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BRIEF ARTICLE

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Anticancer Potency of Cytotoxic Drugs after Exposure to High-Intensity Focused Ultrasound in the Presence of Microbubbles and Hematoporphyrin

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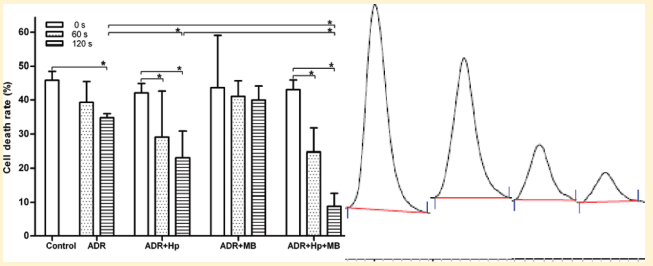
ABSTRACT: Chemotherapy is undertaken perioperatively to improve the efficacy of high-intensity focused ultrasound (HIFU) for solid tumors. HIFU at a sufficient intensity for tissue ablation has recently been applied for drug delivery; ultrasonic cavitation plays an important part in HIFU and drug delivery. Hematoporphyrin and microbubbles are adjuncts because they aid cavitation. The effect of HIFU (1.0 MHz; 12,999 W/cm^2 in continuous waves), in the presence of hematoporphyrin and/or microbubbles, on the anticancer potency of 5-fluorouracil, cisplatin, paclitaxel, mitomycin C or adriamycin, was investigated. Insonated adriamycin resulted in a lower death rate of human cancer cells HO-8910 (45.85 ± 2.65% vs 34.84 ± 1.21%, $p < 0.05$), which was exacerbated when employing hematoporphyrin (34.84 ± 1.21% vs 23.09 ± 7.82%, $p < 0.05$) or hematoporphyrin combined with microbubbles (34.84 ± 1.21% vs. 8.79 ± 3.69%, $p < 0.05$); the therapeutic activity was not affected when adding microbubbles alone. High-performance liquid chromatography detected a smaller peak area after subjecting adriamycin to HIFU with the use of hematoporphyrin alone or combined with microbubbles. The other drugs were not affected. Hematoporphyrin, microbubbles and adriamycin increased the throughput of hydroxyl radicals resulting from cavitation as determined by iodine and methylene blue assays. These data suggested that the anticancer activity of a drug may be decreased by HIFU exposure (particularly in the presence of hematoporphyrin and microbubbles). Cavitation produced reactive species that attacked drug molecules, thereby decreasing their antitumor potency; this process was enhanced if the drug itself generated free radicals under insonation.



KEYWORDS: focused ultrasound, anticancer drug, hematoporphyrin, microbubble, potency

INTRODUCTION

High-intensity focused ultrasound (HIFU) is a noninvasive technique for the treatment of solid tumors. This method involves the ablation of cancerous tissue *via* heat and cavitation. Anticancer agents are usually administered before, during, or after HIFU exposure. They serve as adjuvant chemotherapy and deactivate residual lesions.[1,2] However, the combination does not always lead to the expected synergism (unpublished data). This is consistent with the data of quantifying the interaction between an antitumor drug and ultrasound in sonochemotherapy against ovarian cancers: antagonism sometimes occurs.[3] A drug molecule may be attacked by reactive radicals, thereby decreasing therapeutic potency. Indeed, reactive species attributable to ultrasonic cavitation have been shown to decompose a molecule and/or change the conformation.[4,5] HIFU therapy involves an intensity of up to 20 kW/cm^2.[2,6,7] Such a high intensity undoubtedly results in cavitation within insonated tissues. HIFU treatment is lengthy; the use of microbubbles improves ablation efficiency (necrosis rate), and enhanced cavitation plays a very important part.[8−10] The therapeutic potency of a cytotoxic drug in the HIFU field therefore needs to be investigated.

HIFU provides a means of drug delivery in which cavitation is the determining mediator. Cavitation permeabilizes the cell membrane and vessels, thereby facilitating the transportation of drugs into tissues. Drugs can be efficiently delivered into the lesion because ultrasound can be focused on a target volume within the body without harming overlying tissues. Ultrasonic drug delivery usually employs a lower intensity, but a higher intensity within the range of HIFU ablation has been applied in recent studies (5000 W/cm^2 and 900 W/cm^2 in the investigations of Seip et al. and Chen et al., respectively).[11,12] These data imply that anticancer drugs can be concurrently released into tissues within the focus and within a definitive area outside

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the focus in HIFU exposure to improve therapeutic effects (i.e., intraoperative chemotherapy).[5] Microbubbles are used to assist ultrasonic delivery (i.e., coadministration of drugs and microbubbles). Ultrasound is applied to rupture microbubbles, thereby increasing the uptake of drugs into the lesion.[13−15]

Hematoporphyrin is a sonosensitizer for sonodynamic therapy. It increases the yield of free radicals, thereby enhancing ultrasound-induced tissue damage.[16] Chemotherapy is undertaken to improve the outcome in sonodynamic therapy for cancers.[17] Microbubbles enhance the sonodynamic effects *via* triggering acoustic cavitation.[18] Hematoporphyrin or microbubble benefits cavitation, so administering two agents concurrently will generate large amounts of reactive species. This must be considered if employing an anticancer drug.

The aim of the present study was to determine the effect of HIFU exposure on the anticancer potency of a cytotoxic agent in the presence of hematoporphyrin and/or microbubbles. Five frequently applied antitumor drugs (5-fluorouracil, adriamycin, mitomycin C, cisplatin and paclitaxel) were tested. Preliminary data suggested that only the potency of adriamycin was decreased.

MATERIALS AND METHODS

Chemicals. 5-Fluorouracil (5-FU) was purchased from Tianjin Jinyao Amino Acid Company (Tianjin, China). Cisplatin (DDP) was obtained from Qilu Pharmaceutical Company (Jinan, China). Paclitaxel (TAX) was purchased from Hainan Haiyao Company (Haikou, China). Mitomycin C (MMC) and adriamycin (ADR) were acquired from Zhejiang Hisun Pharmaceutical Company (Taizhou, China). The reference reagents for these anticancer drugs for high-performance liquid chromatography (HPLC) assay and hematoporphyrin (Hp) were from Fluka (Buchs, Switzerland). A microbubble (MB) agent for clinical contrast ultrasound images, SonoVue, was obtained from Bracco (Milan, Italy).

Cells. Human ovarian cancer cells HO-8910 were cultured in RPMI 1640 medium (HyClone Laboratories, Logan, UT) enriched with 10% fetal calf serum (MDgenics Incorporated, St. Louis, MO) at 37 °C and an atmosphere of 5% CO_2.[3]

Ultrasound Exposure. Ultrasonic exposure was undertaken as previously described with an ultrasound-guided HIFU system (JC; Chongqing Haifu Technology, Chongqing, China).[1,2,6,9,10] This device comprised a therapeutic ultrasound unit and a diagnostic ultrasound unit. The focal length of the 150 mm diameter therapeutic transducer was 150 mm, and it emitted continuous waves at a frequency of 1.0 MHz. An intensity of 12,999 W/cm^2 (I_{SATA}) was applied, which was within the range of clinical HIFU treatment.[2,6,7] Chemicals were added into a polyethylene chamber and then exposed to HIFU; insonation was guided with the diagnostic ultrasound unit (Figure 1).

Anticancer drugs were dissolved in phosphate-buffered saline (PBS), and 2-mL aliquots added into the chamber. Drugs, in the presence/absence of Hp and/or MB, were subjected to HIFU. The exposure duration was 60 or 120 s, and the control received sham insonation (0 s). Concentrations were 30 μg/mL, 20 μg/mL, 1000 μg/mL, 50 μg/mL, 60 μg/mL and 100 μg/mL for ADR, MMC, 5-FU, DDP, TAX and Hp, respectively; 30 μL SonoVue was added. The use of ultrasound achieved several-10-fold-higher local drug concentration in insonated tissues.[14,19] Thus, drugs in 10× peak plasma concentrations were insonated.[20] The Hp level was selected based upon the (i) human pharmacokinetics of the photosensitizer porfimer sodium and (ii) concentration of a sonosensitizer in cancer tissues being several times higher than that in plasma.[21,22]

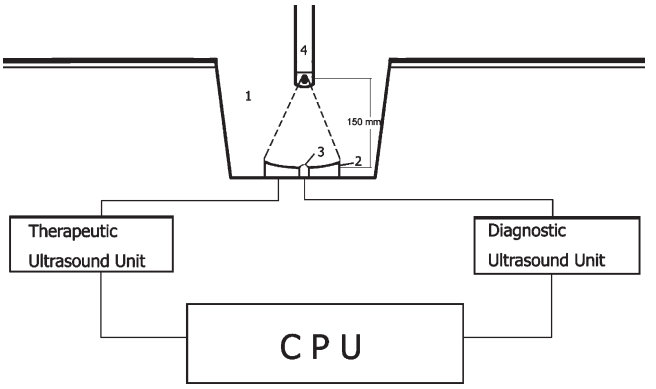

Figure 1. Illustration of the experimental setup. A clinical ultrasound-guided HIFU device was used. (1) tank containing degassed water; (2) therapeutic ultrasound transducer (150 mm diameter) with a focal length of 150 mm; (3) diagnostic ultrasound transducer; (4) chamber with chemicals. CPU, central processing unit.

Insonated drugs were filtered through a 0.22 μm membrane. They then underwent assays to measure the anticancer potency as well as HPLC. All procedures were undertaken in the dark to avoid the photoactivation and phototoxicity of Hp.

Anticancer Potency. The anticancer potency of a drug was determined using the tetrazolium assay.[23] Cells were subjected to a drug for 4 h. Chemicals were then washed away with PBS. Fresh medium was added; cells were seeded onto a 96-well plate and kept at 37 °C. Cell viability was determined at 48 h. The rate of cell death was used to assess the antitumor potency (cell death rate = 1-cell survival rate).

The final concentrations of ADR, MMC, 5-FU, DDP and TAX were 3 μg/mL, 2 μg/mL, 100 μg/mL, 5 μg/mL and 6 μg/mL, respectively. These were the peak plasma concentrations in clinical pharmacokinetics.[20] The present study indicated that the concentration and the area under the concentration−time curve (concentration × time) were consistent with those in humans.[24]

Determination of Drug Level Using HPLC. The level of ADR, 5-FU, MMC or TAX was determined using HPLC (1100; Agilent Technologies, Waldbronn, Germany).[25−28] A TC-C18 analytical column (150 × 4.6 mm; particle size, 5 μm; Agilent) was used and the drug quantified by measurement of the peak area.

DDP was analyzed with HPLC after derivatization with sodium diethyldithiocarbamate (DDTC) and nickel chloride as an internal standard. The ratio of DDTC(Pt)/DDTC(Ni) was used for quantification.[29]

Quantifying Ultrasonic Cavitation. Ultrasonic cavitation was quantified by measuring the yield of hydroxyl radicals with the iodine assay and methylene blue assay.[30,31]

In the iodine assay, drugs were added to a dosimeter solution (0.01 M potassium iodine, 0.1 M chloral hydrate, 1 M sodium chloride, 0.3 g/L amylum) with a final volume of 2 mL, and then insonated. Absorbance was determined by spectrophotometric means at 555 nm (8453; Hewlett-Packard Company, Waldbronn, Germany).

In the methylene blue assay, chemicals were added to methylene blue solution (20 μM) to a final volume of 2 mL, and then exposed to HIFU. Absorbance was measured at 664 nm.

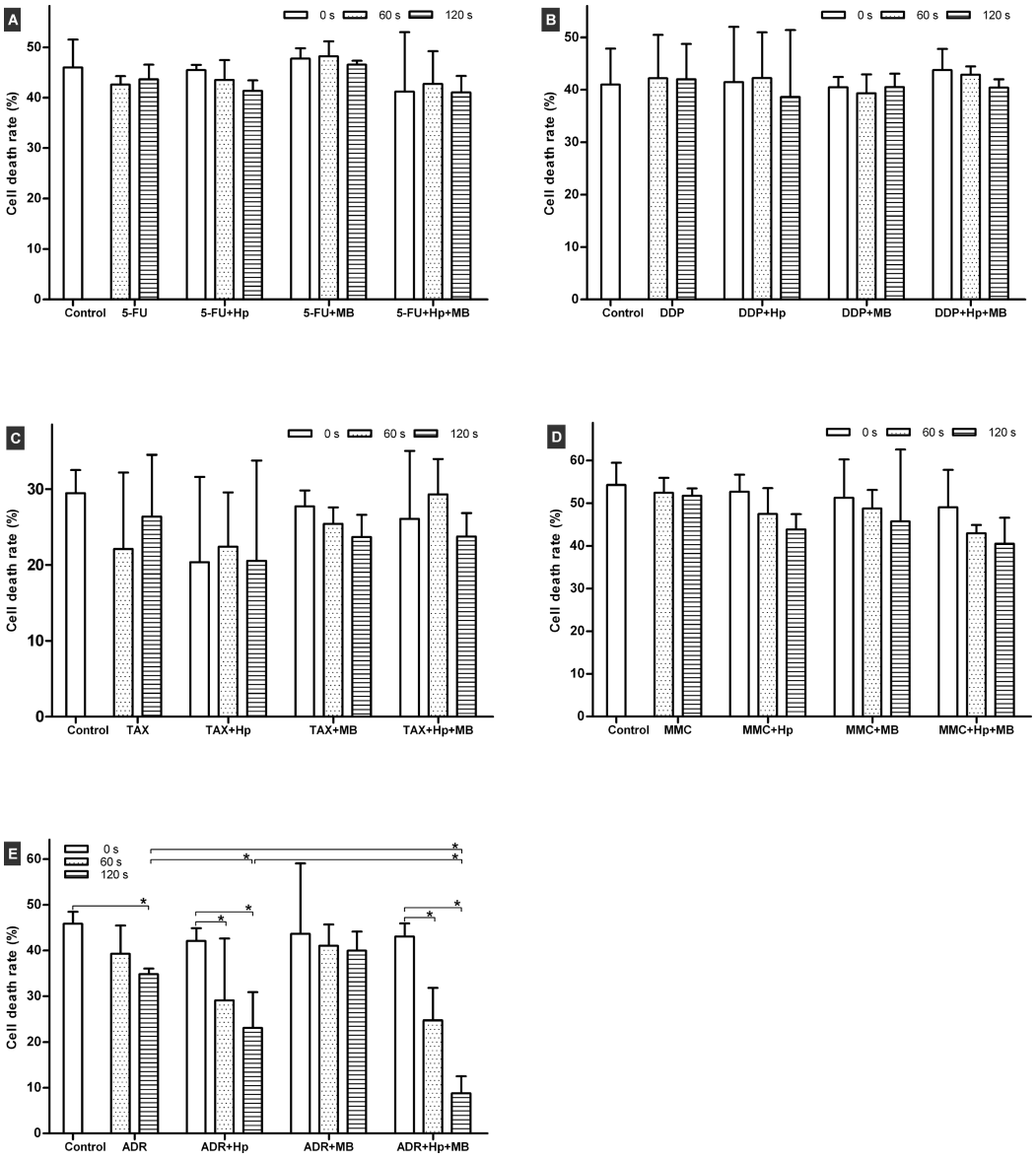

Figure 2. Cell-death rates attributable to insonated 5-fluorouracil (A), cisplatin (B), paclitaxel (C), mitomycin C (D) and adriamycin (E) (n = 5). A lower fraction was observed after subjecting adriamycin to HIFU in the presence of hematoporphyrin, or hematoporphyrin combined with microbubbles. There were no variations in the other drugs tested. A drug that underwent 0 s insonation alone served as the control. 5-FU, 5-fluorouracil; DDP, cisplatin; TAX, paclitaxel; MMC, mitomycin C; ADR, adriamycin; Hp, hematoporphyrin; MB, microbubble. *$p < 0.05$.

The decrease in absorbance reflected the throughput of hydroxyl radicals.

Concentrations of anticancer drugs were identical to those in the anticancer potency assay. The level of Hp was 100 μg/mL and 30 μL SonoVue was used.

Statistical Analyses. Data were processed with the statistical software SAS (SAS Institute, Cary, NC). Analysis of variance (ANOVA) was used. $p < 0.05$ was considered significant.

RESULTS

Anticancer Potency. Without HIFU exposure, the cell-death rate attributable to a cytotoxic drug was not modulated with the addition of MB and/or Hp.

The cell-death fraction due to 5-FU, MMC, DDP or TAX was not decreased after subjecting an antitumor drug to insonation in the presence of Hp, MB or both agents ($p = 0.1392$, $p = 0.1183$, $p = 0.9075$, $p = 0.2906$) (Figure 2A−D).

Anticancer potencies of ADR varied between groups ($p < 0.0001$), and there was no difference at 0 s ($p = 0.9881$). ADR at 120 s insonation led to a lower cell-death rate ($p < 0.05$). Rates in the ADR + Hp and ADR + Hp + MB groups were decreased ($p < 0.05$), and a longer duration of exposure resulted in a lower fraction; rates were 24.71 ± 7.13% at 60 s and 8.79 ± 3.69% at 120 s in the ADR + Hp + MB group ($p < 0.05$). The cell-death rate in the ADR + Hp + MB group was less than that in the ADR + Hp group (8.79 ± 3.69% vs 23.09 ± 7.82% at 120 s, $p < 0.05$). Anticancer potency was not affected in the ADR + MB group (Figure 2E).

Drug Molecule. There were no differences in the peak areas of 5-FU, MMC or TAX between groups ($p = 0.4274$, $p = 0.3770$, $p = 0.3043$) (Figure 3A,C,D). Ratios of DDTC(Pt) to DDTC(Ni)

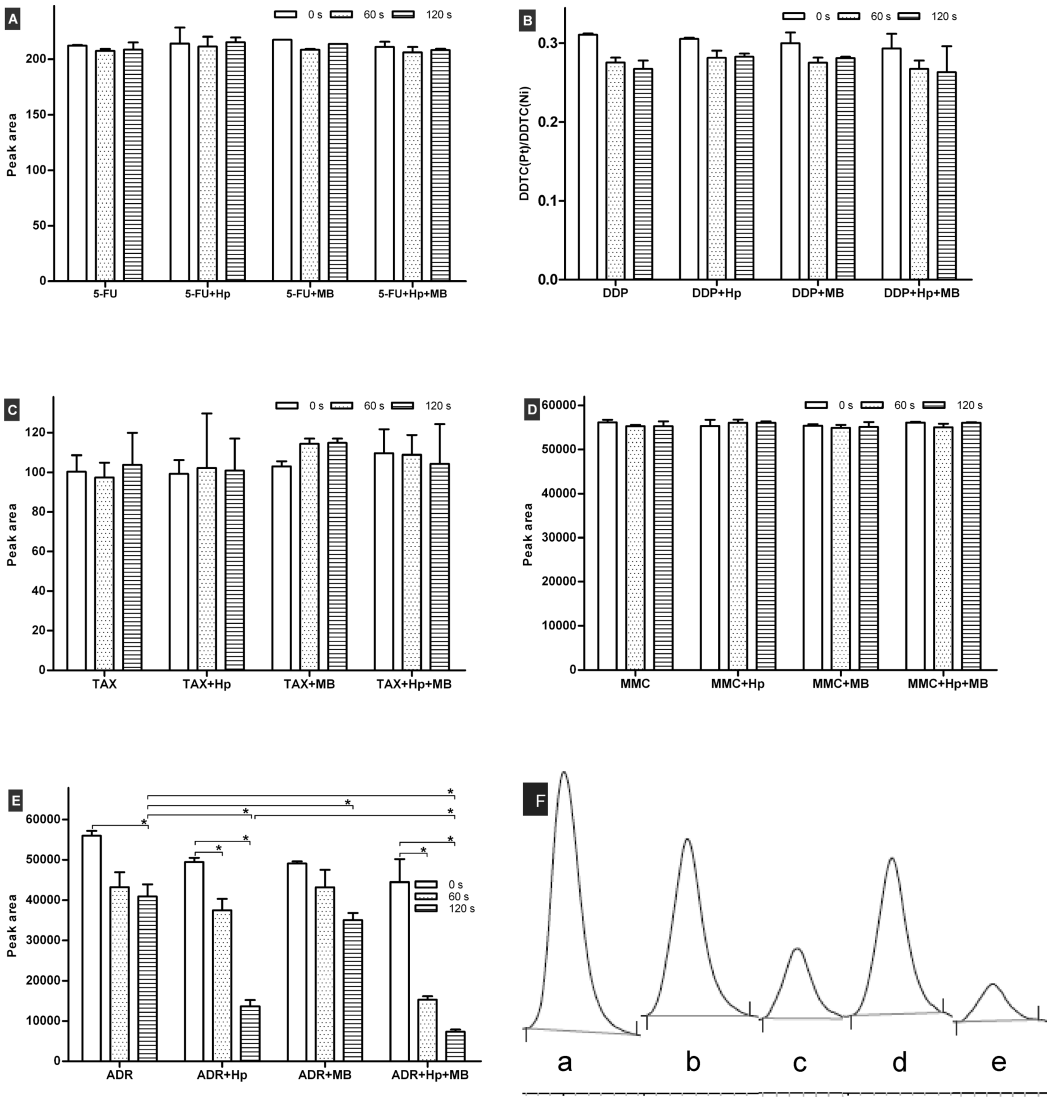

Figure 3. Contents of 5-fluorouracil (A), cisplatin (B), paclitaxel (C), mitomycin C (D) and adriamycin (E), and the chromatogram of adriamycin (F) after exposure to HIFU as determined by HPLC ($n = 3$). No variations were detected in 5-fluorouracil, cisplatin, paclitaxel and mitomycin C, and the exposure of adriamycin to HIFU resulted in a lower peak area. Chromatogram for adriamycin in the control (a), ADR (b), ADR + Hp (c), ADR + MB (d) and ADR + Hp + MB (e) groups at 120 s. 5-FU, 5-fluorouracil; DDP, cisplatin; TAX, paclitaxel; MMC, mitomycin C; ADR, adriamycin; Hp, hematoporphyrin; MB, microbubble. $^*p < 0.05$.

were not modulated at 60 s ($p = 0.5513$) and 120 s ($p = 0.6239$) (Figure 3B).

The peak area of ADR did not vary between groups at 0 s ($p = 0.0768$). HPLC detected a slight decrease in the peak area after 120 s insonation ($p < 0.05$). The area was decreased in the ADR + Hp + MB group at 60 s ($p < 0.05$) and in the ADR + Hp, ADR + MB and ADR + Hp + MB groups at 120 s ($p < 0.05$). The value in the ADR + Hp + MB group was less than that in the ADR + Hp group at 120 s (7324 ± 532 vs 13598 ± 1593, $p < 0.05$) (Figure 3E,F).

Yield of Free Radicals. *5-FU.* The iodine assay detected higher absorbance values in other groups at 60 s ($p < 0.05$) and in the Hp, MB, 5-FU + Hp, 5-FU + MB and 5-FU + Hp + MB groups at 120 s ($p < 0.05$) compared with ultrasound (US) alone. In the methylene blue assay, the decrease in absorbance in each group was higher than that in the US group at 60 s ($p < 0.05$) or 120 s ($p < 0.05$) (Figure 4A).

DDP. The absorbance in the Hp, DDP + Hp or DDP + Hp + MB group was higher than that in the US group in the iodine assay ($p < 0.05$). Hydroxyl throughput was increased in other groups compared with that in the US group in the methylene blue assay ($p < 0.05$) (Figure 4B).

TAX. Higher absorbance values were detected in the Hp, TAX + Hp and TAX + Hp + MB groups at 60 s ($p < 0.05$) and in the Hp, MB, TAX + Hp and TAX + Hp + MB groups at 120 s ($p < 0.05$) compared with the US group in the iodine assay. The methylene blue assay showed higher decreases in absorbance in the Hp, MB, TAX + Hp and TAX + Hp + MB groups ($p < 0.05$) (Figure 4C).

MMC. Absorbance values were increased in the Hp, MMC + Hp and MMC + Hp + MB groups at 60 s ($p < 0.05$), and in the Hp, MB, MMC + Hp and MMC + Hp + MB groups at 120 s ($p < 0.05$) compared with insonation alone in the iodine assay. In the methylene blue assay, hydroxyl throughput was increased in the

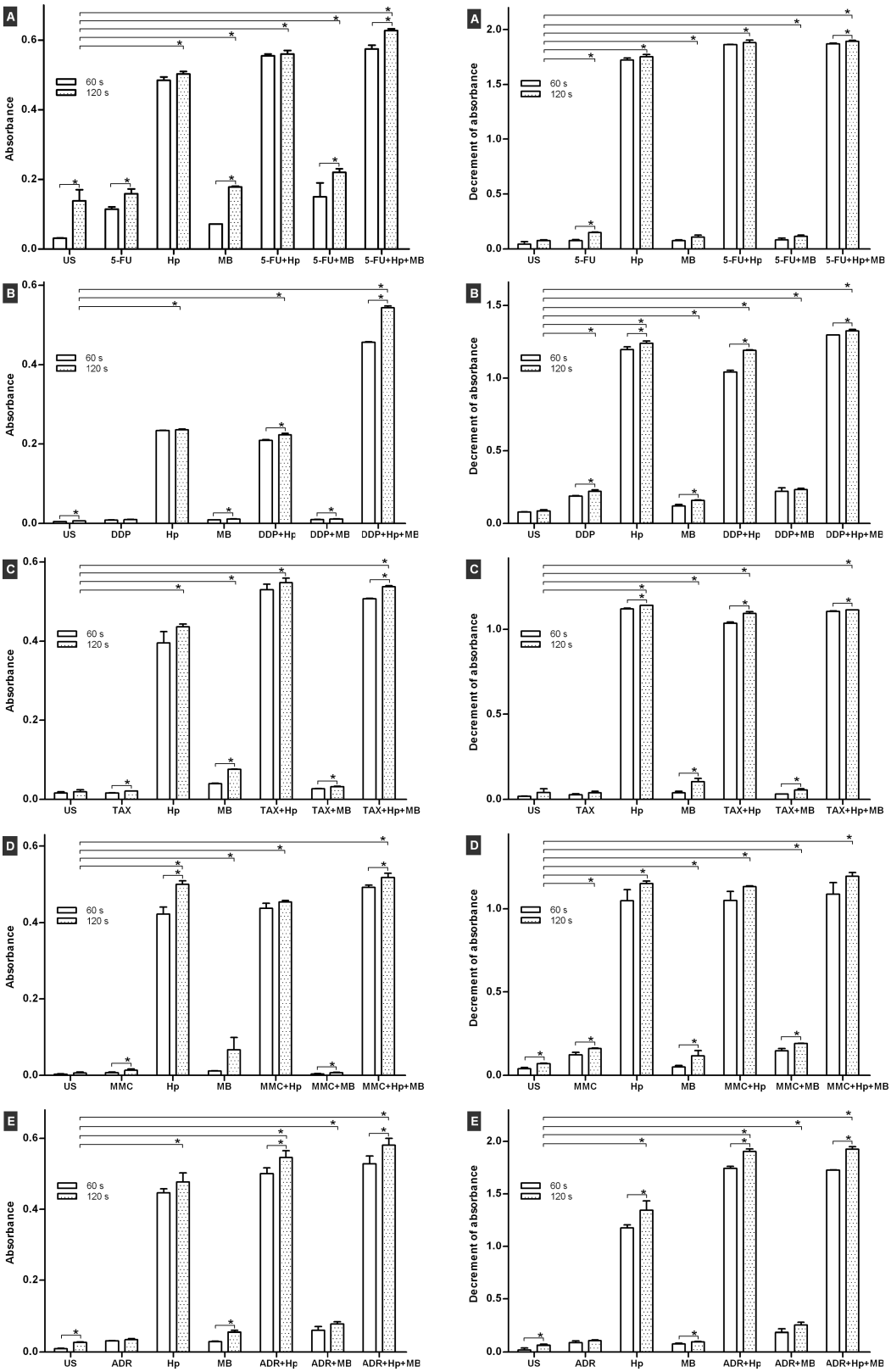

Figure 4. Yield of hydroxyl radicals in 5-fluorouracil (A), cisplatin (B), paclitaxel (C), mitomycin C (D) and adriamycin (E), determined with the iodine (left column) or methylene blue assay (right column) (n = 3). 5-FU, 5-fluorouracil; DDP, cisplatin; TAX, paclitaxel; MMC, mitomycin C; ADR, adriamycin; Hp, hematoporphyrin; MB, microbubble; US, ultrasound. *$p < 0.05$.

Hp, MMC + Hp, MMC + MB or MMC + Hp + MB group at 60 s ($p < 0.05$), and in all other groups at 120 s ($p < 0.05$) (Figure 4D).

ADR. Absorbance values were increased in the Hp, ADR + Hp, ADR + MB and ADR + Hp + MB groups at 60 s ($p < 0.05$) or

120 s ($p < 0.05$) compared with insonation alone in the iodine assay. In the methylene blue assay, hydroxyl throughput was increased in each group at 60 s ($p < 0.05$), and in the Hp, ADR + Hp, ADR + MB or ADR + Hp + MB group at 120 s ($p < 0.05$), compared with the US group (Figure 4E).

DISCUSSION

The anticancer potency of 5-FU, DDP, MMC or TAX was not affected by insonation, but that of ADR was decreased (particularly in the presence of Hp and MB). Insonation of 120 s duration decreased potencies by 24.00%, 45.12% and 79.57% in the ADR, ADR + Hp and ADR + Hp + MB groups, respectively. In ultrasonic chemotherapy, there is a threshold−dose effect in some cell types. A synergism occurs only if an anticancer drug reaches a critical level, and a lower dose leads to an addition or even an antagonism (i.e., the drug level is a determinant for the interaction between a drug and ultrasound).[3] The intensity adopted in the present study was within the range of HIFU therapy. The present data therefore suggested that some anticancer agents should not be employed for intraoperative HIFU chemotherapy; a combination effect cannot be achieved while using those drugs. Cavitation is dependent upon the HIFU intensity, insonation time and sonosensitizer. In some regimens, a higher intensity (up to 20,000 W/cm^2) is applied, and treatment lasts for several hours.[2,6,7] Such regimens lead to stronger cavitation, thereby increasing the risk of decreasing the effect of a drug. The critical acoustic parameter for deactivating a drug should be investigated because such information can be used to select anticancer agents in HIFU therapy.

Ultrasonic delivery of drugs usually involves a lower intensity, so the potency of a drug may not be affected. This concept is consistent with the literature: insonation has been shown to increase the intracellular accumulation of ADR, thereby enhancing cytotoxicity.[13,32,33] HIFU was recently applied for drug delivery at a higher intensity for tissue ablation.[11,12] The effect on the therapeutic potency of a drug should therefore be considered.

Microbubbles can enhance HIFU ablation/drug delivery and sonodynamic therapy. Chemotherapy is usually employed in cancer treatment with HIFU or sonodynamic therapy. Porphyrin has an affinity with cancer tissues. It can be linked to microbubble-encapsulated/free drugs to increase tissue selectivity in ultrasonic delivery.[5,34] The effect of HIFU on the potency of a cytotoxic drug in the presence of Hp and MB was therefore investigated in the present study. The anticancer activity of ADR was decreased with the addition of Hp,and the lowest cell-death rate occurred when adding Hp and MB concurrently. These findings suggested that some cytotoxic agents should not be employed in the hematoporphyrin−microbubble−anticancer drug−HIFU regimen. The present data were limited by the use of free Hp. The therapeutic potency of a drug should be investigated if Hp is linked to (microbubble-encapsulated) drugs (i.e., hematoporphyrin−microbubble−anticancer drug−HIFU regimen).

HPLC detected lower peak areas in the ADR + Hp and ADR + Hp + MB groups. These were in accordance with alterations in anticancer activity. HIFU, in the presence of Hp and MB, decreased the active molecules of ADR debasing the antitumor potency. Only a marginal reduction in peak area in the ADR + MB group at 120 s was observed, which may explain why therapeutic potency was not affected. The anticancer potency of ADR was decreased in the ADR group but almost preserved in the ADR + MB group. The data suggested that coadministration of an anticancer drug with microbubbles may benefit intraoperative chemotherapy in cancer HIFU treatment. The underlying mechanisms remain unclear and should be explored. Investigations under much lower intensities demonstrated that the efficiency of enhancing a cytotoxic agent with (microbubble-assisted) ultrasound was reliant upon cell type and the drug, and on occasion even lacked potentiation.[3,35] Anticancer drugs therefore should be individually selected for a specific cancer when applying chemotherapy during HIFU therapy.

Cavitation was quantified by measuring hydroxyl throughput. That the addition of Hp increased the yield of hydroxyl confirmed that Hp was an efficient sonosensitizer.[5,16] Interestingly, a smaller increase was observed when using MB. Investigations under a lower intensity and shorter duration of exposure have shown that microbubbles remarkably enhance cavitation.[5,36] The intensity in the present study was very high, so microbubbles were ruptured rapidly. Exhaustion of the supply of microbubbles limited the increase in the number of free radicals. This suggested that the intensity should be optimized in drug delivery using microbubble-HIFU. An excessively high intensity did not benefit cavitation.

The absorbance was increased in the ADR group at 60 s or in the MMC group at 120 s, compared with the US group. This observation was consistent with another study: ADR and MMC enhanced the production of free radicals attributable to cavitation.[37] However, only ADR was affected by insonation. A higher absorbance value indicated that ADR was a stronger cavitation sensitizer. An unsaturated bond and a hydroxyl radical can be oxidized by reactive species resulting from ultrasonic cavitation;[4,38] DNA experiments manifested that ultrasound frequently splits the carbon−oxygen bond.[39] A molecule of ADR contains hydroxyl ions and double bonds, making it prone to such bond splitting. Furthermore, ADR itself increased the yield of reactive radicals under sonication, thereby intensifying and accelerating the breakage of a molecule (a process similar to self-catalysis). MMC was a weaker sensitizer and limited the reaction rate. Thus, no variations in anticancer potency were observed in the present study with respect to MMC. Unsaturated bonds lie between carbon and nitrogen atoms in a molecule of 5-FU, and DDP lacks a vulnerable ion, thereby reducing the susceptibility to ultrasound. TAX was not deactivated even though it had vulnerable groups. Low solubility in water may provide a shelter: few molecules can be attacked by hydrophilic free radicals. The stability of a molecule within the HIFU field, therefore, is reliant upon the molecular structure.

Adriamycin favored cavitation increasing the throughput of free radicals. Investigations under lower intensities suggested that the enhancement of ADR with ultrasound was partly mediated *via* enhancing cavitation by ADR increasing the production of free radicals; reactive species were involved in the antitumor action of ADR and increased the sensitivity of cells to the mechanical stress of ultrasound.[32,33] More free radicals were generated when insonating ADR with HIFU, which may result in more severe damage to cells if cells were in the medium. However, as demonstrated in the present study, abundant free radicals induced by ADR-enhanced HIFU cavitation can attack ADR molecules, thereby decreasing anticancer potency. This should be considered because the cytotoxicity of ADR is free radical-independent in some cell types.[40] For a particular cancer in which free radicals play a critical part in the action of ADR, the

two aspects mentioned above should be considered when applying ADR for HIFU therapy *in vivo*.

In summary, our preliminary data suggested that the anticancer activity of a drug may be decreased in the HIFU field (particularly in the presence of Hp and/or MB). Such drugs should not be employed for intraoperative HIFU chemotherapy and HIFU-mediated drug delivery. Hp- and MB-enhanced ultrasonic cavitation produces a large amount of free radicals which attack a drug molecule, thereby decreasing antitumor potency.

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Author Contributions

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