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Sonodynamically-induced Anticancer Effects of Polyethylene Glycol-Modified Carbon Nano Tubes

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Abstract. *Background/Aim: Sonodynamic cancer therapy is based on the preferential uptake and/or retention of a sonosensitizing drug (sonosensitizer) in tumor tissues and the subsequent activation of the drug by ultrasound irradiation. In the present study, we investigated the sonodynamically-induced antitumoral effect with functionalized carbon nanotubes, such as poly-ethylene glycol-modified carbon nanotubes (PEG-modified CNTs). Materials and Methods: Antitumor effects were evaluated using histological observation and assessing tumor growth following sonodynamic exposure to PEG-modified CNTs. Results: The combined treatment of 100 μM PEG-modified CNT and ultrasound induced a 2-fold cytotoxicity. Sodium azide, which quenches singlet oxygen, significantly inhibited ultrasonication induced cell damage in the presence of PEG-modified CNTs. This suggests that singlet oxygen produced by the combined use of PEG-modified CNTs and ultrasound is involved in the induction of antitumoral effects. The destruction of tumor tissue was observed with the ultrasonic treatment in combination with PEG-modified CNTs, while neither the treatment with PEG-modified CNTs alone nor ultrasound alone caused any necrosis. Conclusion: These results indicate that PEG-modified CNT functions as a sonosensitizer and is effective for sonochemical treatment of solid tumors.*

There is a new promising strategy for cancer treatment using the synergistic effect of ultrasound and chemicals, which can efficiently cause crushing (cavitation), as a result of repeated expansion and contraction of microbubbles by ultrasound irradiation. In this way the biological effects of some chemicals can be enhanced. Substances activated *via* this non-thermal action are called ultrasound sensitizers (1-5). However, compared to the thermal effects of an ultrasound-absorbing tumor treatment, there are only few research reports on non-thermal effects, such as sonochemical effects based on cavitation (2, 6-7).

We have previously reported that photochemically active compounds, such as hematoporpyrin, ATX-70, pheophorbide A, rose bengal, adriamycin FAD104, THP-adriamycin, ATX-S10, and porfimer sodium, may be sonosensitizers that can be activated by ultrasound irradiation (2-4, 6, 8-16). Porphyrins have been observed to accumulate in tumor tissues following intravenous administration (5, 7, 17-18). The administration of such compounds followed by ultrasound to treat implanted murine tumors has been shown to significantly suppress tumor growth, whereas the application of ultrasound alone only slightly inhibits growth (5, 7, 19-26). This suggests that combinational use of sonodynamically active porphyrins and ultrasound may have antitumoral effects. We have proposed that this potential modality be called sonodynamic therapy (2, 26).

Nanomedicine is a medical application of nanotechnology for the diagnosis and treatment of human illnesses, using precisely designed materials (nanoparticles) with a diameter of typically 1-100 nm (27). Nanomaterials, such as functionalized carbon nano tubes (CNTs), have unique physicochemical properties, for example, a suitable size, a large surface area-to-mass ratio and a high reactivity, which are used to overcome some of the limitations of the traditional therapeutic agents (28). Nanomaterials have the potential to provide maximum therapeutic effects as a result of improved drug delivery. Nanomaterials can penetrate pathological areas, such as solid tumors and infarct areas, with high precision, due to their small in size (29-30). We

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have previously investigated the sonodynamically-induced antitumoral effects of functionalized fullerenes and their activation by ultrasound (31-32). These results have confirmed that the combined use of the functionalized fullerene and the ultrasound induces some kind of cell damage mediated by the generation of reactive oxygen species.

Functionalized CNTs have recently received considerable attention as multifunctional nanodrugs for their use as DDSs due to their interesting sensitizing properties, such as their favorable pharmacokinetics and toxicology, which have been exploited in the field of medicine (27-30, 33). Functionalized CNTs have the ability to catalyze the generation of singlet oxygen, which is invaluable for the destruction of cellular targets, particularly of nucleic acids and cell membranes (27). Therefore, functionalized CNTs can are unaverage sonosensitizers in sonodynamic cancer therapy.

It has been reported that, the removal of foreign particles by the reticuloendothelial system (RES) dramatically decreases the half-life and efficiency of nano particles (27). Nano particles are typically protected from removal by the RES by modifying their surfaces with uncharged chemical molecules, such as hydrophilic polymers and nonionic surfactants, of which polyethylene glycol (PEG) is among the most effective one, due to its high hydrophilicity (27).

PEG-modified CNTs (Figure 1) are likely to be activated by ultrasound as well as by functionalized nanoparticles, thus, the combinational use of PEG and US may assist their interaction for an effective anti-tumoral therapy.

Here, we report *in vitro* and *in vivo* antitumoral effects induced sonodynamically by PEG-modified CNTs on experimental tumors, using a 2 MHz standing wave ultrasound.

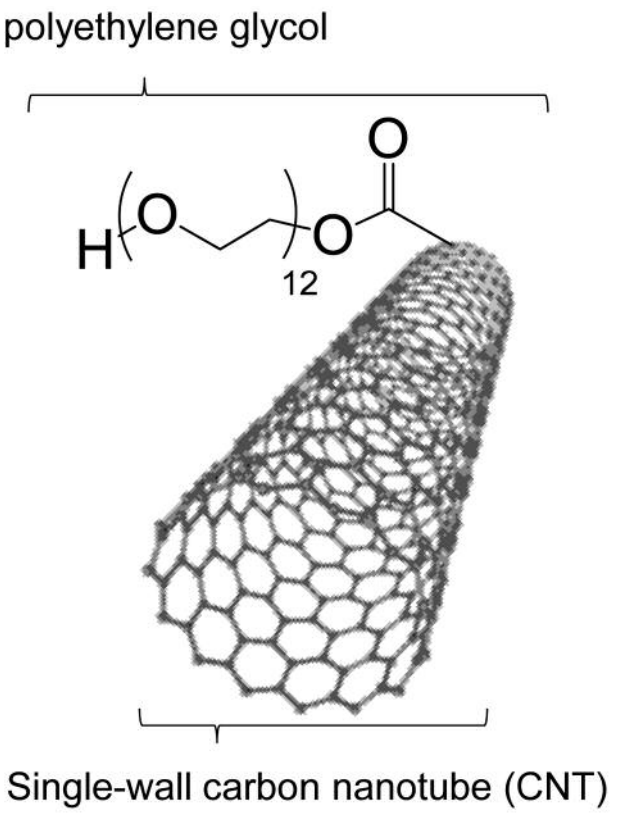

Figure 1. *Chemical structure of PEG-modified CNTs.*

Materials and Methods

Chemicals. Polyethylene glyco (PEG)-modified CNTs (PEG-modified CNTs) were purchased from Nanocs Inc. (New York, NY, USA). 2,2,6,6-Tetramethyl-4-piperidone-1-oxyl, 2,2,6,6-tetramethylpiperidine, sodium azide (NaN3), histidine (His), mannitol (Man), sorbitol (Sor) and superoxide dismutase (SOD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents of analytical grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Evaluation of the in vitro effect. Sarcoma 180 cells were donated by Meiji Seika Kaisha (Tokyo, Japan). The cells were passaged weekly through male ICR mice (Japan SLC, Shizuoka, Japan) in the form of ascites cancer. Briefly, sarcoma 180 cells were collected from the abdominal cavity of tumor-bearing mice 7 to 10 days following their transplantation there. First, the ascites cells were suspended in air-saturated phosphate buffer (PBS) at pH 7.4, collected by centrifugation at 100 × *g* for 1 min, and then resuspended in PBS at 4×10^6 cells/ml, and stored on ice until use for the experiments. The viability of the adjusted cells was evaluated using trypan blue staining (2-4, 6, 8-14). Cell suspension aliquots of 0.5 ml were mixed with 0.5 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 (Fuchs-Rosenthal Haemosytometer) using an optical microscope (OLYMPUS, T041, Tokyo, Japan). Viability was checked before each treatment, and cell suspensions with an integrity above 99% were used in the following stag of experiments. The number of intact cells before treatment was regarded as the standard for the integrity of the cells following insonation. An aliquot of cell suspension (3.0 ml) was then transferred to an exposure chamber and insonated. The extent of ultrasonically-induced cell damage in the presence or absence of 100 μM PEG-modified CNTs in suspensions with and without potential active oxygen scavengers was determined by comparing the integrity before and immediately after insonation (2-4, 6, 8-16, 32). Each result represents the mean and standard deviation (SD) of four determinations.

Evaluation of the antitumoral effect. Colon 26 carcinoma cells were obtained from the Cancer Institute (Tokyo, Japan). The cell lines were maintained weekly through 5-week-old male BALB/c mice (Japan SLC). Carcinoma cells were transplanted subcutaneously into a fresh tumor of about 1 mm3 in the left dorsal scapula region of 5-week-old male CDF1 mice (Japan SLC). The treatment was initiated when the tumors grew to a diameter of about 10 mm (approximately 14 days after implantation). Tumor-bearing mice

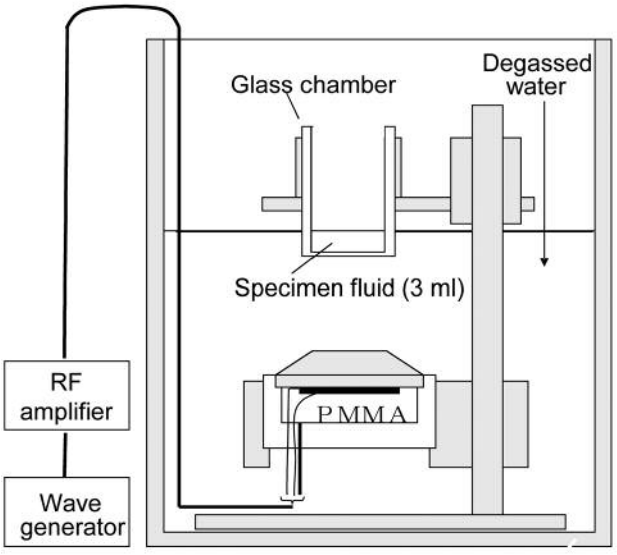

Figure 2. *Cross-sectional view of the in vitro insonation set-up. In an in vitro study set-up of ultrasonic sonodynamic therapy in a cell injury experiment, the glass chamber containing each sample is put in place and the ultrasound focuses on the target area in the tumor cell solution in the glass chamber. RF amplifier: Radio frequency amplifier; PMMA: polymethyl methacrylate.*

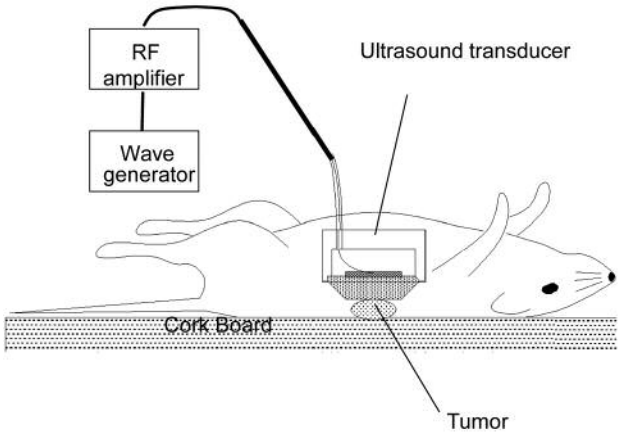

Figure 3. *The in vivo insonation set-up. A cross section of the transducer is shown. Experimental setup of ultrasound sonodynamic therapy in a rat solid tumor model, each rat was placed in a position and the ultrasound is focused in the solid tumor. RF amplifier: Radio frequency amplifier; PMMA: polymethyl methacrylate.*

were divided into four groups: i) untreated, and treated with ii) PEG-modified CNTs, iii) ultrasound alone, or iv) PEG-modified CNTs and ultrasound. In the treatment with the PEG-modified CNT, a dose of 25 mg/kg of PEG-modified CNT was administered to the cancer model animal, and in the combinational treatment, the ultrasonic irradiation was performed 1 h after the administration of PEG-modified CNT (5, 21-26).

The long and short diameters (a and b, respectively, in mm) of each tumor were measured using 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 of four mice. The values were compared using Student's *t*-test, with 0.05 as the minimum level of significance. After the experiment, the tumor tissue was stained with hematoxylin and eosin.

In vitro insonation. The apparatus for ultrasonic exposure is shown schematically in Figure 2. The ultrasound transducer uses a piezoelectric ceramic disk 24 mm in diameter and was driven at its resonance frequency (1.93 MHz). The sinusoidal drive signal of the transducer was monitored by an oscilloscope during ultrasonic exposure.

The transducer was submerged in degassed water facing upward at room temperature, with its acoustic surface parallel to the water surface. An insonation glass container 31 mm in diameter with a flat bottom layer 1.5 mm thick was placed 30 mm away 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 adjusted to approximately the level of the suspension or solution in the container. Ultrasound attenuation through the bottom layer of the container was estimated using a needle-type hydrophone in propagation mode, 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, amplitude was attenuated less than 10%. This small value may have been due to the thickness of the layer being close to half a wavelength, and because the acoustic field was close to the plane wave field. The ultrasonic intensity was calculated by dividing the measured acoustic power by the projected area. The temperature rose in 2.5 ml air-saturated water in the container during exposure at the highest ultrasonic intensity used in the experiments was less than 1˚C (2-4, 6, 8-16).

In vivo insonation. The *in vivo* ultrasonic exposure set-up is shown in Figure 3. The air-backed transducer used a lead-zirconate-titanateceramic disk of 12 mm diameter, purchased from Fuji Ceramics (Fujinomiya, Shizuoka, Japan), and was tightly bonded onto an aluminum layer. The transducer was cooled by circulating water at 25˚C to keep the tumor from overheating. The driving resonance frequency of the transducer was 1.93 MHz. Under the anesthesia with sodium pentobarbital, a solid tumor was formed by inoculating 26 colon cells into mice. The hair over the solid tumor was shaved under anesthesia and an 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 a standing wave mode at the free-field intensity of 3 W/cm^2 for 15 min. A thermistor probe was inserted into the center of the solid tumor to confirm that the intratumoral temperature was maintained below 35˚C.

Electron spin resonance (ESR) measurements. Ultrasound-induced nitroxide production in the presence and absence of PEG-modified CNTs was measured using 2,2,6,6-tetrame-thylpiperidine (TEMP) as a trapping agent in the presence and absence of oxygen scavengers. The generated 2,6,6-tetrame-thylpiperidine-N-oxyl (TEMPO), which is a reaction product of nitroxide compound and TEMP, was measured using ESR. The TEMP solution was air-saturated and adjusted to pH 9.0. Samples were collected over time

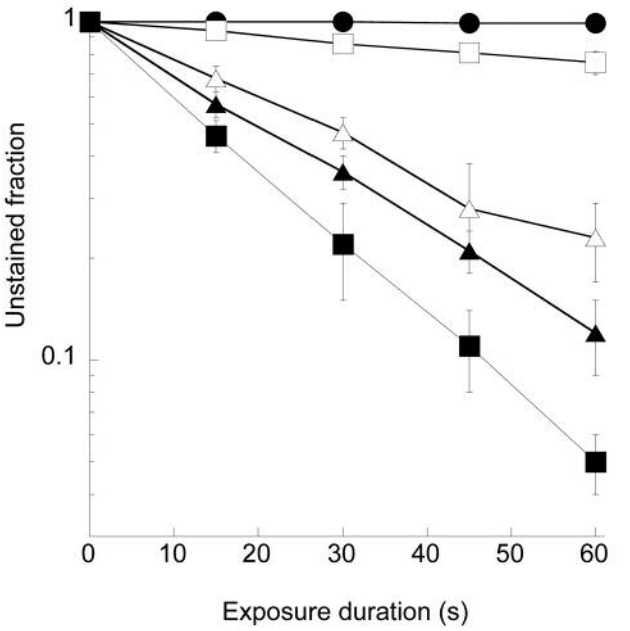

Figure 4. *In vitro effect of ultrasound with and without PEG-modified CNTs on isolated sarcoma 180 cells. Each point and vertical bar represent the mean±SD of four insonation experiments. ●: PEG-modified CNTs alone, □: ultrasound, △: 25 μM PEG-modified CNTs + ultrasound, ▲: 50 μM PEG-modified CNTs + ultrasound, ■: 100 μM PEG-modified CNTs + ultrasound.*

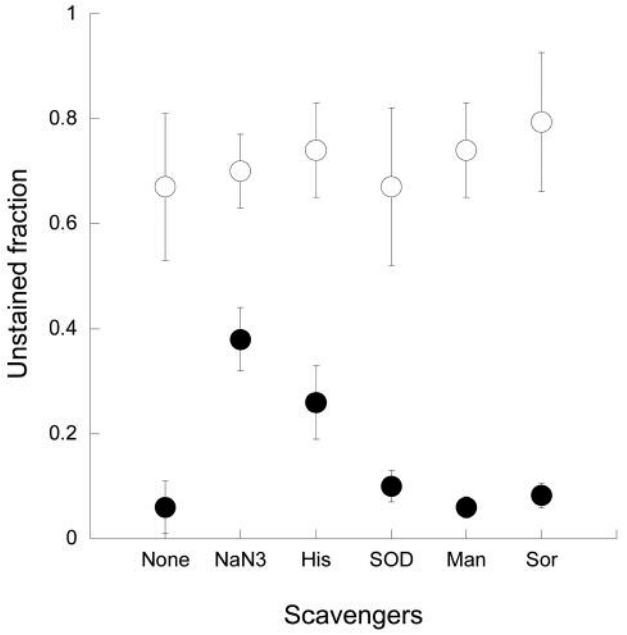

Figure 6. *Effect of active oxygen scavengers on cell damage with and without PEG-modified CNTs after 60 s of insonation. Each point and vertical bar represent the mean±SD of four insonation experiments. ○: without PEG-modified CNTs, ●: with PEG-modified CNTs.*

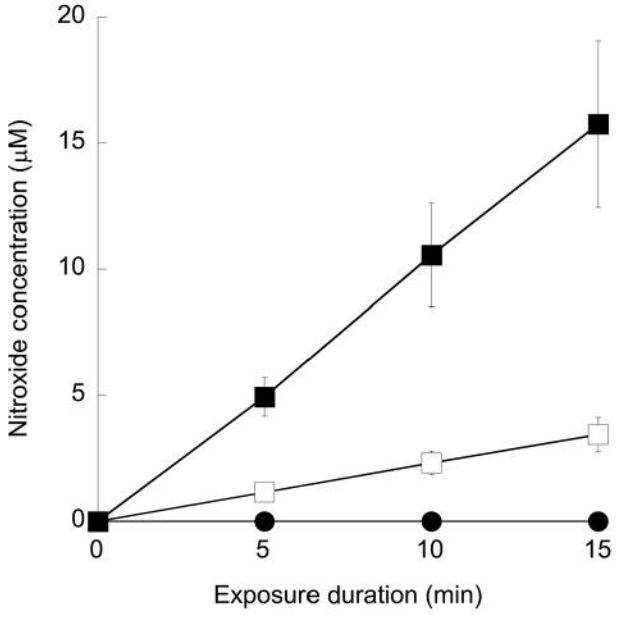

Figure 5. *Nitroxide generation in an air-saturated solution of 50 mM TMPone during exposure to ultrasound in the presence and absence of PEG-modified CNTs. Values represent the mean±SD of four independent experiments. ●: control, □: ultrasound alone, ○: 100 μM PEG-modified CNTs, ■: 100 μM PEG-modified CNTs + ultrasound.*

and placed in glass capillaries with an inner diameter of 1.1-1.2 mm, a wall thickness of 0.2 mm, and a length of 75 mm. The ESR spectra were recorded using a JEOL JES-FE3XG X-band spectrometer (JEOL Ltd., Tokyo, Japan) operating at a modulation frequency of 100 kHz and a microwave frequency of 9.26 GHz. The concentration of nitroxide produced was determined by comparison to the peak-to-peak ESR signal amplitude of 1 mM TEMPO.

Results

In vitro effect. The viable cell ratio of sarcoma 180 cells (trypan blue unstained cells) in the presence and absence of 100 μM PEG-modified CNTs following exposure to ultrasound for a fixed duration is plotted *versus* exposure duration in Figure 4, as are the results obtained with the 100 μM PEG-modified CNTs without ultrasound. The fraction of unstained cells plotted on a logarithmic scale decreased in proportion to the ultrasound irradiation time. Ultrasound-induced cytotoxicity was enhanced approximately 2-fold with the PEG-modified CNTs. The percentage of unstained cells after 60 s of sonication decreased to 76% in the absence of PEG-modified CNT and 5% in the presence of PEG-modified CNT. Cell damage was not observed with the PEG-modified CNTs alone.

Nitroxide generation. The ESR and spin trapping techniques were carried out to determine whether active oxygen species,

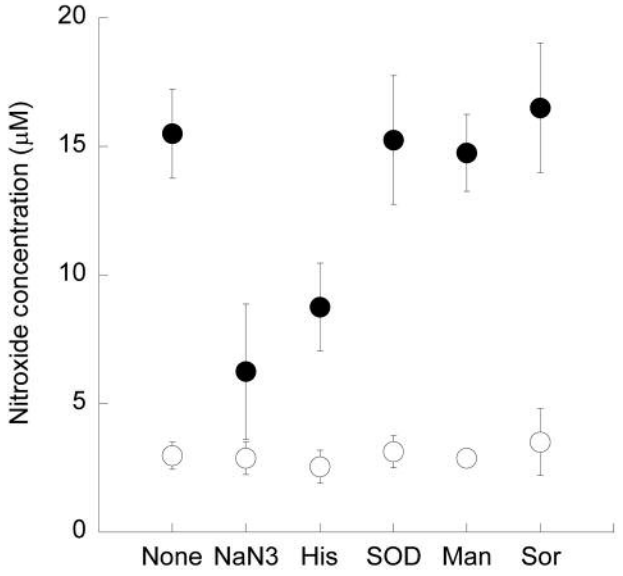

Figure 7. *Effect of active oxygen scavengers on nitroxide generation with and without PEG-modified CNTs after 60 s of insonation. Each point and vertical bar represent the mean±SD of four insonation experiments.* ○*: without PEG-modified CNTs,* ●*: with PEG-modified CNTs.*

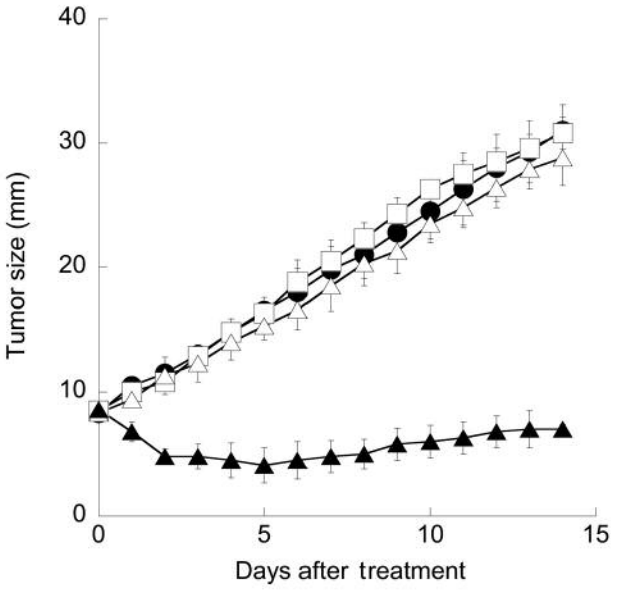

Figure 8. *Effect of ultrasound with and without PEG-modified CNTs on the growth of colon 26 solid tumors. Each point and vertical bar represent the mean±SD of four mice.* ◆*: control,* □*: ultrasound alone,* △*: PEG-modified CNTs alone,* ▲*: PEG-modified CNTs + ultrasound.*

including singlet oxygen and hydroxyl radicals, participate in the induction of cytotoxicity by ultrasound in the presence of PEG-modified CNTs. Figure 5 shows the amount of 4-oxoTEMPO produced sonodynamically with 50 mM TMPone aqueous solution containing PEG-modified CNTs, which was saturated with air under the same ultrasound irradiation conditions that were used in the cell experiments. Nitroxide production was determined from the ESR signal amplitude and was plotted against sonication time. The amount of nitroxide generated by the ultrasonic irradiation increased in proportion to the duration of ultrasonic irradiation. While 100 μM PEG-modified CNTs increased the nitroxide production rate by about three-fold, treatment with PEG-modified CNT alone did not produce any nitroxide.

Effect of reactive oxygen scavengers. To determine whether active oxygen species participate in the induction of apoptosis by ultrasound, we examined the effect of active oxygen scavengers (10 mM histidine, 100 μg/mL SOD, and 100 mM mannitol) on the fraction of cells showing morphological changes associated with apoptosis (Figure 6) and on the production of nitroxide (Figure 7). Histidine significantly reduced the apoptosis induction and nitroxide generation caused by exposure to ultrasound in the presence of PEG-modified CNTs. In contrast, SOD and mannitol did not produce an effect to these measurements at all.

Antitumoral effect in vivo. Figure 8 compares the effect of each treatment on the growth of colon 26 solid tumors, plotting weekly tumor size growth for five weeks following inoculation. PEG-modified CNTs alone had no inhibitory effect on the tumor growth, ultrasound alone had a slight inhibitory effect, while the combined treatment of ultrasound and PEG-modified CNT had a synergistic anti-tumoral effect. In the combinational treatment group, significant suppression of tumor growth after treatment was observed compared to the untreated control with $p<0.05$.

Figure 9 shows histologic sections of the tumors. Tumors treated with PEG-modified CNT alone or ultrasound alone did not show significant histological changes compared to untreated control tumors. (Figures 9B, C and A, respectively). On the other hand, combined treatment with PEG-modified CNT and ultrasound resulted in massive necrosis in the tumor area (Figure 9D).

Discussion

Here we demonstrated significant increases in sonochemically-induced *in vivo* anti-tumoral effects and biomechanically induced *in vitro* cytotoxicity using PEG-modified CNTs. PEG-modified CNTs enhanced the ultrasound-induced cytotoxicity against solitary sarcoma 180 cells, similar to porfimer sodium at the same concentration (2-4, 6, 8-16). The combination of

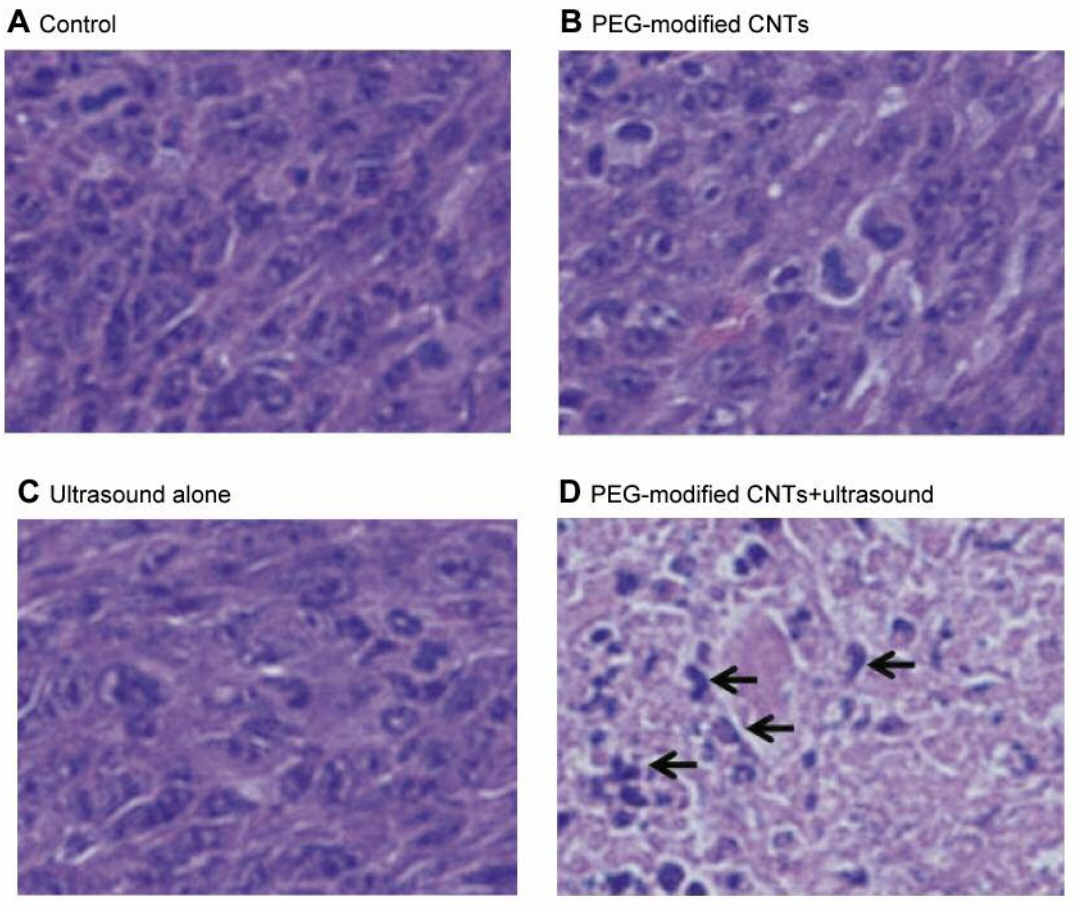

Figure 9. *Effect of ultrasound and/or PEG-modified CNTs on colon 26 carcinoma. Histological sections (×400) of the tumors using hematoxylin and eosin staining. The arrows point to necrotic areas in the tumor due to the combined treatment of PEG-modified CNTs and ultrasound.*

PEG-modified CNTs at a dose of 20 mg/kg with ultrasonic exposure has been shown to suppress the growth of implanted colon 26 cell tumors (5, 7, 19-26). In this study we showed the destruction of the tumor tissue following treatment with PEG-modified CNTs + ultrasound. Liquefied membranes were observed in the destructed colon cells. Colon 26, a syngeneic tumor model, is particularly well-suited to assess tumor responses to checkpoint inhibitors and/ or chemotherapy agents compared to allogeneic tumors. Colon 26 cells belong to the type of the colorectal cancer models that are characterized by extremely aggressive malignancy and a high metastatic potential (5, 21).

Sarcoma 180 cells were used to study cytotoxicity *in vitro* because it is difficult to achieve adequately high survival rates with isolated colon 26 cells (5, 21-26). CNTs can be synthesized as single-walled (SWCNTs) or multi-walled (MWCNTs), with the former comprising a single graphene sheet, and the latter comprising several concentric and nested graphene sheets. Both have diameters between 0.5 to 5 nm and lengths of up to 1 μm. Approximately two-thirds of as-produced SWCNTs show semiconducting properties while their band gap structure causes intrinsic fluorescence emission in the near-infrared (NIR) range (34).

SWCNTs nano-particles are suitable for imaging applications in a wide variety of biomedical fields. In addition, CNTs have ROS-generating ability, and ROS generation increases when these are used in combination with ultrasound. The formulation of reactive oxygen species (ROS) through electron transfer from single-walled CNTs in water has been reported in lung epithelial cells (35).

The implicit biological applications of functional CNTs are related to sonosensitizing or photosensitizing effects when used in combination with ultrasound or visible light, respectively (22-26). Similar to the photodynamic chemical reactions of reactive oxygen species, sonodynamically-induced reactions are classified into two types: i) a type I reaction, where the exit state of the sensitizer reacts with the substrate, and ii) a type II reaction that reacts with excited

oxygen to produce highly ROS, such as hydroxyl radicals, singlet oxygen ($^1O_2$), and superoxide radicals. We observed a substantial enhancement of generated 4-oxo-TEMPO radicals with the PEG-modified CNTs under the same acoustic conditions as the ones used in our *in vitro* experiments. Histidine and sodium azide are singlet oxygen scavengers. Thus, it is suggested that the significant inhibition of PEG-modified CNT-enhanced sonodynamically-induced cytotoxicity by histidine and sodium azide is due to increased sonication of active oxygen by PEG-modified CNT (27-30, 33). This result suggests that both the *in vitro* study and the *in vivo* antitumoral effect of PEG-modified CNTs was induced sonochemically by the additional application of ultrasound, although further studies are required to verify this. The concentration of 100 mM mannitol used in the current study is higher than the concentration reported as being efficient for scavenging the induction of ultrasonically hydroxyl radicals (36-40), yet we observed no significant change in the sonodynamically-induced cytotoxicity with mannitol plus PEG-modified CNTs. This suggests that hydroxyl radicals likely do not play an important role in this cytotoxicity. Superoxide radicals may not be important because no inhibitory effects were observed with SOD. Our results suggest that singlet oxygen most likely mediates the ultrasonically-induced cytotoxicity that is enhanced by PEG-modified CNTs, consistent with a previous hypothesis that singlet oxygen is the mediator for porphyrin derivatives, such as porfimer sodium and ATX-70 (31-32).

Sonochemically active cavitation inducing active oxygen formation is much less likely to happen inside cells compared to extracellular space (41). The resonance size of cavitation bubbles in an aqueous medium with ultrasonic frequencies in the order of megahertz is a few micrometers (42, 43), that is comparable to the size of most mammalian tissue cells. In addition, the cytoplasmic oxygen concentration is much lower compared to the extracellular fluid oxygen concentration, and the general diffusion range of reactive oxygen species is less than 100 nm (42). From the above, the cell membrane is considered as the most likely target site for the sonochemical effect of the ultrasound on cells.

According to results from experiments using cells and on living animals, sonodynamic therapy is non-cytotoxic or of very low toxicity when combined with ultrasound, and sono-sensitizers can efficiently destruct malignant cells or tissues. Most acoustic sensitizers are either porphyrin-derivatives or microparticles (26), while some of them have physical or chemical properties that significantly limit clinical applications, such as low water solubility (44). These drawbacks can be addressed using nanotechnology.

The results of the present study clearly suggest that PEG-modified CNTs exposed to ultrasound are cytotoxic to sarcoma 180 cells, and that this cytotoxicity is due to the generation of singlet oxygen, resulting in cell death. An antitumoral effect of combinational treatment was observed in colon 26 solid tumors, strongly supporting the potential of this approach towards cancer treatment using sonodynamic therapy in combination with PEG-modified CNTs (31-32, 36-38, 41, 45). In future research, in using focused ultrasound instead of plain waves, it will be necessary to build models for human applications and perform further experiments.

Conflicts of Interest

No conflicts of interest to be declared.

Authors' Contributions

NY executed the study, analyzed the findings and prepared the article. YI interpreted and analyzed the results and was involved in the writing of the manuscript. SU built the ultrasound irradiation apparatus. FC interpreted and analyzed the results and was involved in the writing of the manuscript. YM designed the study and prepared the article.

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