

High-Intensity Focused Ultrasound Enhanced Anti-Tumor Activities of Paclitaxel in Breast Cancer *in vitro* and *in vivo*

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Background: Paclitaxel (PTX) is an important oncologic chemotherapeutic agent against breast cancer, but breast cancer patients develop significant resistance to PTX during chemotherapy. Alterations in tubulin and associated proteins have been implicated in resistance to PTX. High-intensity focused ultrasound (HIFU) induces deep tumor penetration of anti-tumor agents in solid tumors.

Methods: We investigated the influence of HIFU on the anti-tumor activities of PTX in breast cancer. Both *in vivo* and *in vitro* experiments were performed in this research: mice were treated with 2 mg/Kg PTX through tail vein injection, while breast cancer cells were treated with 400 nM PTX. Cell viability was analyzed through Cell Counting Kit-8. Cell apoptosis was evaluated through Annexin-V/PI Apoptosis Analysis Kit. The activities of catalase (CAT) and superoxide dismutase (SOD) and the concentration of malondialdehyde (MDA) were evaluated by relative commercial kits.

Results: HIFU enhanced PTX-inhibited breast cancer cell viability and PTX-induced cell apoptosis. Simultaneous treatment of HIFU and PTX decreased the activities of CAT and SOD and increased the concentration of MDA. In mice bearing MDA-MB-231 tumors, the treatment of HIFU and PTX significantly decreased tumor size, increased body weight and elevated animal survival. HIFU enhanced the distribution of PTX in tumor tissues.

Conclusion: The performance of HIFU promoted the distribution of PTX and enhanced its anti-tumor activities in breast cancer.

Keywords: breast cancer, paclitaxel, high-intensity focused ultrasound, cancer therapy

Introduction

Breast cancer accounts for about 7–10% of tumors in female population, and is the number one malignant tumor in Chinese women aged 30–59 years.¹ Chemotherapy is an effective therapy for breast cancer, but many patients experience chemotherapeutic reactions such as nausea, hair loss, abnormal sensation, and bone marrow suppression when undergoing chemotherapy. These side effects hinder clinical treatment and increase patients' fear of the chemotherapy.² Till now, diverse treatments are performed to enhance the anti-tumor activities of chemotherapeutic agents and to decrease the extent of side effects, including crocin administration, silymarin administration, and melatonin coadministration.^{3–6}

Paclitaxel (PTX) is an important oncologic chemotherapeutic agent and has become the first-line drug in chemotherapy for breast cancer patients.⁷ PTX can bind to β -microtubulin and affect the dynamic balance between microtubulin dimers and microtubules. PTX promotes the assembly of microtubulin into microtubules, as well as aggregates and blocks the depolymerization of assembled ones. Abnormal microtubule formation inhibits cell mitosis and leads to cell death.^{8–10} PTX resistance in breast cancer patients has received increasing attention. PTX is 30–60% effective as a first-line agent, while it is 20–40% effective as a second-line agent or single-agent in chemotherapy.¹¹ The development of resistance to PTX in breast cancer involves multiple molecular mechanisms. Altered expression of β -tubulin isotypes and changes in microtubule-associated proteins, such as microtubule-associated protein-4 and tau, affect microtubule dynamics and modulate the resistance to PTX.¹² PTX and the microtubule-binding protein Tau bind to microtubules

through the same site. Tau competes with PTX to inhibit its role in promoting microtubule aggregation, thereby causing resistance to PTX.¹³ Altered expression levels of specific isotype microtubule proteins can also lead to PTX resistance.¹⁴

Because of its non-ionizing and noninvasive properties, high-intensity focused ultrasound (HIFU) is a relatively safe treatment.^{15,16} Since the repeated treatment of HIFU is possible and the complication occurrence rate is low, HIFU has become an attractive option for breast cancer therapy.¹⁷ The combination of HIFU with nanoparticles has a substantial potential to enhance the efficacy of drug delivery and reduce side effects of drugs in the therapy of tumors and other diseases.¹⁸ Moreover, HIFU technology provides the targeted release of drugs encapsulated in a low-temperature thermo-sensitive liposome PTX coated in animal model.¹⁹ Thus, HIFU may also promote PTX delivery and its anti-tumor activity.

In this research, we aimed to investigate whether HIFU could enhance the anti-tumor effect of PTX in breast cancer.

Methods

Cell Culture

MDA-MB-231 and MCF-7 cells (ATCC, Manassas, VA) were used in this research. DMEM (Invitrogen, MA, USA) containing 10% fetal bovine serum (Gibco, MA, USA) and 100 U/mL penicillin and streptomycin (Thermo Fisher, MA, USA) was used for cell culture.

PTX was purchased from Aladdin (Shanghai, China). Cells were separated into the control group, control + PTX group, HIFU group, and HIFU + PTX group. After PTX treatment, cell viability was analyzed.

HIFU

1×10^6 cells were placed in the test tube and radiated at 1 MHz of frequency for 30s. For mouse model, HIFU was applied at 1.5 MHz of frequency for 5 min at the tumor site (interval 2 mm, time per spot 30s). Mice were anesthetized with inhalation isoflurane throughout the HIFU exposure process. Each mouse was positioned in a holder.

Measurement of Cell Viability

1000 cells per well were plated in 96-well plates. Cell Counting Kit-8 assay (Bimake, Shanghai, China) was employed for evaluating cell viability according to the manufacturers' instruction.

Measurement of Cell Apoptosis

Cell apoptosis was analyzed by Annexin-V/PI Apoptosis Analysis Kit (Yeasen, Shanghai, China) according to the manufacturer's protocol.

Detection of Antioxidant Activities

Superoxide dismutase (SOD) was measured by SOD analysis kit (KeyGen Biotech, Nanjing, China) using the Xanthine oxidase method, measured at 550 nm, and expressed as U/mL. Catalase (CAT) was evaluated by CAT analysis kit (KeyGen Biotech) using H₂O₂ and ammonium molybdate, measured at 405 nm, and expressed as U/g protein. The concentration of malondialdehyde (MDA) was measured by MDA analysis kit (KeyGen Biotech) by thiobarbituric acid (TBA) method, measured at 532 nm, and expressed as nmol/L.

Animal Model

Female BALB/c mice (5-week old) were xenografted with 5×10^6 MDA-MB-231 cells into the right flank. Mice were kept until tumor size reached 5 to 8 mm. Mice bearing tumors were treated with 2 mg/Kg PTX through tail vein injection. Mice were separated into the saline group, PTX group, HIFU group, and HIFU + PTX group. The tumor size, body weight, and survival rate were analyzed. Animal studies were performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (8th edition, NIH), and were approved by the ethics committee of Cangzhou Central Hospital (2021.06.17.c4).

Pharmacokinetics of PTX

Blood samples (0.3 mL) and tumor tissue samples were collected at 0.2, 0.5, 1, 2, 4, 8, 12, and 24 h after PTX treatment. Tissues were homogenized by Omni Bead Ruptor 24 Homogenizer (Kennesaw, GA, USA). To quantify the PTX concentration in samples, solid phase extraction was applied before the measurement. PTX concentration was detected by liquid chromatography/mass spectrometry (LC/MS).

Statistical Analysis

In this research, statistical analysis was performed by SPSS Statistics Version 11.0. Data were shown as mean \pm standard deviation (SD). Statistical significance was analyzed by one-way ANOVA or two-way ANOVA followed by appropriate post hoc tests, and defined by $P < 0.05$.

Results

Effect of HIFU on PTX-Inhibited Breast Cancer Cell Viability

In Figure 1A and B, 400 nM or 1000 nM PTX significantly inhibited the viability of MDA-MB-231 (400 nM: 81.0% \pm 7.5%, $p=0.030$; 1000 nM: 56.1% \pm 8.2%, $p<0.001$) and MCF-7 (400 nM: 85.8% \pm 6.8%, $p=0.048$; 1000 nM: 72.1% \pm 7.8%, $p=0.009$) cells. Thus, the PTX concentration used in subsequent experiments was chosen as 400 nM. HIFU inhibited the viability of MCF-7 cells (Figure 2A) (86.5% \pm 6.6%, $p=0.049$). In both cell lines, cells treated with the

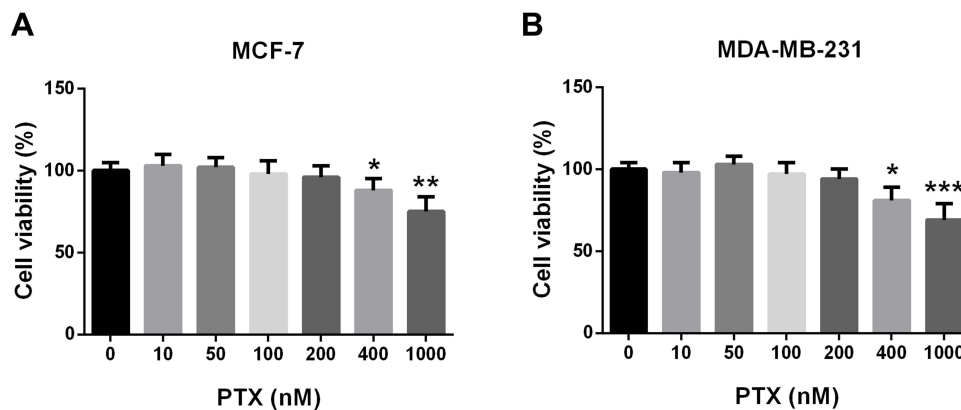


Figure 1 Effects of different concentrations of paclitaxel on cell viability of MCF-7 (A) and MDA-MB-231 (B). Data are expressed as the mean \pm SD. $n=3$ for each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with the control group.

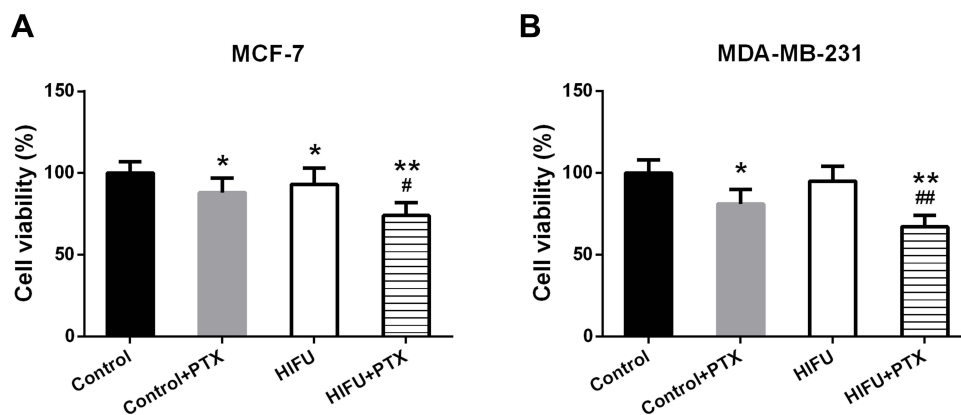


Figure 2 Cell viability of MCF-7 (A) and MDA-MB-231 (B) with the treatment of 400 nM paclitaxel and HIFU. Data are expressed as the mean \pm SD. $n=3$ for each group. * $p<0.05$, ** $p<0.01$ compared with the control group; # $p<0.05$, ## $p<0.01$ compared with the control + PTX group.

combination of HIFU and PTX exhibited significantly lower cell viability than those treated with PTX alone (MCF-7: $65.7\% \pm 7.4\%$, $p=0.024$; MDA-MB-231: $60.1\% \pm 6.4\%$, $p=0.008$) (Figure 2A and B).

Effect of HIFU on PTX-Induced Oxidative Stress in Breast Cancer Cells

In both cell lines, both HIFU and PTX reduced the activity of CAT (Figure 3A, -13.1 U/g protein, $p=0.028$; -27.8 U/g protein, $p<0.001$ and Figure 3D, -12.3 U/g protein, $p=0.047$; -36.1 U/g protein, $p<0.001$). Furthermore, cells in the HIFU + PTX group exhibited significantly lower CAT activity than those in the control + PTX group (Figure 3A, -12.2 U/g protein, $p=0.039$ and Figure 3D, -9.3 U/g protein, $p=0.049$). The MDA concentrations of both cell lines in the control + PTX group were significantly elevated compared with the control group (Figure 3B, 3.5 nmol/L, $p=0.028$; Figure 3E, 4.8 nmol/L, $p=0.008$). Importantly, the MDA concentration in the HIFU + PTX group was significantly higher than in the control + PTX group (Figure 3B, 3.8 nmol/L, $p=0.049$; Figure 3E, 3.0 nmol/L, $p=0.047$). Furthermore, the activity of SOD in the HIFU + PTX group was significantly lower than in the control + PTX group (Figure 3C, -8.5 U/mL, $p=0.048$ and Figure 3F -8.1 U/mL, $p=0.040$). Thus, HIFU and PTX exhibited synergistic effect on the induction of oxidative stress in breast cancer cells.

Effect of HIFU on PTX-Induced Breast Cancer Cell Apoptosis

In MCF-7 cells, both HIFU ($14.8\% \pm 2.4\%$ vs $7.8\% \pm 2.4\%$, $p=0.047$) and PTX ($17.1\% \pm 3.2\%$ vs $7.8\% \pm 2.4\%$, $p=0.011$) enhanced cell apoptosis (Figure 4A and B). Compared with the control + PTX group, MCF-7 cells in the HIFU + PTX group ($26.6\% \pm 2.8\%$, $p=0.009$) showed significantly elevated extent of apoptosis (Figure 4A and B). MDA-MB-231 cells in the HIFU + PTX group ($38.4\% \pm 3.6\%$ vs $24.2\% \pm 2.7\%$, $p=0.001$) also exhibited significantly higher apoptosis than in the control + PTX group (Figure 4A and C). Thus, HIFU and PTX showed synergistic effect on breast cancer cell apoptosis.

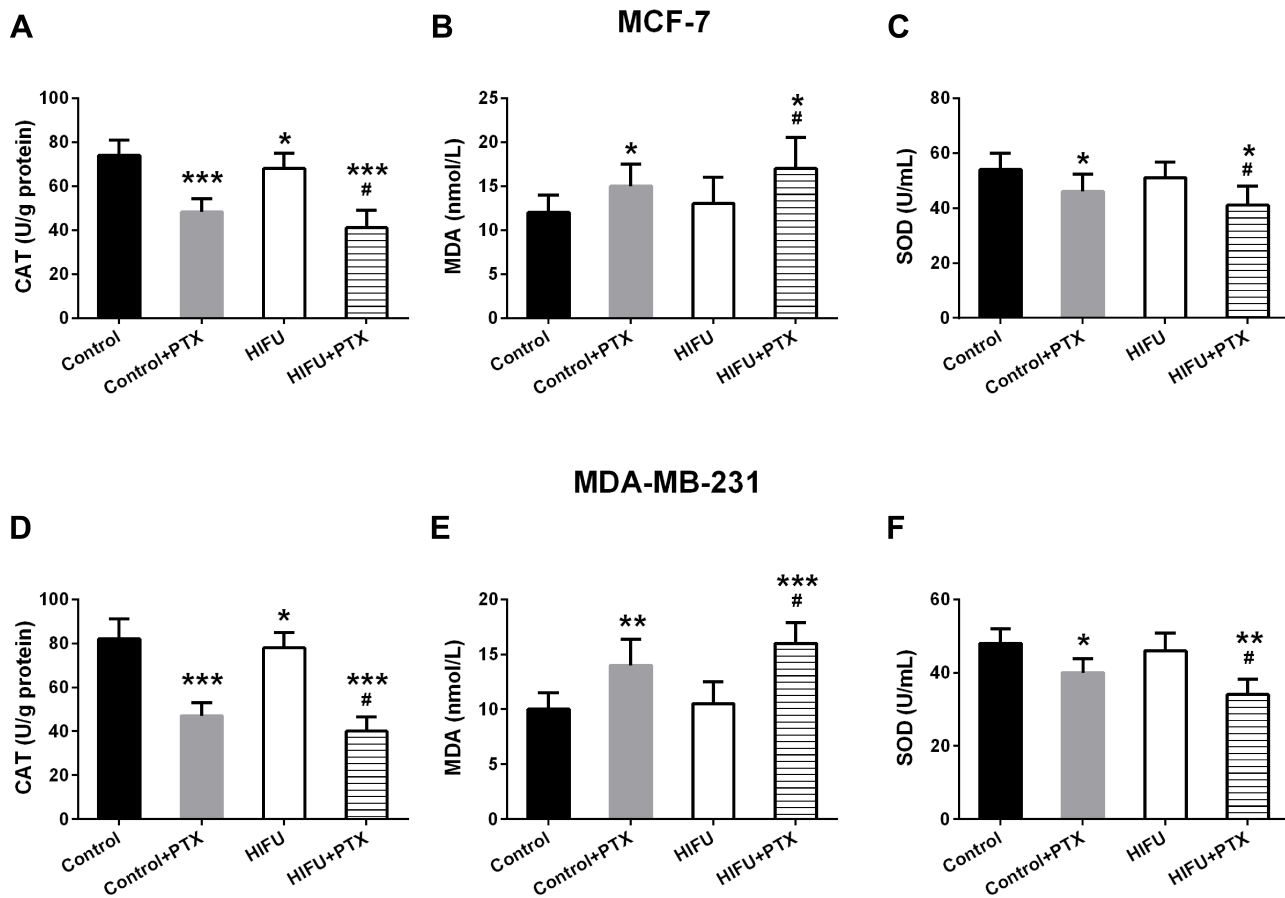


Figure 3 Levels of CAT (A and D), MDA (B and E) and SOD (C and F) in MCF-7 and MDA-MB-231 induced by paclitaxel were detected. Data are expressed as the mean \pm SD. $n=3$ for each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with the control group; # $p<0.05$ compared with the control + PTX group.

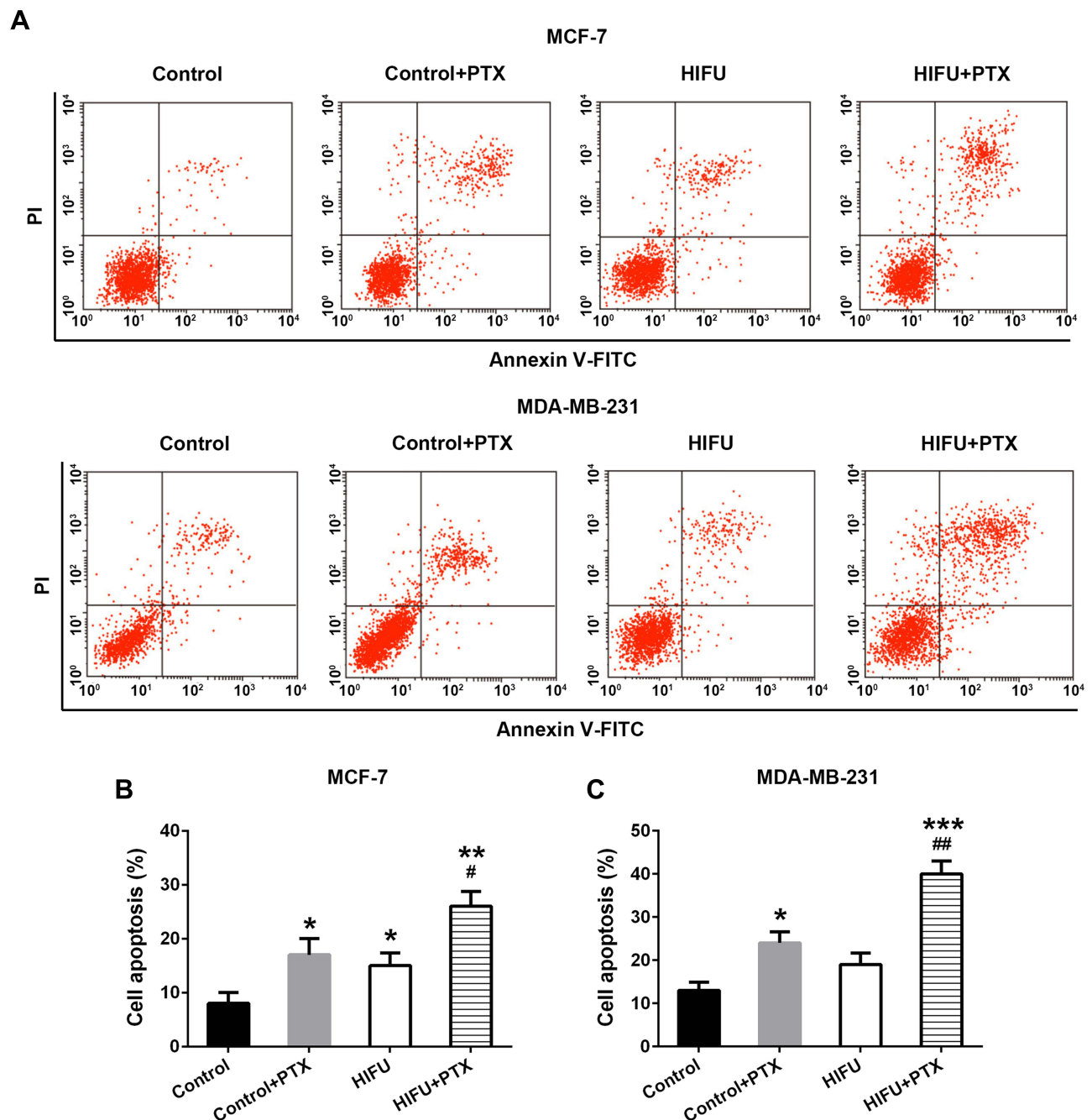


Figure 4 Cell apoptosis of MCF-7 and MDA-MB-231 induced by paclitaxel were enhanced by HIFU. (A) Representative images and the percentages of cell apoptosis in MCF-7 (B) and MDA-MB-231 cells (C). Data are expressed as the mean \pm SD. $n=3$ for each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared with the control group; # $p<0.05$, ## $p<0.01$ compared with the control + PTX group.

Effect of HIFU on the Anti-Tumor Effect of PTX in Mice Bearing MDA-MB-231 Tumors

In mice bearing MDA-MB-231 tumors, the effects of HIFU and PTX on tumor tissues were also evaluated. Tumor size in the PTX group ($218.63 \pm 32.98 \text{ mm}^3$ vs $282.14 \pm 36.71 \text{ mm}^3$, $p=0.001$) was significantly smaller than that of the saline group (Figure 5A). In the HIFU + PTX group ($166.74 \pm 30.11 \text{ mm}^3$ vs $218.63 \pm 32.98 \text{ mm}^3$, $p=0.012$), the tumor size was significantly smaller than that in the PTX group (Figure 5A). Compared with mice in the saline group, those in the HIFU + PTX group displayed significantly higher body weight ($20.9 \pm 2.2 \text{ g}$ vs $17.2 \pm 1.9 \text{ g}$, $p=0.017$) (Figure 5B).

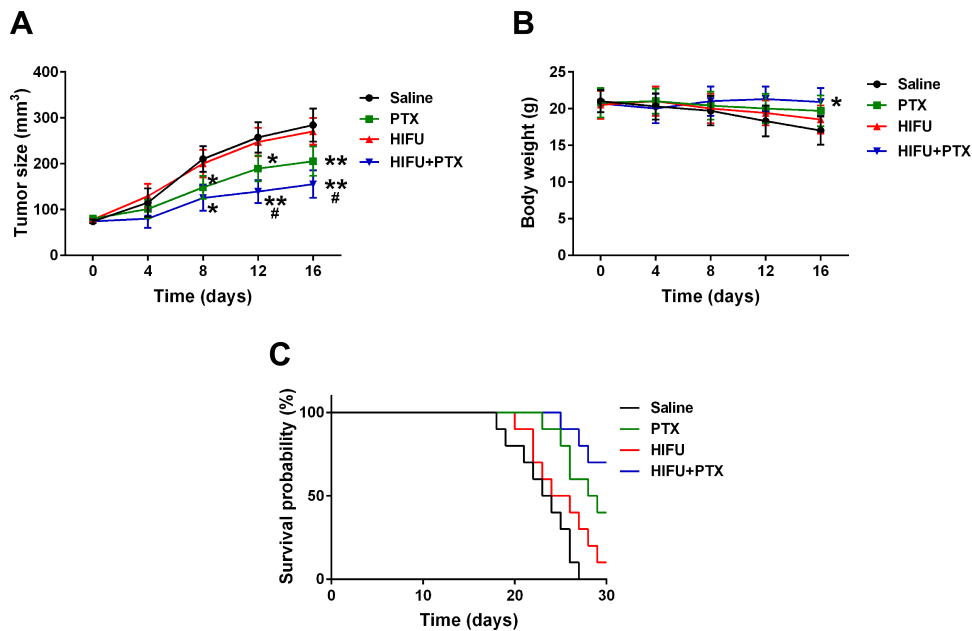


Figure 5 Antitumor efficacy using HIFU and PTX. Tumor volume (**A**), body weight (**B**) and survival probability (**C**) in different groups were monitored. Data are expressed as the mean \pm SD. $n=5$ for each group. * $p<0.05$, ** $p<0.01$ compared with the saline group; # $p<0.05$ compared with the PTX group.

Furthermore, the HIFU + PTX group exhibited the highest survival rate among all four groups (Figure 5C). Thus, HIFU and PTX exhibited synergistic anti-tumor effect against breast cancer.

Effect of HIFU on the Distribution of PTX in Tumor Tissues

Pharmacokinetic studies of PTX with and without HIFU were conducted. In both the PTX group and the HIFU + PTX group, PTX concentrations in the plasma dropped rapidly and showed no significant difference (Figure 6A). In mice bearing MDA-MB-231 tumors, PTX concentrations in the tumor tissues were increased in the first 4 hours after injection and subsequently decreased (Figure 6B). 24 hours post PTX injection, the HIFU + PTX group showed significantly higher PTX concentrations in tumor tissues than the PTX group (108.66 ± 20.17 ng/g vs 72.15 ± 12.04 ng/g, $p=0.012$) (Figure 6B).

Discussion

In this research, we investigated the therapeutic and drug delivery efficacy of HIFU-triggered PTX against breast cancer through both in vivo and in vitro experiments. Intravenously administered PTX could be well accumulated in the tumor

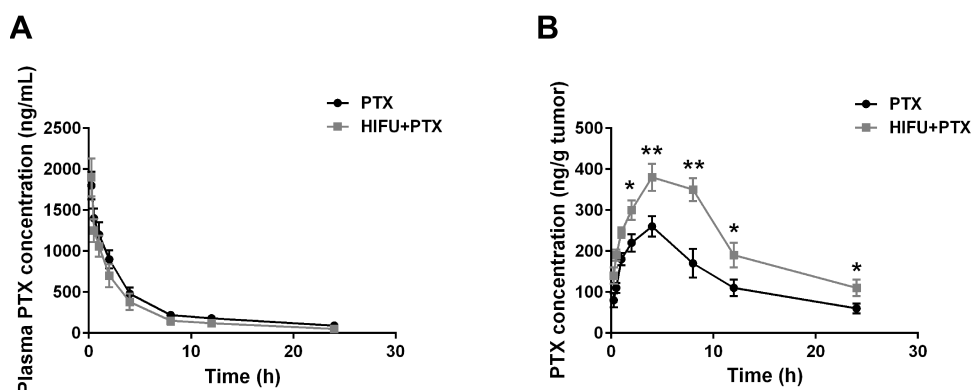


Figure 6 Pharmacokinetics of PTX after injection of PTX in different groups. PTX concentration in plasma (**A**) and tumors (**B**). Data are expressed as the mean \pm SD. $n=5$ for each group. * $p<0.05$, ** $p<0.01$ compared with the PTX group.

tissues of mice bearing MDA-MB-231 xenograft tumors through HIFU treatment. Compared to PTX without HIFU treatment, *in vitro* HIFU-triggered PTX in breast cancer cells displayed enhanced effects in inhibiting cell viability, inducing oxidative stress, and promoting apoptosis. According to these results, treatment of HIFU could promote the accumulation efficiency of PTX in breast cancer tissues and its anti-tumor effect.

Rapid growth of tumor tissues leads to leakage from the vascular system. In the complex tumor microenvironment, limited deep tumor penetration greatly hinders the delivery of anti-tumor drugs to tumor sites.²⁰ It is well known that heterogeneous tumors have different vascular structures and perfusion rates.²¹ In tumor tissues, the thick extracellular matrix (ECM) can generate a physical barrier to inhibit drug accumulation.²²

Several studies have attempted to promote anti-tumor drug delivery by remodeling ECM in the tumor microenvironment.²³ Matrix metalloprotease (MMP) can break down the structure of ECM thereby improve anti-tumor drug delivery and therapeutic efficacy.²⁴ HIFU can physically break down dense tumor ECM structures with no toxicity²⁵ and improve the delivery of high molecular weight antibodies and anti-tumor drugs to tumor tissues.^{26,27} The exact mechanism of HIFU-mediated nanoparticle delivery in tumors has been reported.^{25,28} The dense ECM structure of tumor tissues can be successfully disrupted by exposure to noninvasive pulsed HIFU. In addition, normalizing the tumor vasculature can reduce the interstitial flow pressure in ECM-rich tumors.²⁹ Intravenously administered drugs can successfully accumulate in tumor tissues exposed to HIFU.³⁰

In addition to radiotherapy, surgery and hormonal therapy, chemotherapy has become an effective treatment for breast cancer.³¹ Most patients respond to the initial chemotherapy, but the sensitivity and response rate against chemotherapeutic agents gradually decline during the process of chemotherapy as a result of the development of multidrug resistance, which can eventually lead treatment failure.³² The mechanisms by which drug resistance arises are complex and can be mediated by a variety of factors. Evidence suggests that drug resistance is closely associated with increased drug efflux transporter protein activity, reduced levels of cellular drug uptake, upregulation of the detoxification system, or adaptation to metabolic reprogramming.^{33–35}

There is growing interest in the use of neoadjuvant treatments for earlier inhibition of micro-metastasis to improve the treatment outcome.³⁶ Neoadjuvant chemotherapy is the gold standard treatment for breast cancer to reduce the extent of subsequent surgical treatment.³⁷ HIFU is an option for neoadjuvant therapy against pancreatic cancer and prostate cancer.^{38,39} In preclinical data, higher concentration of chemotherapy in tumors is correlated with increased tumor response.⁴⁰ Research has reported that mild local hyperthermia induced by magnetic resonance guided-HIFU and lyso-thermosensitive liposomal doxorubicin could be used in the neoadjuvant chemotherapy of patients with breast cancer.⁴¹

As a taxane compound, PTX has been widely used for the chemotherapy of breast cancer. Through interfering with microtubule polymerization, PTX inhibits cell division and induces the apoptosis of tumor cells.⁴² Evidence has shown that PTX has potent therapeutic effect against breast cancer. However, approximately 50% of breast cancer patients develop significant resistance to PTX within 6–10 months of chemotherapy.⁴³ We therefore explored whether HIFU could promote the anti-tumor activity of PTX in breast cancer.

PTX binds to microtubules, stabilizes microtubule polymerization and promotes the prolongation of tubulin polymers.^{44,45} PTX polymerizes free microtubule unattached or preexisting ones in the microtubule tissue center.⁴⁶ The ability of cell division is inhibited by insufficient requirement for mitotic checkpoints.⁴⁷ PTX interferes with the kinetics of microtubule polymerization and delays mitotic progression, which ultimately leads to mitotic arrest and apoptosis.^{48,49} In this research, we investigated the effect of PTX or HIFU alone or the combination of both on breast cancer viability and apoptosis. Results demonstrated that treatment of HIFU had no effect on the viability or apoptosis of MDA-MB-231 cells, whereas inhibited the viability and enhanced the apoptosis of MCF-7 cells. The combination of HIFU and PTX conferred more potent inhibition of cell viability and induction of cell apoptosis than PTX alone in breast cancer cells.

It has been illustrated that treatment with PTX can induce the production of hydroperoxides and cause oxidative stress.⁵⁰ Oxidative stress is an imbalance of oxidative and anti-oxidant factors, which peroxidizes membrane lipids, damages organelles, and leads to cellular damage.⁵¹ It is caused by overproduced oxidative stress species or reduced anti-oxidant capacity.⁵² The key parameters are anti-oxidant enzymes such as SOD and CAT, as well as end products of lipid peroxidation such as MDA.⁵³ In this research, we also evaluated the oxidative stress in breast cancer cells. In MCF-7 and MDA-MB-231 cells, the administration of PTX inhibited the activity of SOD and CAT and increased the level of MDA.

Meanwhile, the combination of HIFU and PTX exerted more potent inhibition of SOD and CAT and elevation of MDA level than PTX alone in breast cancer cells. These results indicated that HIFU could increase the anti-tumor activity of PTX in breast cancer cells.

Furthermore, we also evaluated the effect of HIFU on PTX treatment in mice bearing MDA-MB-231 tumors. These *in vivo* experiments showed that the combination of HIFU and PTX significantly enhanced the effect of PTX in decreasing tumor size and increasing body weight and survival rate. Intravenously administered drugs can successfully accumulate in ECM-rich tumors exposed to noninvasive HIFU treatment. In this research, we also analyzed PTX pharmacokinetics in mice bearing MDA-MB-231 tumors. Based on these results, the accumulation of PTX in the tumor tissues was significantly enhanced by the treatment of HIFU.

However, there were some limitations in this study. The number of mice used in the *in vivo* experiments was relatively small. We have already observed the effects of HIFU on the anti-tumor activities of PTX in both cell and mouse models, but difference still exists between laboratory and clinical trials, and results in this research were not directly applicable in humans therefore should be further validated.

In conclusion, treatment of HIFU promoted the distribution of PTX and enhanced the anti-tumor activities of PTX in breast cancer cell lines and mouse model. These findings will provide an experimental basis for HIFU as an adjuvant therapy in patients with breast cancer.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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