Original Article

Ultrasound-stimulated Microbubbles for Treatment of Pancreatic Cancer Cells with Radiation and Nanoparticles: *In vitro* Study

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Abstract

Purpose: This study aims to investigate the radiation enhancement effects of ultrasound-stimulated microbubbles (USMB) with X-rays and nanoparticles on pancreatic cancer cells *in vitro*. **Methods:** Sonazoid™ microbubbles were used for USMB treatment with a commercially available ultrasound unit. The characterization of the microbubbles before and after ultrasound exposure with different mechanical parameters was evaluated microscopically. Two pancreatic cancer cell lines, MIAPaCa-2 and PANC-1, were treated with different concentrations of microbubbles in combination with 150 kVp X-rays and hydrogen peroxide-modified titanium dioxide nanoparticles. Cell viability was evaluated using a water-soluble tetrazolium dye and a colony formation assay. In addition, intracellular reactive oxygen species (ROS) induced by the combined treatment were assessed. **Results:** The number of burst microbubbles increased with ultrasound's higher mechanical index and the exposure time. A significant radiation enhancement effect with a significant increase in ROS levels was observed in MIAPaCa-2 cells treated with USMB and 6 Gy X-rays, whereas it was not significant in PANC-1 cells treated with the same. When a higher concentration of USMB was applied with X-rays, no radiation enhancement effects were observed in either cell line. Moreover, there was no radiation enhancement effect by USMB between cells treated with and without nanoparticles. **Conclusions:** The results indicate that USMB treatment can additively enhance the therapeutic efficacy of radiation therapy on pancreatic cancer cells, while the synergistic enhancement effects are likely to be cell type and microbubble concentration dependent. In addition, USMB did not improve the efficacy of nanoparticle-induced radiosensitization in the current setting.

Keywords: Microbubbles, nanoparticles, radiation therapy, radiosensitizer, ultrasound

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Introduction

Microbubbles have been used clinically as contrast agents to enhance ultrasound diagnostic imaging. Ultrasound pulses vibrate or disrupt the microbubbles, resulting in scattered echoes that can be visualized through blood flow, the cardiovascular system and the perfusion of blood in organs such as the liver and pancreas.[1-3] Clinically available microbubbles currently contain gas with low solubility and high molecular weight, namely octafluoropropane or sulfur hexafluoride, encapsulated by lipid or albumin shells to enhance their stability.[4,5] While microbubbles are commonly used for diagnostic purposes, they also show potential for therapeutic applications in cancer treatment when combined with ultrasound stimulation. Ultrasound and microbubbles can interact mechanically, causing microbubble cavitation and destruction that can generate strong forces in the vicinity of cells. This can lead to cell membrane permeabilization and disruption, a process known as sonoporation.[6-8] Although the mechanisms of ultrasound and microbubble-induced

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sonoporation are complex due to the multiple factors involved in physical, chemical, and biological reactions of microbubbles,[9] this phenomenon can induce various effects on cells, including apoptotic and necrotic cell death.[6,7,10,11] Ultrasound-stimulated microbubbles (USMBs) have also been studied for their ability to facilitate drug or gene delivery into cells through small pores in the cell membrane formed by sonoporation.[6,9,12]

The USMB has been explored as radiosensitizers for radiation therapy, one of the most common treatments for cancer. X-rays, which are widely used in current radiation therapy, can ionize water molecules and induce free radicals and reactive oxygen species (ROS), which can cause intracellular DNA damage and cell death.[13,14] Radiosensitizers are designed to enhance the cytotoxic effects of radiation on cancer cells while minimizing the dose to surrounding normal tissues. Nanoparticles, for example, have been extensively studied as potential radiosensitizers because they can generate secondary electrons and ROS when interacting with X-rays, which can enhance the cellular damage caused by X-rays.[15] With regard to USMB, several studies have reported the combined effect of USMB and X-rays as radiosensitizers, both *in vitro* and *in vivo*.[16-20] Our recent study evaluated cell viability *in vitro* using three different cell lines, human umbilical vein endothelial cells (HUVECs), metastatic follicular thyroid carcinoma cells (FTC-238), and nonsmall-cell lung carcinoma cells (NCI-H727). The results showed that cell viability decreased in all cell lines treated with the combination of USMB and X-rays compared to those treated with X-rays alone; however, the decrease was significant only for the NCI-H727 cells.[21] The study indicates that the radiation enhancement effect of USMB may be dependent on cell type and radiation doses, which is consistent with a second study.[22] Nevertheless, there is still a lack of data to fully understand the impact of USMB on radiation enhancement, as previous studies have been conducted by a limited number of research groups under restricted experimental conditions.

This study aims to verify the potential radiation enhancement effect of USMB treatment on pancreatic cancer cells *in vitro* using commercially available microbubbles and clinical hardware. Pancreatic cancer cells were selected for this study as they are one of the target cancers for radiation research due to their radiation resistance.[23] Moreover, we evaluated the efficacy of USMB in combination with nanoparticles as radiosensitizers to determine whether USMB can enhance the radiation effect of nanoparticles in cells.

Methods

Preparation of microbubbles

Sonazoid™ perfluorobutane microbubbles (GE Healthcare Japan, Tokyo, Japan) were prepared according to the manufacturer's instructions before each experiment. The microbubble solution was diluted with phosphate-buffered saline (PBS, Nakalai Tesque, Kyoto, Japan) to achieve the desired concentration for each experiment. The size of microbubbles was determined by randomly measuring 120 bubbles from three different lots of Sonazoid™ vials using light microscopy images (BZ-9000, KEYENCE, Osaka, Japan) and the ImageJ software (version 1.51k, Wayne Rasband, National Institution of Health, USA). The number of microbubbles was also obtained by counting bubbles in areas of 6.25×10^{-3} mm^3 randomly selected from 20 light microscopy images of three different lots of vials.

Ultrasound exposure

The LOGIQ E portable ultrasound unit, equipped with the 4C-RS transducer (GE Healthcare Japan, Tokyo, Japan), was used to stimulate microbubbles. Ultrasound was applied to microbubbles contained in a 24-well flat bottom plate from the bottom using Aquasonic 100 Ultrasound Transmission Gel (Parker Laboratories, NJ, USA), as shown in Figure 1a. During the exposure, the transducer was moved across the sample position, and the color of the microbubble solution changed from milky white to transparent, as shown in Figure 1b. The number of burst microbubbles was counted using a light microscope after ultrasound exposure to 0.2% v/v microbubbles at different frequencies, mechanical indices (MIs), and exposure times. This was done to determine the optimal ultrasound exposure parameters, where the majority of microbubbles can be burst by the ultrasound stimulation for cell experiments.

Cell culture

The MIAPaCa-2 and PANC-1 human pancreatic cancer cell lines were obtained from the American Type Culture Collection, Manassas, VA, USA. MIAPaCa-2 cells were cultured in E-MEM media (FUJIFILM Wako Pure Chemical, Osaka, Japan) with 1% MEM nonessential amino acids solution (Nakalai Tesque), while PANC-1 cells were cultured in RPMI-1640 media (FUJIFILM Wako Pure Chemical). The culture media were supplemented with 10% fetal bovine serum (Sigma-Aldrich, MO, USA) and 1% penicillin-streptomycin (Nakalai Tesque). The cells were maintained at 37°C with 5% CO_2 in T25 cell culture flasks and subcultured when the cultures reached 80%–90% confluence.

Ultrasound-stimulated microbubbles treatment

Cell viability was determined using a water-soluble tetrazolium (WST) dye (Cell Counting Kit-8, Dojindo Laboratories,

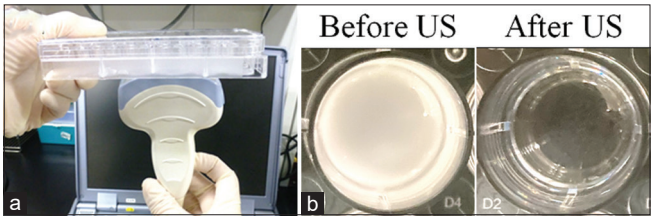

Figure 1: (a) Image of the ultrasound exposure setup for samples in a 24-well flat bottom plate. (b) The color of the microbubble solution was changed from milky white to transparent by ultrasound exposure. US: Ultrasound

Kumamoto, Japan). The cells were harvested from the culture flasks using trypsin-EDTA (Gibco, NY, USA) and seeded at a density of 2.0×10^5 cells/well into 24-well low-attachment surface plates. Different concentrations of microbubbles were added to the cells in a total volume of 600 µL/well, followed by direct application of ultrasound to the plates with the cells in suspension. The cells were washed twice with PBS and then seeded into a 96-well plate with 100 µL of fresh tissue culture media per well. MIAPaCa-2 and PANC-1 cells were seeded at a density of 1.4×10^4 and 2.4×10^4 cells per well, respectively. After incubating for 24 h, the media were removed, and 10 µL of WST reagent was added to each well along with 100 µL of fresh medium. The cells were then returned to the incubator for 4 h to allow for WST development. The absorbance was measured at 450 nm using the EnSpire multimode plate reader (PerkinElmer, MA, USA). Cell viability was calculated as a percentage relative to the untreated control group, which was considered 100%. In addition, the treated cells were stained using 0.5% Trypan blue dye (Nakalai Tesque) immediately after the ultrasound exposure to observe dead cells under a light microscope.

Combined treatment with ultrasound-stimulated microbubbles and radiation

A colony formation assay was used to determine the radiation enhancement effect of the USMB. Following USMB treatment, cells were washed twice with PBS and seeded into a 6-well plate with 3 mL/well of fresh tissue culture media before being exposed to either 3 or 6 Gy of X-rays. X-ray irradiation was performed using an MBR-1505R2 X-ray irradiator (Hitachi Power Solutions, Ibaraki, Japan) at a voltage of 150 kVp and a current of 5 mA with a dose rate of approximately 1.2 Gy/min at the target position. The cells were allowed to grow for 10 days. Afterward, colonies were fixed in a fixation solution for 30 min and stained with 0.5% crystal violet. The stained colonies were counted independently by two individuals. Cell survival curves for each treatment group were obtained as a fraction relative to the unirradiated control group. Dose enhancement factors (DEFs) were calculated as the ratio of the area under the survival curve for cells treated with X-rays alone relative to cells treated with USMB and X-rays.

Reactive oxygen species evaluation for ultrasound-stimulated microbubbles and radiation treatments

Intracellular ROS levels were measured using carboxy 2',7'-dichlorodihydrofluorescein diacetate (c-H_2DCFDA, Thermo Fisher Scientific, MA, USA). The detached cells were incubated with 20 µM c-H_2DCFDA in Hank's balanced salt solution (Nakalai Tesque) for 30 min. Subsequently, the cells were resuspended in 0.01% v/v microbubble solution diluted with PBS and exposed to ultrasound and 6 Gy X-rays. After the treatment, the cells were transferred into 96-well plates at a density of about 3.0×10^4 cells/well with 100 µL/well of PBS. The fluorescence intensity of c-H_2DCFDA was measured using the EnSpire multimode plate reader (PerkinElmer) with excitation and emission wavelengths of 495 and 525 nm, respectively. The obtained fluorescence intensities were corrected using those of nontreated cells.

Triple treatment with ultrasound-stimulated microbubbles, radiation, and nanoparticles

Hydrogen peroxide-modified titanium dioxide nanoparticles (TiO_xNPs) at a final concentration of 1.0 mg/mL were added to cells with microbubbles before ultrasound exposure. The characterization and radiosensitizing effects of the nanoparticles have been demonstrated in our previous studies.[24,25] After the ultrasound stimulation, the cells were incubated at 37°C with 5% CO_2 for 1 h, washed twice with PBS, and then seeded into a 6-well plate with 3 mL/well of fresh tissue culture media. The cells were then exposed to 3 and 6 Gy X-rays and allowed to grow for 10 days. The cell survival curves were obtained using the colony formation assay, as previously described.

Statistical analysis

All cell experiments were conducted with at least three independent replicates, and the data were expressed as mean ± standard deviation calculated from the replicates. Statistical comparisons were performed using Student's *t*-test in SPSS Statistics software (version 28.0.1.0, IBM, Armonk, NY, USA), and $P < 0.05$ was considered statistically significant.

Results

Characterization of sonazoid™ microbubbles

Microscopy images confirmed that the Sonazoid™ microbubbles were spherical and had a mean diameter of 2.0 ± 0.6 µm with a narrow unimodal size distribution [Figure 2]. The number of microbubbles in a 0.01% v/v solution was approximately 1.2×10^4 particles per µL. These results were consistent with previous studies.[26,27]

Effect of ultrasound exposure settings on microbubble bursting

Microbubbles were exposed to ultrasound at different frequencies ranging from 2.0 to 5.5 MHz, with different MI ranging from 0.4 to 1.0. The number of microbubbles that burst after 3 min of ultrasound stimulation increased with the value of MI in each setting [Figure 3a]. The mean burst rate of microbubbles stimulated with 4.0 MHz and an MI of 1.0 was 97.2% ± 1.7%. When the exposure time was reduced using this setting, the mean burst rates decreased to 87.6% and 84.0% for exposure times of 1 and 2 min, respectively, as shown in Figure 3b. Therefore, for the following cell experiments in this study, the ultrasound setting with 4.0 MHz, an MI of 1.0, and an exposure time of 3 min was used as it could burst more than 90% of microbubbles.

Cytotoxicity of ultrasound-stimulated microbubbles treatment

The viability of MIAPaCa-2 and PANC-1 cells exposed to 0.01, 0.04, 0.1, and 0.4% v/v microbubbles and ultrasound was assessed using a WST assay. There was no significant decrease in cell viability for either cell line when exposed to microbubbles alone, even at the highest concentration of 0.4% v/v [Figure 4a and b]. Furthermore, no cytotoxicity was observed in cells treated with ultrasound exposure alone

in the absence of microbubbles. When the microbubbles were stimulated with ultrasound, cell viability significantly decreased in a microbubble concentration-dependent manner in both cell lines. The viability of MIAPaCa-2 and PANC-1 cells treated with 0.01% USMB was 88% and 81%, respectively, compared to 34% and 16% for cells treated with 0.4% USMB. The number of dead cells increased when the cells were treated with USMB, as confirmed by trypan blue staining [Figure 4c].

Radiation enhancement effect of ultrasound-stimulated microbubbles combined with X-rays

The effectiveness of USMB on the X-ray dose was evaluated using a colony formation assay. The viability of cells treated with 0.01% and 0.4% USMB decreased when combined with 3 and 6 Gy X-rays, compared to those treated with X-rays alone [Figure 5a and b]. The viability of MIAPaCa-2 cells was significantly lower when treated with 0.01% USMB and 6 Gy X-rays compared to those treated with 0.4% USMB and the same dose of X-rays, whereas viability decreased with increasing X-ray dose and microbubble concentration in PANC-1 cells. The survival curves showed that USMB had a significant radiation enhancement effect on MIAPaCa-2 cells treated with 6 Gy X-rays and on PANC-1 cells treated with 3 Gy X-rays when the concentration of microbubbles was 0.01% [Figure 5c and d]. However, MIAPaCa-2 cells treated with 3 Gy X-rays and PANC-1 cells treated with 6 Gy X-rays at 0.01% USMB showed only a slight enhancement, which was not statistically significant. No radiation enhancement effect of USMB with X-rays was observed in MIAPaCa-2 and PANC-1 cells when the concentration of microbubbles was 0.4% v/v. The DEFs for each treatment group were summarized in Table 1.

Reactive oxygen species generated by ultrasound-stimulated microbubbles with X-rays

The intracellular ROS induced by USMB upon X-ray irradiation was assessed using a c-H_2DCFDA fluorescent probe. The fluorescence intensities increased significantly in both MIAPaCa-2 and PANC-1 cells when treated with USMB and X-rays compared to those treated with X-rays alone [Figure 6].

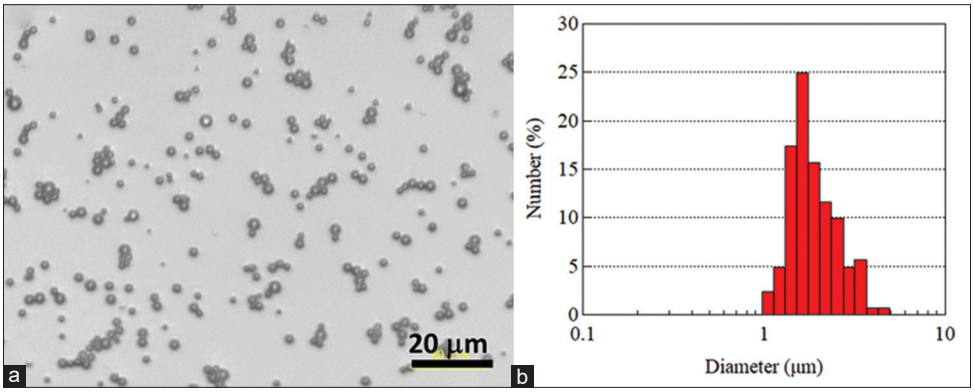

Figure 2: (a) A representative microscopy image of Sonazoid™ microbubbles. (b) Size distribution was obtained from 120 single microbubbles measured using microscopy images

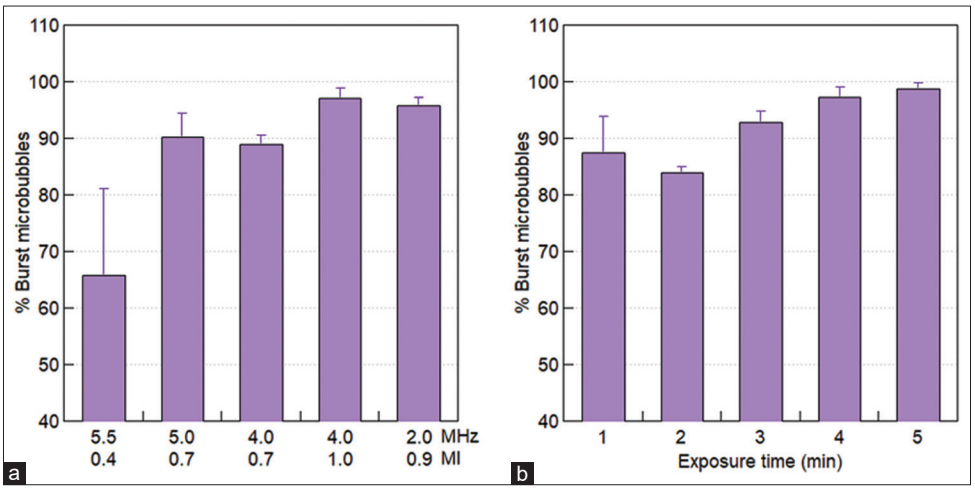

Figure 3: Number of microbubbles burst by ultrasound at different exposure parameters. (a) Different frequencies and mechanical indexes (MIs) with an exposure time of 3 min, and (b) different exposure times with 4.0 MHz and an MI of 1.0. Microbubbles were counted using a microscope before and after exposure. Data are represented as the means ± standard deviations obtained from nine independent microscopy images for each exposure setting. MI: Mechanical index

The combination of USMB and X-rays generated 1.24 and 1.38 folds higher ROS levels than X-rays alone in MIAPaCa-2 and PANC-1 cells, respectively. Fluorescence intensities increased in cells treated with USMB alone compared to untreated cells, but the increases were much smaller than those observed in cells treated with either USMB plus X-rays or X-rays alone.

Impact of ultrasound-stimulated microbubbles combined with nanoparticles on radiation treatment

The combination effect of USMB and TiO_xNPs as radiosensitizing agents was evaluated using a colony formation assay. Cells treated with 0.01% USMB in the absence of nanoparticles showed a radiation enhancement effect, which was consistent with the result observed in Figure 5; however, there was no difference in the cell survival curve between cells treated with USMB in the presence and absence of TiO_xNPs in both of cell lines [Figure 7]. The DEFs for USMB with TiO_xNPs were not greater than those for USMB without TiO_xNPs, as summarized in Table 1. The cells treated with TiO_xNPs but not exposed to USMB showed a slight radiation enhancement effect, which was not statistically significant as the duration of nanoparticle-cell interactions was short in this study.

Table 1: Radiation dose enhancement factors for ultrasound-stimulated microbubbles and titanium dioxide nanoparticles treatments in MIAPaCa-2 and PANC-1 cells

Cells	0.01% USMB	0.4% USMB	TiO_xNPs	0.01% USMB + TiO_xNPs
MIAPaCa-2	1.13	1.06	1.06	1.13
PANC-1	1.10	0.96	1.05	1.04

USMB: Ultrasound-stimulated microbubbles, TiO_xNPs: Titanium dioxide nanoparticles

Discussion

Cell viability of both MIAPaCa-2 and PANC-1 cells decreased when treated with the combination of USMB and X-rays compared to those treated with X-rays alone. However, the significant radiation enhancement effect of USMB was found only in MIAPaCa-2 cells treated with 6 Gy of X-rays and PANC-1 cells treated with 3 Gy using a low concentration of microbubbles. This result suggests that USMB may induce the additive effect to radiation, whereas the synergistic effect (i.e., radiation enhancement effect) may depend on multiple factors such as cell type, radiation dose, and microbubble concentration, which is consistent with previous findings in the literature.[21,22,27,28]

Previous studies have reported the combined effect of USMB and radiation on a variety of cancer cell lines. One of the studies reported by Karshafian *et al*., which appears to be the first study to report USMB combined radiation therapy, evaluated cell viability *in vitro* using acute myeloid leukemia cells with 0%–3% v/v microbubbles stimulated by ultrasound and 160 kVp X-rays.[16] They showed that cell viability decreased

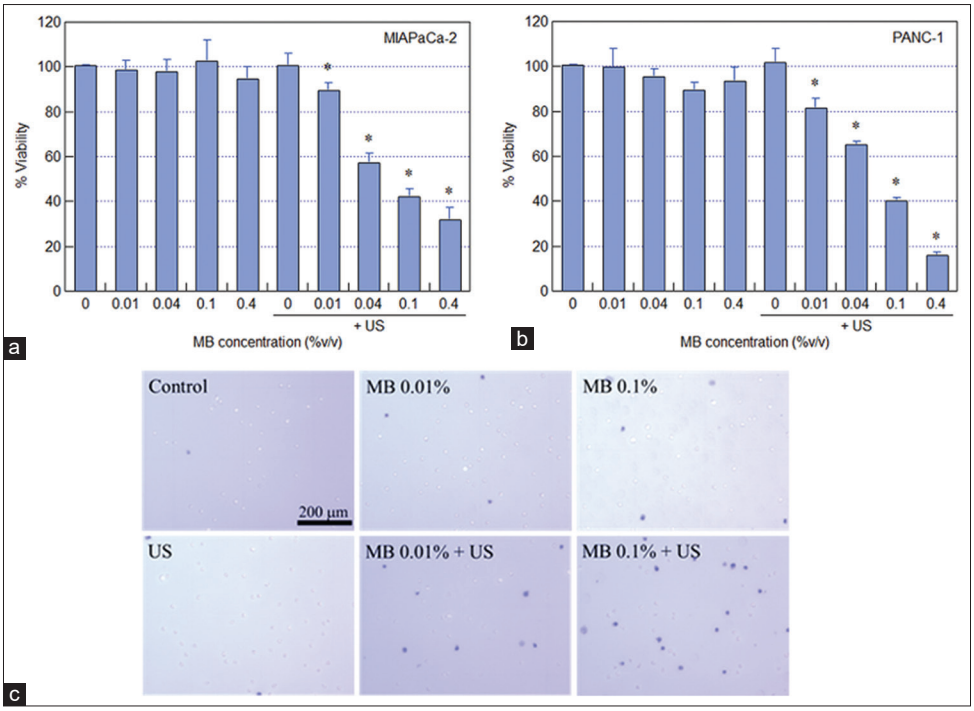

Figure 4: Effect of ultrasound-stimulated microbubbles on the viability of (a) MIAPaCa-2 and (b) PANC-1 cells. Cells were treated with 0%–0.4% v/v microbubbles ± ultrasound. Cell viability was measured 24 h after treatment using a water-soluble tetrazolium assay. Each data are normalized to the untreated control samples (cells without both microbubbles and ultrasound) and represented as the means ± standard deviations obtained from at least three independent measurements. *$P < 0.05$ compared to cells treated with ultrasound alone. (c) Detection of dead cells by trypan blue staining for MIAPaCa-2 cells treated with 0.01% and 0.1% v/v microbubbles and ultrasound. MB: Microbubbles; US: Ultrasound

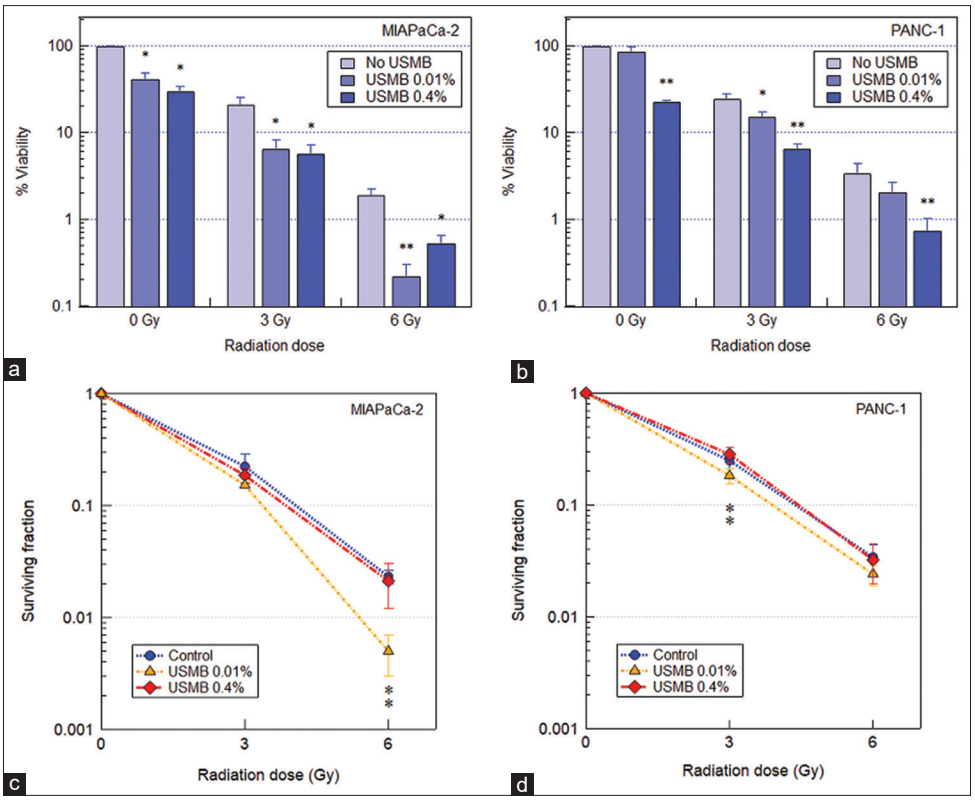

Figure 5: Effect of ultrasound-stimulated microbubbles (USMB) on the viability and survival curve of (a and c) MIAPaCa-2 and (b and d) PANC-1 cells treated with 0–6 Gy X-ray irradiation. Cells were treated with 0.01% or 0.4% v/v USMB before X-ray irradiation. Cell viability was evaluated using a colony formation assay. Each data of cell viability are normalized to the untreated control samples (cells without both USMB and X-rays) (a and b), and each survival curve was obtained by normalizing the viability to the unirradiated control samples for each USMB treatment (c and d). Data are presented as the means ± standard deviations obtained from at least three independent measurements. *$P < 0.05$ compared to cells treated with X-rays alone for each radiation dose, and **$P < 0.05$ compared to cells treated with 0.01% or 0.4% v/v USMB for each radiation dose. USMB: Ultrasound-stimulated microbubbles

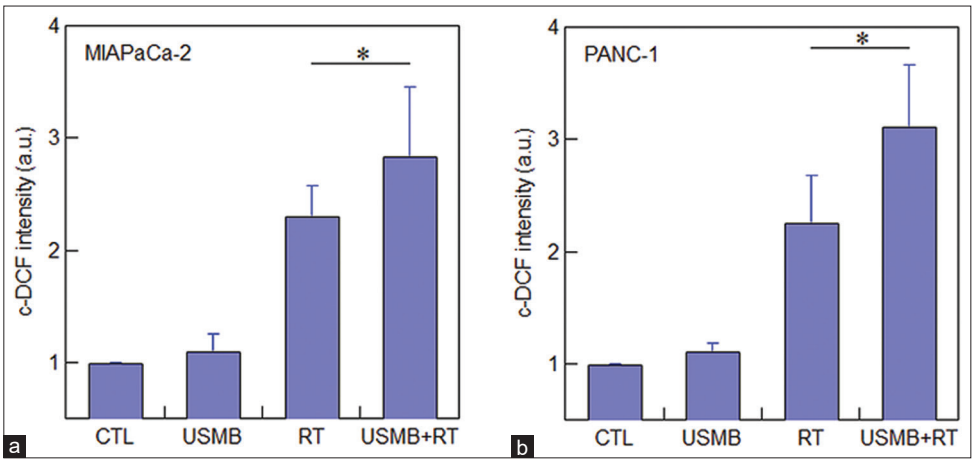

Figure 6: Reactive oxygen species generation in (a) MIAPaCa-2 and (b) PANC-1 cells treated with 0.01% v/v ultrasound-stimulated microbubbles and 6 Gy of X-rays. Each fluorescence intensity of carboxy 2',7'-dichlorodihydrofluorescein diacetate is normalized to the untreated control samples (cells without both microbubbles and ultrasound) as 1.0 and represented as the means ± standard deviations obtained from three independent measurements. *$P < 0.05$. CTL: Control; USMB: Ultrasound-stimulated microbubbles; RT: Radiation

in cells treated with the combined treatments compared to those treated with USMB alone or X-rays alone. Subsequent *in vitro* studies by their research team also demonstrated the additive effect of USMB combined radiation treatment using human prostate cancer cells, murine fibrosarcoma cells, and HUVECs.[29-31] Contrary to these reports, a study done by Lammertink *et al*. showed no effect of USMB on the efficacy of radiation treatment in human pharyngeal squamous carcinoma

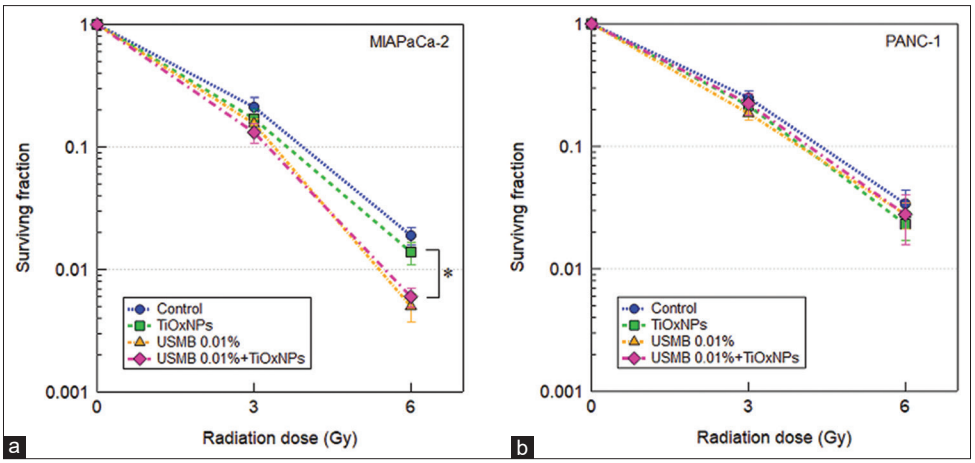

Figure 7: Effect of ultrasound-stimulated microbubbles (USMB) on the survival of (a) MIAPaCa-2 and (b) PANC-1 cells treated with titanium dioxide nanoparticles (TiO_xNPs) and 0–6 Gy X-ray irradiation. Cells were treated with 0.01% v/v USMB and 1.0 mg/mL TiO_xNPs before X-ray irradiation. The survival rate was evaluated using a colony formation assay and presented as the means ± standard deviations obtained from at least three independent measurements. $*P < 0.05$

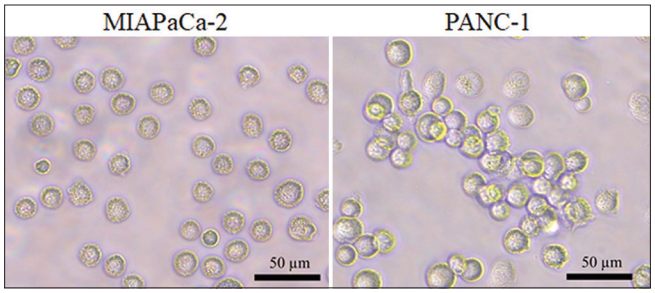

Figure 8: Difference in suspended cell state between MIAPaCa-2 and PANC-1 cells. Microscopy images of cells were obtained after cell detachment using trypsin

cells, while a significant decrease in survival fraction was found when cisplatin was combined with USMB and radiation treatment.[32] Moreover, our previous study using different types of cancer cell lines showed insignificant differences in normalized survival for FTC-238 and HUVEC cells treated with USMB and radiation.[21] Although the experimental settings were different between these studies, given the conflicting results reported, cell type is likely to be one of the dominant factors determining the effect of USMB on radiation treatment, which is in alignment with the findings observed in this study.

The difference in cellular characteristics between MIAPaCa-2 and PANC-1 cells may also influence the response to USMB. In our experiment, cells were exposed to USMB in a suspended state where PANC-1 cells tended to aggregate into clumps after trypsinization, whereas MIAPaCa-2 cells maintained cellular separation, as shown in Figure 8. This could affect the efficacy of USMB on PANC-1 cells due to a reduction in the cell surface area in contact with the microbubbles. It has been suggested that microbubble contact with cells is necessary for effective USMB sonoporation. Several studies have demonstrated that cell survival was reduced for suspended cells compared to adherent cells,[22,33] possibly because microbubbles float on the surface of the solution where they cannot make direct contact with adherent cells.[34] It should also be noted that the suspended cell state has been used in previous studies.[16,21,29,31] Furthermore, different cellular responses between MIAPaCa-2 and PANC-1 cells to USMB sonoporation and their dependence on ultrasound settings and microbubble concentration have been reported.[27] The discrepancy in radiation enhancement effect between MIAPaCa-2 and PANC-1 cells observed in our study may be due to these differences too.

In addition to being cell type dependent, the radiation enhancement effect of USMB could be microbubble concentration dependent. Cell viability significantly decreased with increasing microbubble concentration [Figure 4], which is consistent with the literature.[16,28] However, the radiation enhancement effect was only observed at the low microbubble concentration and there was no radiation enhancement effect at the high microbubble concentration [Figure 5]. It is speculated that at the high microbubble concentration where the cellular cytotoxicity of USMB is significantly high, as seen in Figure 4, USMB may induce lethal damage to most of the interacted cells before the X-ray irradiation, resulting in only the additive effect of X-rays. At the low microbubble concentration, cells may be sublethally damaged rather than lethally damaged by USMB, and then radiation may cause additional injury to these sublethally damaged cells, leading to enhanced cell death as the synergistic effect of USMB and radiation. The result suggests that the low microbubble concentration may be preferable to enhance the radiation effects, which is also supported by an *in vivo* study by Kim *et al*. who investigated the effect of USMB using different microbubble concentrations in combination with X-rays.[28] They showed that cell death and disruption increased with USMB treatment as the microbubble concentration increased, but when it was combined with 2 or 8 Gy of X-rays, the level of cell death was the same between low and high microbubble concentrations. This was more apparent when the pressure of the ultrasound was increased.

There was no enhancement effect by USMB combined with TiO_x NPs as radiosensitizers, contrary to our expectation that USMB could create transient pores on the cell membrane to promote cellular uptake of nanoparticles and then enhance the radiation effects with nanoparticles. A study by Lu *et al*. investigated USMB and radiation in combination with gold nanoparticles using human hepatocellular carcinoma cells, showing a significant radiation-induced decrease in cell viability for the combined treatment compared to radiation alone.[35] They synthesized gold nanoparticle-encapsulated microbubbles for their experiments, whereas in our study, the nanoparticles were simply mixed with microbubbles. Proximity between microbubbles, nanoparticles and cells has been suggested to be important for improving the efficacy of nanoparticle delivery into cells, as the pores on the cell membrane created by USMB could close before the nanoparticles reach them if the nanoparticles are far from the cells.[36] Given the previous literature with our result, the conjugation of nanoparticles with microbubbles may be necessary to achieve significant radiation enhancement. A similar result to ours was reported in a study using gemcitabine added to USMB on pancreatic cancer cells.[37] It was shown that USMB did not increase the cellular uptake of gemcitabine, regardless of the ultrasound pressure, resulting only in an additive effect with gemcitabine on cell viability.

ROS induced by USMB in combination with radiation may contribute to the radiation enhancement effect caused by USMB. Ionizing radiations are known to be able to generate ROS through interactions with surrounding water molecules, a process termed radiolysis,[38] whereas USMB has been reported to generate ROS as microbubble cavitation could transfer energy to the surrounding oxygen molecules.[39,40] Our data showed that ROS levels were significantly increased by the combination of USMB and X-rays compared to either USMB alone or X-rays alone in both cell lines, suggesting that USMB may enhance intracellular ROS production by the X-rays. Despite the significant increase in ROS induced by the USMB with 6 Gy of X-rays, the significantly enhanced effect on cell viability was found only in MIAPaCa-2 cells, not in PANC-1 cells in our study. The contradictory results in PANC-1 cells indicate that the amount of ROS induced by USMB and X-rays is not sufficient to achieve the significantly enhanced effect on cell viability. Although the different cellular characteristics between MIAPaCa-2 and PANC-1 cells described earlier may also affect the resulting cell viability, other mechanisms of radiosensitization by USMB, such as oxygen effects and ceramide production in cells, should be investigated to explain this discrepancy.[41,42]

One of the limitations of our study was that only one ultrasound exposure setting was used in the cell experiment. The different responses of the microbubbles result from different ultrasound settings, i.e., different frequencies, MIs, and exposure times, which could possibly lead to different biological effects of USMB combined with radiation.[6,28,34,43] Indeed, a study by Kotopoulis *et al*. showed different responses in MIAPaCa-2 and PANC-1 cells when exposed to different ultrasound conditions with Sonazoid™ microbubbles.[27] Another *in vivo* study using MIAPaCa-2 xenografted mice treated with different ultrasound pressures and Sonazoid™ microbubbles revealed that the higher ultrasound power was more effective in reducing tumor volume and increasing vascular damage.[44] However, *in vivo* experiments were not performed in this study. The results of USMB treatment in animal models could be affected by tumor vascular disruption induced by USMB sonoporation and radiation damage.[42] Taken together, further comprehensive studies with different experimental settings are warranted to clarify the effect of USMB on radiation therapy as well as the mechanisms.

Conclusions

The current study demonstrates the synergistic radiation enhancement in addition to the additive effect of USMB on radiation therapy for pancreatic cancer, which is likely to depend on cell type, microbubble concentration, and radiation dose. These trends are consistent with previous literature using different cancer cell lines. On the other hand, USMB showed no differences in the cell survival curve in the presence or absence of nanoparticles with radiation. This indicates that optimal strategies to improve nanoparticle delivery to cells, including optimization of ultrasound settings, should be considered in combination with USMB. Although more extensive studies with different experimental conditions are required to confirm the efficacy of USMB in radiation therapy, current findings suggest that USMB may potentially enhance the effect of radiation as radiosensitizers on pancreatic cancer cells.

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Conflicts of interest

There are no conflicts of interest.

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