

Sonodynamically-induced Anticancer Effects by Functionalized Fullerenes

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Abstract. *Background: Functionalized fullerenes, such as polyhydroxy fullerenes (PHF), have attracted particular attention due to their water solubility and their potential application in tumor imaging and therapy as carbon nanomaterials. In this study, the sonodynamically-induced antitumor effect of PHF was investigated. Materials and Methods: Sonodynamically-induced antitumor effects of PHF in combination with ultrasound were investigated using isolated sarcoma 180 cells and solid tumor from colon 26 carcinoma cells. Results: The cell damage induced by sonication was enhanced by two-fold in the presence of 80 μ M PHF. Histidine significantly inhibited this enhancement. This inhibitory effect suggests that the sonodynamically-induced antitumor effect was mediated by sonodynamically-generated reactive oxygen species. The combined treatment of ultrasonic exposure with PHF suppressed the growth of implanted colon 26 tumors. The destruction of tumor tissue was observed with the ultrasonic treatment in combination with PHF, while neither the treatment with PHF alone nor that with ultrasound alone caused necrosis. Conclusion: These results suggest that PHF is a potential sonosensitizer for sonodynamic treatment of solid tumors.*

Ultrasound has an appropriate tissue attenuation coefficient for penetrating intervening tissues to reach non-superficial objects while maintaining the ability to focus energy into small volumes. This is a unique advantage when compared to electromagnetic modalities such as laser beams in the

application to non-invasive treatment of non-superficial tumors. Although the use of ultrasound for tumor treatment has been relatively well-investigated with respect to the thermal effects due to ultrasound absorption, only a few studies have been reported with respect to non-thermal effects, such as the sonochemical effects due to ultrasound cavitation (1).

Recently, we found that photochemically-active porphyrins such as hematoporphyrin and porfimer sodium, can induce significant cell damage when activated by ultrasound. When implanted murine tumors are treated after the administration of such chemicals, tumor growth is significantly inhibited at an intensity where ultrasound alone shows only a slight inhibitory effect. Therefore, photochemically-active porphyrins may be useful for sensitizing tumors to ultrasound. We have proposed that this potential modality be called 'sonodynamic therapy' (2-15).

Nanomedicine is the medical application of nanotechnology for the diagnosis and treatment of human diseases. It uses precisely engineered materials, known as nanoparticles generally in the 1-100-nm dimension range. Nanomaterials such as functionalized fullerenes, have unique physicochemical properties, such as small size, large surface area to mass ratio and high reactivity which are used to overcome some limitations of traditional therapeutic agents (16-18). One of the most important features of nanomaterials is the potential for improvement of drug delivery to the target area to provide the maximum therapeutic efficacy. Due to their size, nanomaterials are capable of accumulating in pathological areas, such as many solid tumors and infarcted sites (19, 20).

Recently, water soluble functionalized fullerenes, such as polyhydroxy fullerenes (PHF, Figure 1) have received considerable attention particularly due to their interesting sensitizing properties, which have been exploited in many biological fields. For example, a potential biological application of functionalized fullerenes is related to their photosensitization by either UV or visible light (21, 22). The

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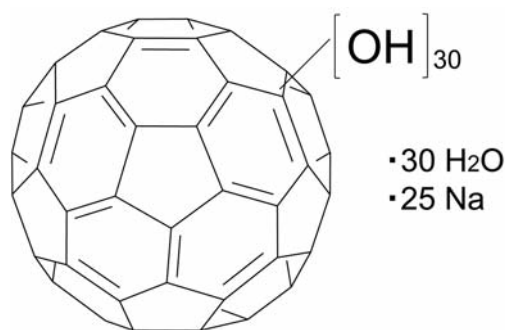


Figure 1. Chemical structure of polyhydroxy small gap hydrated fullerenes.

resulting excited-state singlet-state fullerene molecule is readily converted to the long-lived triplet state fullerene *via* intersystem crossing. In the presence of molecular oxygen, fullerene may decay from its triplet to the ground state, transferring its energy to oxygen molecules and generating reactive oxygen species (ROS) such as singlet oxygen ($^1\text{O}_2$) and superoxide radicals, known to be a highly cytotoxic species (23). The ability of fullerenes to catalyze the production of singlet oxygen is invaluable in the destruction of cellular targets, particularly of nucleic acids and cell membranes. Therefore, functionalized fullerenes constitute an excellent photosensitizer for use in photodynamic therapy (PDT) of tumors.

It would not be unnatural to expect that polyhydroxy fullerene can also be activated by ultrasound as well as the above described porphyrins and that its use in combination with ultrasonic exposure may also be effective for tumor treatment. In this article, sonodynamically-induced *in vitro* and *in vivo* antitumor effects of PHF were investigated on experimental tumors using ultrasound at 2 MHz in standing wave mode.

Materials and Methods

Chemicals. Polyhydroxy small gap hydrated fullerenes, (PHF), histidine, mannitol, and superoxide dismutase (SOD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other reagents were commercial products of analytical grade.

Evaluation of *in vitro* effect. Sarcoma 180 cells were supplied by Meiji Seika Kaisha (Tokyo, Japan). The cells were passaged weekly through male ICR mice (Japan SLC, Japan) in the form of ascites. Cells were harvested from the peritoneal cavity of tumor-bearing animals 7 to 10 days after inoculation. The tumor cells harvested from mice were suspended in an air saturated phosphate buffer solution (PBS, pH 7.4) and were packed by light centrifugation ($100 \times g$ for 1 min). The cells were then resuspended in PBS at a density of 4×10^6 cells/ml. The cell suspensions were stored on ice until used in the experiments.

The viability of the isolated cells was determined by staining of the cells with trypan blue dye. A 1-ml aliquot was taken from the cell suspension and mixed with 1 ml of 0.5% trypan blue solution. The integrity of the cells was determined by counting the number of unstained cells on a hemocytometer glass plate using an optical microscope. This was checked before every treatment, and cell suspensions with integrity above 99% were used in a series of experiments. This number of intact cells before treatment was regarded as the standard for the integrity determination after insonation. A 2.5-ml portion of the cell suspension was transferred to an exposure chamber and insonated. The extent of ultrasonically-induced cell damage in the presence and absence of $80 \mu\text{M}$ PHF in suspensions with and without potential active oxygen scavengers was determined by comparing the integrity before and immediately after insonation. Each result is presented as the mean with the standard deviation (SD) of four insonation experiments.

Evaluation of antitumor effect. Colon 26 carcinoma cells were supplied by the Cancer Institute (Tokyo, Japan). The cell lines were passaged weekly through male BALB/c mice (Japan SLC, Japan) (5 weeks old). Transplanted tumors were initiated by subcutaneous trocar-injection of approximately 1 mm^3 pieces of fresh tumor into the left dorsal scapula region of male 5-week-old CDF1 mice (Japan SLC, Japan). When the tumor grew to diameter of about 10 mm approximately 14 days after implantation, the treatment study was started. The tumor-bearing mice were divided into four groups of four mice: the control group, and those treated with PHF, ultrasound, or PHF with ultrasound. For the treatments with PHF, PHF was administered to the tumor at a dose of 25 mg/kg. For the combined treatment, the insonation was carried out 1 h after the administration of PHF.

The long and short diameters (*a* and *b* respectively in mm) of the tumor were measured with a slide caliper every 7 days after inoculation. The tumor size was calculated as $(a+b)/2$. The mean and SD were calculated for each group consisting of four mice. The values were compared by Student's *t*-test with 0.05 as the minimum level of significance. Fourteen days after the treatment, the mice were sacrificed and the tumors were dissected out and weighed, and stored in fixative solution (10% buffered formalin). The fixed tumors were later stained with hematoxylin and eosin (H & E) for histological examination.

***In vitro* insonation.** The *in vitro* exposure set-up is shown in Figure 2. The air-backed transducer used a lead-titanate piezoelectric ceramic disk of 24 mm diameter, purchased from Hitachi Metals (Tokyo, Japan), and was tightly bonded onto an aluminum layer with a low heat-expansion epoxy adhesive. The overall resonant frequency of the transducer was 1.92 MHz. Sine waves were generated by a wave generator (model MG442A; Anritsu Electric, Tokyo, Japan) and amplified by an RF amplifier (model 210L; ENI, Rochester, NY, USA). The sinusoidal drive signal of the transducer was monitored by an oscilloscope during the ultrasonic exposure. The transducer was submerged in degassed water at room temperature facing upward, with its acoustic surface parallel to the water surface. An insonation glass container of 31 mm diameter with a flat bottom layer of 1.5 mm thickness was placed 30 mm from the transducer. A 2.5 ml aliquot of air-saturated cell suspension was placed in the container. The level of the degassed water was approximately adjusted to the level of the suspension or solution in the container. The ultrasound attenuation through the bottom layer

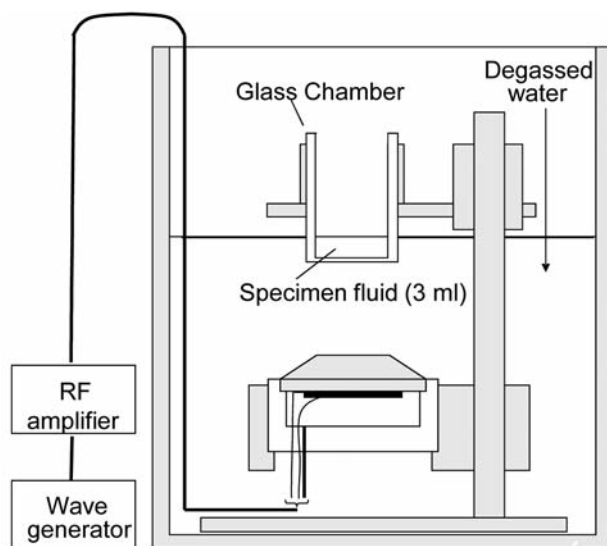


Figure 2. Cross-sectional view of *in vitro* insonation set-up.

of the container for insonation was also estimated in a propagation mode, using a needle-type hydrophone by comparing the acoustic pressure on the axis with and without the layer between the transducer and the hydrophone. When the layer was parallel to the transducer surface, the attenuation in amplitude was less than 10%. It may have been small because the thickness of the layer was close to a half wavelength and the acoustic field was close enough to the plane wave field. The temperature rise in 2.5 ml air-saturated water in the glass container during the insonation was checked using a 0.25 mm diameter Chromel-Almel thermocouple. It was less than 1°C for 1 min insonation at the free field intensity of 4.5 W/cm².

***In vivo* insonation.** The *in vivo* ultrasonic exposure set-up is shown in Figure 3. The air-backed transducer used a lead-zirconate-titanate ceramic disk of 12 mm diameter, purchased from Fuji Ceramics (Fujinomiya, Sizuoka, Japan), and was tightly bonded onto an aluminum layer, which was cooled by circulating water to keep the transducer and tumor temperature below a 35°C. The overall resonant frequency of the transducer was 2.0 MHz. A tumor-bearing mouse one week after tumor cell inoculation was anesthetized with sodium pentobarbital (40 mg/kg, *i.p.*). The hair over the tumor was shaved and ultrasound gel was applied to the naked skin. The mouse was fixed on a cork board and the transducer was placed tightly on the tumor. The tumor was insonated in standing wave mode at a free-field intensity of 3 W/cm² for 15 min. The transducer was cooled by circulating water at 25°C during insonation. The tumor temperature was measured by inserting a thermistor probe (Anritsu Electric) into the central region of the tumor. It was kept below 35°C, much lower than the hyperthermia level.

Electron spin resonance (ESR) measurements. Ultrasonically induced nitroxide production in the presence and absence of PHF was measured by ESR spectroscopy in an air-saturated aqueous solution of 50 mM 2,2,6,6-Tetramethylpiperidine in the presence and absence of oxygen scavengers. The pH of the solutions was adjusted to 9.0

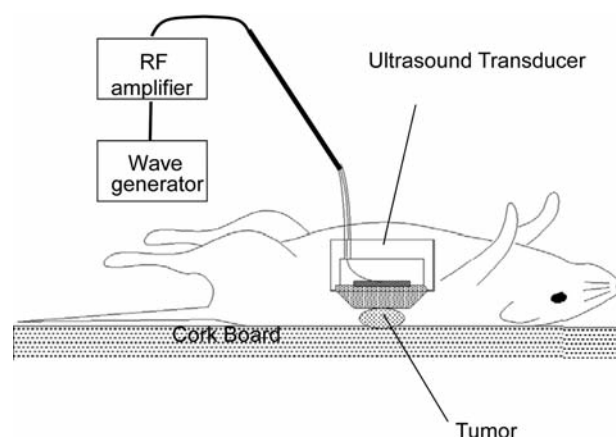


Figure 3. *In vivo* insonation set-up. A cross-section is shown for the transducer.

with 50 mM PBS. At appropriate time points, samples were taken for ESR measurement and placed in glass capillary tubes with an inner diameter of 1.1-1.2 mm, a wall thickness of 0.2 mm, and a length of 75 mm (Allied Corporation Fisher Scientific, Pittsburgh, PA, USA). The ESR spectra were recorded using a JEOL JES-FE3XG X-band spectrometer (JEOL Ltd., Tokyo, Japan) operating at 100 kHz modulation frequency and at 9.26 GHz microwave frequency. The modulation amplitude of the magnetic field was set at 1.0×10^{-4} T, and the microwave power was 10 mW. The concentration of the produced nitroxide was determined by comparison with the peak-to-peak ESR signal amplitude of a 1 mM 2,2,6,6-tetramethyl-4-piperidone-1-oxyl solution. The ratio of the amplitude to the nitroxide concentration was also verified by comparison with the peak-to-peak ESR signal amplitude of a 1 mM 2,2,6,6-tetramethyl-4-piperidol-1-oxyl solution.

Results

***In vitro* effect.** The unstained fractions of the isolated sarcoma 180 cells in the air-saturated suspensions, in the presence and absence of 80 μM PHF after fixed duration of insonation, are plotted versus insonation time in Figure 4. The results with 80 μM PHF without ultrasound are also plotted versus time. The unstained fractions plotted on a logarithmic scale decreased linearly with insonation time. The ultrasonically-induced cell-damaging rate was enhanced by PHF by approximately two-fold. After 60 s insonation, the unstained fraction decreased to 31% without PHF, while it was only 1.8% in the presence of PHF. No cell damage was observed with PHF alone.

Nitroxide generation. Reactive oxygen species were measured by ESR and spin trapping technique to determine whether reactive oxygen species including singlet oxygen and hydroxyl radicals participate in the induction of apoptosis by ultrasound. Figure 5 shows the amounts of nitroxide

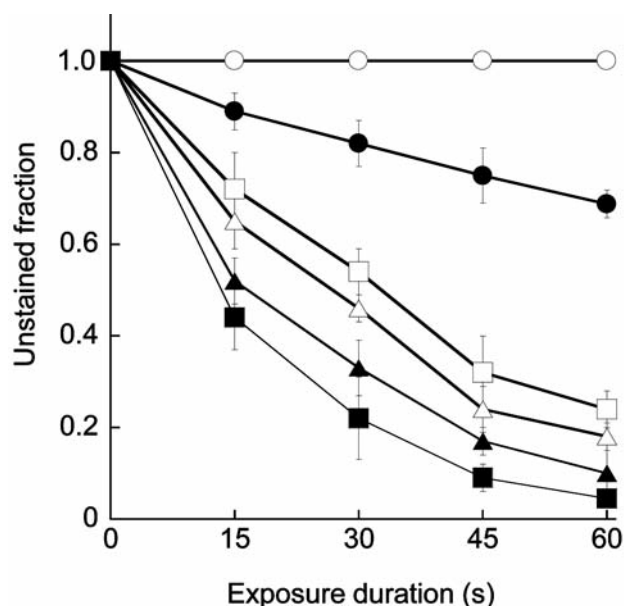


Figure 4. *In vitro* effect of ultrasound with and without PHF on isolated sarcoma 180 cells. ●, PHF alone; □, ultrasound alone; △, 20 μ M PHF + ultrasound; ▲, 40 μ M PHF + ultrasound; ■, 80 μ M PHF + ultrasound. Each point and vertical bar represent the mean \pm SD of four insonation experiments.

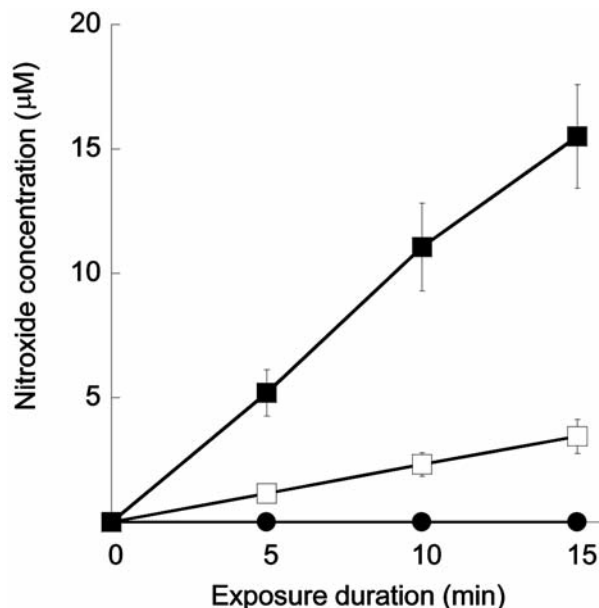


Figure 5. Nitroxide generation in an air-saturated solution of 50 mM TMPone during exposure to ultrasound in the presence and absence of PHF. ○, Control; ●, 80 μ M PHF alone; □, ultrasound alone; ■, 80 μ M PHF + ultrasound. Values represent the means \pm S.D. of four independent experiments.

ultrasonically generated in air-saturated aqueous solutions of 50 mM TMPone with and without PHF under the same acoustic conditions as employed in the cellular experiments. The nitroxide levels were determined from the ESR signal amplitudes and plotted versus the insonation time. The amount of ultrasonically generated nitroxide increased linearly with the insonation time. PHF at a concentration of 80 μ M enhanced the rate by approximately three-fold. Nitroxide generation was not observed with PHF alone.

Effect of reactive oxygen scavengers. To determine whether reactive oxygen species including singlet oxygen and hydroxyl radicals participate in sonodynamical cell damage, we examined the effect of reactive oxygen scavengers (10 mM histidine, 100 μ g/ml SOD, and 100 mM mannitol) on the cell damage (Figure 6) as well as on the production of nitroxide (Figure 7). Histidine significantly reduced the cell damage and nitroxide generation caused by exposure to ultrasound in the presence of PHF. In contrast, SOD and mannitol did not effect these measurements.

Antitumor effect *in vivo*. The effect of each treatment on the growth of colon 26 solid tumors is compared in Figure 8 by plotting the tumor size for five weeks after the inoculation. PHF alone had no inhibitory effect on tumor growth. Ultrasound alone had a slight inhibitory effect. PHF with

ultrasound had a marked synergistic antitumor effects. Significant suppression of tumor growth after the treatment was observed in the combined treatment. Histological sections of the tumors are compared in Figure 9. No significant histological change was observed in the tumors treated with PHF alone or ultrasound alone (Figure 9b and c). In contrast, the combination treatment with PHF and ultrasound revealed a massive necrosis in the tumor region (Figure 9d).

Discussion

A significant ultrasonically-induced antitumor effect, as well as significant enhancement of ultrasonically-induced *in vitro* cell damage, was demonstrated with PHF. PHF enhanced the ultrasonically-induced damage on isolated sarcoma 180 cells by approximately the same factor as hematoporphyrin at the same concentration (2). In the experimental treatment combined with ultrasonic exposure, PHF inhibited the growth of the inoculated colon 26 cell tumors at a dose of 25 mg/kg. Colon 26 is a syngeneic experimental tumor model and is more suitable for evaluation of antitumor effects than sarcoma 180, which is allogeneic. This was the reason why colon 26 cells were used in the present *in vivo* experiment. On the other hand, sarcoma 180 was chosen for the *in vitro* experiment because it is difficult to obtain an adequately high viability of colon 26 cells as isolated cells.

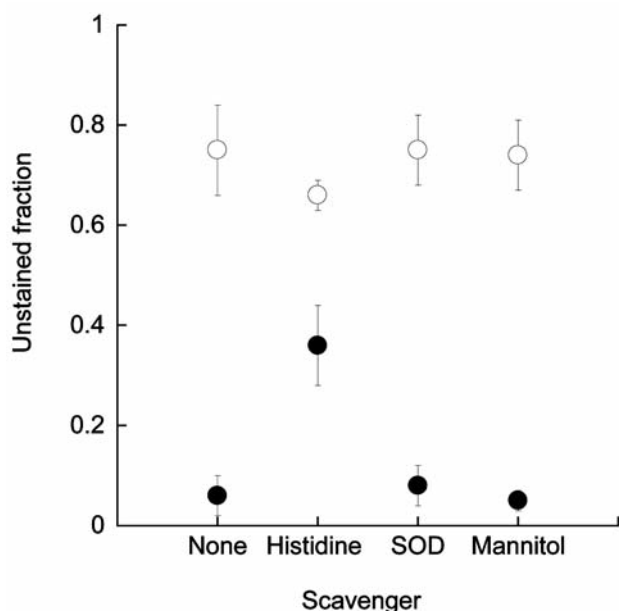


Figure 6. Effect of active oxygen scavengers on (A) cell damage with (●) and without (○) PHF after 60 s insonation. Each point and vertical bar represent the mean±SD of four insonation experiments.

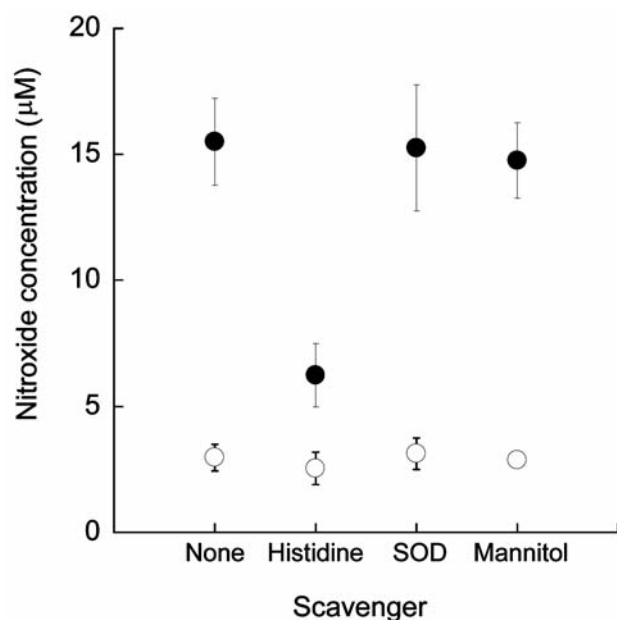


Figure 7. Effect of active oxygen scavengers on nitroxide generation with (●) and without (○) PHF after 60 s insonation. Each point and vertical bar represent the mean±SD of four insonation experiments.

Like photodynamic reactions, sonodynamic reactions are classified as either type I or type II. In type I reactions, the excited state of the sensitizer reacts with the substrate, whereas in type II reactions, it reacts with oxygen, yielding

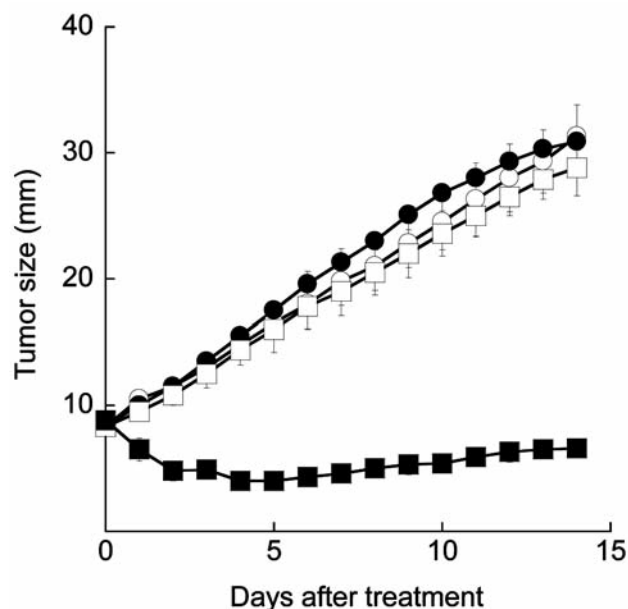


Figure 8. Effect of ultrasound with and without PHF on growth of colon26 solid tumor cells. ○, Control; ●, PHF alone; □, ultrasound alone; ■, PHF + ultrasound. Each point and vertical bar represent the mean±SD of four mice.

radicals, radical ions, or singlet oxygen. We observed a substantial enhancement of nitroxide generation in the presence of PHF and under the sonodynamic conditions used in the cellular experiments. Histidine is known to act as a scavenger of singlet oxygen and possibly of hydroxyl radical. Thus, the significant reduction by histidine of the PHF-enhanced ultrasonically-induced cell damage suggests that the enhancement was due to ultrasonic generation of active oxygen enhanced by PHF. The result may further suggest that not only the *in vitro* enhancement but also the ultrasonically-induced *in vivo* antitumor effect with PHF was induced sonochemically. This should be investigated further studies.

Since the mannitol concentration of 100 mM is more than the concentration reported as being effective at scavenging ultrasonically induced hydroxyl radicals (24) and no significant change in ultrasonically induced cell damage was observed with 100 mM mannitol, thus hydroxyl radical is not likely to be an important mediator for the damage. Superoxide radical may also not be important either, since SOD had no significant effect either. Among the active oxygen species of singlet oxygen, hydroxyl radical, and superoxide radical, singlet oxygen is most likely to have mediated the ultrasonically-induced cell damage enhanced with PHF. Basically the same hypothesis goes for singlet oxygen as the mediator has also been proposed for porphyrin derivatives such as hematoporphyrin and ATX-70 (6, 7).

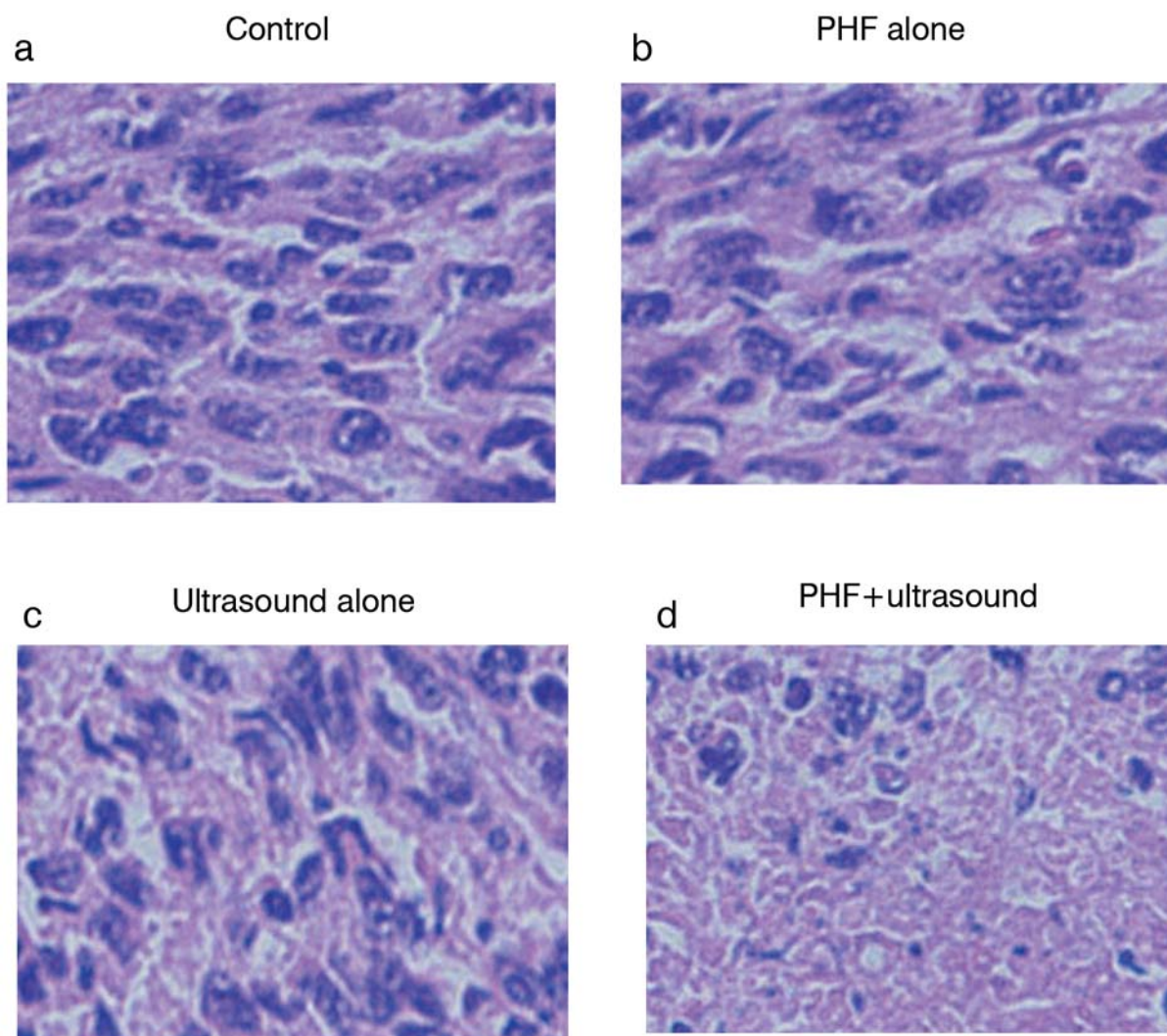


Figure 9. Effect of ultrasound with and without PHF on colon 26 carcinoma cells. Histological sections ($\times 400$) of the tumors are compared for (a) control, (b) PHF alone, (c) ultrasound alone, and (d) PHF+ ultrasound.

Sonochemically-active cavitation inducing active oxygen generation is much less likely to take place inside the cells than outside. The resonant size of a microbubble in an aqueous medium at an ultrasonic frequency in the order of a megahertz is several micrometers. This is in the same order of magnitude as the size of most mammalian tissue cells. Furthermore, the oxygen content in cytoplasm is lower by at least an order of magnitude than that in the extracellular fluid, and the typical diffusion distance of active oxygen species is less than 0.1 mm. Therefore, the cell membrane is most likely the site of action for sonochemical effects on the cells subjected to ultrasound.

A series of *in vitro* and *in vivo* trials confirmed that sonodynamic therapy, where either ultrasound or

sonosensitizer had no or very low cytotoxicity, was efficacious in destroying malignant cells/tissues, and most sonosensitizers are either porphyrins or their derivatives (25). Unfortunately, some sonosensitizers have physico-chemical properties that seriously limit their clinical application, such as poor water solubility (*e.g.* hypocrellin), and nanotechnology is one method that can be adopted to solve such problems. The results reported here may be preliminary, but they significantly support the possibility of clinical application of sonodynamic treatment using water-soluble functionalized fullerenes, such as polyhydroxy fullerenes. Further investigations using experimental animals of a size similar to humans will be needed before such application is feasible.

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