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● *Original Contribution*

HIGH-INTENSITY FOCUSED ULTRASOUND-INDUCED, LOCALIZED MILD HYPERTHERMIA TO ENHANCE ANTI-CANCER EFFICACY OF SYSTEMIC DOXORUBICIN: AN EXPERIMENTAL STUDY

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Abstract—The aim of this study was to evaluate the enhancement of the efficacy of systemic doxorubicin by pulsed high-intensity focused ultrasound (HIFU)-induced, localized mild hyperthermia. For the *in vitro* study, the intranuclear uptake of doxorubicin by squamous cell carcinoma (SCC)-7 cells incubated at different temperatures was compared. For the *in vivo* study, mice with SCC-7 tumors were assigned to either the control, conventional hyperthermia, HIFU hyperthermia, doxorubicin-alone, conventional hyperthermia + doxorubicin or HIFU hyperthermia + doxorubicin group. Conventional hyperthermia was induced by immersing the tumor in warm water (42.5°C), and HIFU hyperthermia was induced by HIFU after optimizing the parameters with direct temperature measurements (frequency = 1 MHz, pulse repetition frequency = 5 Hz, power = 12 W, duty cycle = 50%). In the *in vitro* study, fluorescence was more intense at 42°C than at 37°C and was time dependent. In the *in vivo* study, tumor growth in the HIFU hyperthermia + doxorubicin group was most prominently suppressed with the highest apoptotic index compared with all other groups ($p < 0.05$). Pulsed HIFU-induced localized mild hyperthermia enhanced the anti-cancer efficacy of systemic doxorubicin more than conventional mild hyperthermia.
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Key Words: **Mild hyperthermia, High-intensity focused ultrasound, Doxorubicin, Chemotherapeutic efficacy.**

INTRODUCTION

Mild hyperthermia, raising the tissue temperature to 42°C–43°C, enhances the efficacy of anti-cancer chemotherapy (Bull 1984; Dahl 1988) by increasing blood flow (Song et al. 1996; Griffin and Corry 2009), increasing vascular permeability (Hariharan et al. 2007; Jain 2005; McDonald and Choyke 2003), improving cellular uptake of the drug (Gerweck 1985; Urano et al. 1999) and increasing direct cytotoxicity (Herman 1983a, 1983b). Increased blood flow induced by mild hyperthermia and vascular permeability improves drug delivery to the target tissue (Yudina and Moonen 2012). The delivered drug is taken up by malignant cells more readily, and the cells are more susceptible to the drug than in a normal environment.

Conventionally, whole-body systems, such as hot tubs, infrared rays or heating mats, have been used to induce mild hyperthermia. Conventional hyperthermia therapy using these non-specific external heat sources has drawbacks. Systemic hyperthermia can induce damage to normal tissues anywhere in the body (Overgaard and Suit 1979; Reinhold and Endrich 1986), and the toxic effect of concurrently administered chemotherapeutic agents is increased not only in diseased target cells, but also in normal cells (Bull et al. 1982; Kerner et al. 1999). Moreover, conventional methods are critically limited in clinical use, as treatment usually lasts 1 to 2 h and frequently deteriorates the general condition of the patient by elevating body temperature. Therefore, conventional mild hyperthermia in anti-cancer treatment has not been successful in clinical use.

As a result, the need for mild hyperthermia localized to the target tissue has arisen. Because localized hyperthermia, if feasible, would raise the temperature only at the target tissue (*i.e.,* malignant tumor), there may be

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few side effects while enhancing the efficacy of the chemotherapeutic agent. Therefore, to localize the heat as much as possible, researchers and industry have developed radiofrequency-based regional hyperthermic devices. Although these efforts have improved efficacy, they have not been able to completely avoid the limitations.

To completely overcome the limitations, an internal heating source seems to be the most effective solution. Internal heating can be induced by several clinically available therapeutic modalities, such as radiofrequency, microwaves, lasers and high-intensity focused ultrasound (HIFU). Among these, HIFU, which focuses high-energy ultrasound waves on a small spot, appears to be the optimal modality for inducing localized mild hyperthermia, because HIFU can localize heating with sharp margins, can be meticulously controlled by magnetic resonance (MR) thermometry and can be performed completely non-invasively (Curra et al. 2000; Kennedy 2005). HIFU is emerging clinically for ablation therapy, which induces coagulation necrosis by increasing temperatures to >65°C (Wijlemans et al. 2012). By adjusting ultrasound parameters, such as acoustic power and/or duty cycle, HIFU can elevate and maintain a stable temperature for a mild hyperthermia of 42°C–43°C (Vaezy and Zderic 2007). Hence, we anticipate that HIFU is the choice modality for inducing mild hyperthermia to enhance the efficacy of chemotherapy.

Therefore, the aims of this experimental study were to assess technical feasibility of HIFU-induced mild hyperthermia localized to the tumor area and to evaluate the enhancement of the efficacy of systemically administered doxorubicin by HIFU-induced localized mild hyperthermia as compared with conventional hyperthermia.

METHODS

Preparations of cells and tumor model

This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of our institute, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International, Protocol No. H-A9-003) and adheres to the guide of the Institute of Laboratory Animal Resource (ILAR).

The mouse squamous cell carcinoma cell line SCC-7 was from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in 150-cm^2 dishes containing 20 mL of culture medium at 37°C in a 5.0% CO_2 atmosphere with 95% relative humidity. The culture medium was Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (Gibco) and 1% antibiotics. When the cells were 70%–80% confluent, they were made to detach with a cell dissociation solution (Sigma, St. Louis, MO, USA) and collected. SCC-7 cells were counted (1×10^6) and suspended in 50 μL in DPBS (Gibco). The suspension was mixed with Matrigel (BD Bioscience, Bedford, MA, USA) in a 1:1 ratio. Therefore, the total cell suspension volume was 100 μL. Balb/c-nude mice (OrientBio, Sungnam, Korea) were used for *in vivo* experiments. The mice were 5-wk-old males and weighed 20 $\pm$ 3 g. For *in vivo* experiments, an SCC-7 cell suspension (1×10^6 cells in 100 μL) was subcutaneously injected into the lateral part of the right thigh, where breathing motions were minimal during HIFU therapy.

In vitro *assessment of cellular doxorubicin uptake*

To assess whether or not cellular doxorubicin uptake is enhanced by hyperthermia, four experimental groups were established: no hyperthermia (control) for 30 min (C-30, n = 3), no hyperthermia for 120 min (C-120, n = 3), hyperthermia for 30 min (H-30, n = 3) and hyperthermia for 120 min (H-120, n = 3).

SCC-7 cells were seeded (1×10^3 cells/well) in chamber slides. After 1 d of growth in a normal environment, cell medium was removed and the cells were washed once with phosphate-buffered saline (Gibco). Cell medium and doxorubicin (4 μg/mL) were then added. Immediately after addition of the doxorubicin, cells were incubated at 37°C or 42°C for 30 min or 120 min depending on the group. The doxorubicin solution was then removed, and cells were fixed in formaldehyde for 10 min. Cells were washed three times with phosphate-buffered saline, and the chamber was eliminated from slides. Fixed nuclei were stained with mounting solution with DAPI (4′,6-diamidino-2-phenylindole) (Sigma). Slides were covered immediately.

Doxorubicin fluorescence in each group was assessed by confocal microscopy (LSM 700, Carl Zeiss, Jena, Germany) to compare doxorubicin uptake (594 nm). Quantitative fluorescence analysis was conducted using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Fluorescence intensity was determined with the "color histogram" function.

HIFU system

An animal HIFU system (Therapy Imaging Probe System, Phillips Research, Briarcliff Manor, NY, USA) was used to generate pulsed HIFU. The natural focus and diameter of the annular array transducer (eight elements) were both 80 mm. The focal zone was $1.5 \times 1.5 \times 6.0$ mm (by the center sonication frequency of 1.0 MHz, at −6 dB). Tumor-bearing mice were fixed on a custom-made treatment bed equipped with an acoustic absorber. The HIFU transducer and part of the mouse's body were submerged in water so that ultrasound waves from the HIFU transducer could be