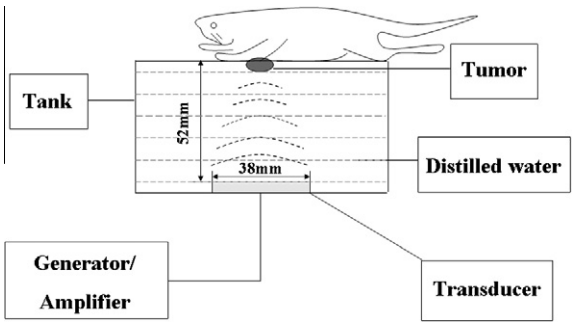

Fig. 1. Ultrasonic exposure equipment.