Comparison between sonodynamic and photodynamic effect on MDA-MB-231 cells

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Abstract
Photodynamic therapy (PDT) and sonodynamic therapy (SDT) are therapeutic modalities for tumors. In this study we investigated the combined cytotoxic effect of 0.36 W/cm² and 0.72 W/cm² ultrasound with various Ce6 concentrations (1, 2, 5, 10 μg/ml), and that of 1 μg/ml Ce6 with different laser light dose (650 nm; 10.4 mW/cm²; 0.3, 0.6, 1.2 and 2.5 J/cm²) on MDA-MB-231 cells. Both high reactive oxygen species (ROS) production and a decline in mitochondrial membrane potential (MMP) were detected with high Ce6 concentrations (5 and 10 μg/ml) combined with 0.72 W/cm² ultrasound and 1.2, 2.5 J/cm² laser light with 1 μg/ml Ce6. In addition, cell membrane integrity was evaluated by using propidium iodide (PI), revealing membrane damage was aggravated with the increasing ultrasound intensity, but no significant difference on cell membrane integrity could be observed after PDT treatment. These results suggest ROS may play an important role both in SDT and PDT. Besides, mitochondria may be an initial target in PDT while SDT can cause multi-site damages in MDA-MB-231 cells.

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1. Introduction

Breast cancer has been one of the most fatal cancers threatening females’ health in the world for decades [1]. Traditional therapies such as radiotherapy and chemotherapy are not the best way to treat breast cancer because of poor prognosis and serious side effects [2]. Although surgical treatment is a relatively effective method for the sufferer, but it has higher recurrence rate and may cause great injury for the physical body and beauty [3]. Therefore, searching for novel therapeutic strategies in tumor treatment, especially noninvasive approaches, is urgently required.

Photodynamic therapy (PDT) has been increasingly used for treatment of various tumors [4]. The basis of this therapy is the photosensitizer preferentially accumulates in malignant tissue and is subsequently activated by light of an appropriate wavelength [5,6]. Following absorption appropriate light wavelengths, the photosensitizer is excited into a high-energy state, from which it is returned, accompanied by the transfer of an electron to adjacent molecules, referred to as a type I photochemical reaction, or energy to ground state of molecular oxygen, type II photochemical reaction [7,8]. The reactive oxygen species (ROS), such as singlet oxygen (¹O₂), superoxide radical anion (O₂⁻), hydroxyl radical (·OH), and hydrogen peroxide (H₂O₂), produced in this process are harmful to cells [9]. As demonstrated by previous studies, PDT has been successfully applied in some clinical cases [4]. However, lasers are unable to penetrate and reach deep tissues to activate the photosensitizer, so the application of PDT is considered to be limited in the future development [6].

Sonodynamic therapy (SDT), another new promising tumor treatment method, which is derived from PDT, applies ultrasound to activate sensitizers and could eventually kill cancer cells [10]. Ultrasound has an appropriate tissue attenuation ability, allowing it to penetrate into tissues and reach non-superficial objects while maintaining the ability to focus energy into small volumes and activate sensitizers [11]. Among noninvasive treatments, this advantage is unique compared to PDT. Recently, in vitro and in vivo experiments have demonstrated the significant anti-tumor effects of SDT, but the mechanisms have not been understood clearly. Hiraoka et al. [12] reported that the mechanism of ultrasound is primarily due to certain mechanical stress, such as physical disruption of cellular membrane, and the synergistic effect of ultrasound and sensitizers is suggested owing to the photo-excitation by the sonoluminescence produced in collapsing cavitation [13]. Studies have suggested that SDT-induced cavitation may be responsible for the sonochemically generated radical production and a quantitative phenomenon [14,15]. Li et al. investigated the combined anti-cancer effect of PDT and SDT which was called Sono-Photodynamic therapy (SPDT) [16], however, the involved mechanisms remain unclear.
In this study, we applied a sensitizer chlorin e6 (Ce6), which has been reported to preferentially accumulate in tumor tissues [17]. By evaluating the antitumor effect of Ce6 mediated PDT and SDT on human breast cancer MDA-MB-231 cells, we attempt to compare the distinct mechanisms between the two anti-cancer therapies.

2. Materials and methods

2.1. Cell culture

Human breast cancer MDA-MB-231 cells were obtained from the cell bank of Chinese Academy of Science, Shanghai, China. The cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Life Technologies, Inc., USA) supplemented with 10% fetal bovine serum (FBS, HYCLONE, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2. Cells in the exponential phase of growth were used in each experiment.

2.2. Chemical

Ce6 was purchased from Sigma chemical (St. Louis, MO, USA) and the purity was greater than 95%. Ce6 was dissolved in sterilized PBS (0.01 M, pH 7.4) at a stock concentration of 2.5 mg/ml, aliquoted and stored in the dark at −20 °C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide tetrazolium (MTT), N-acetylcysteine (NAC), propidium iodide (PI) and rhodamine 123 (Rho123), were also supplied by Sigma. 2′,7′-Dichlorodihydrofluorescein-diacetate (DCFH-DA) was supplied by Molecular Probes Inc. (Invitrogen, CA, USA). All other reagents were commercial products of analytical grade.

2.3. Photodynamic and ultrasound treatment

The laser light (excitation wavelength: 650 nm; manufacturer: Institute of Photonics & Photon Technology, Department of Physics, Northwest University, Shaanxi, China) was used as a source for evocation of the photodynamic effect. Irradiance was measured by the radiometer system (Institute of photonics & photon-technology, Department of physics, Northwest university). For the laser light, the power output: 1.3 mW; power intensity: 10.4 mW/cm²; irradiation time: 30–240 s; emitting aperture area: 0.125 cm²; area of the beam: 9.62 cm²; The power intensity (10.4 mW/cm²) = the output power (1.3 mW)/the detector area (0.125 cm²) and the energy intensity was calculated using the following equation: energy intensity (J/cm²) = power intensity (W/cm²) × irradiation time (seconds), so the final light dose was varied from 0 (dark control) to 2.5 J/cm² in this experiment. When cells reached around 70% of confluence in a 35 mm diameter culture dish, cells were divided randomly into four groups: (1) control, (2) Ce6 alone, (3) laser light alone, (4) Ce6 plus laser light (PDT). For Ce6 alone and PDT groups, cells were sonicated in the serum-free DMEM with 1 μg/ml Ce6 for 4 h, allowing the sensitizer uptake of the cells to reach a maximum level. Instead of Ce6, an equivalent quantity of serum-free DMEM was used in the control and laser light alone groups. The cells in PDT group were irradiated with light from 0.3 J/cm² to 2.5 J/cm².

A 35 mm diameter planar transducer (Institution of Applied Acoustics, Shaanxi Normal University) was submerged in a circular water bath filled with de-gassed water. Continuous-wave ultrasound was generated by the amplifier (T&C Power Conversion, Inc., Rochester, NY). For SDT, cells in 35 mm culture dish were placed in the water bath and 1 cm above the top of the transducer in a horizontal position. Cells were sonicated at intensities of 0.36, 0.72 W/cm² with the frequency of 1.0 MHz, for each intensity the concentration of Ce6 varied from 1 μg/ml to 10 μg/ml. After the treatment procedure, cells were re-suspended in fresh medium and cultured for an additional time as specified in the text and then subjected to different analysis.

Experiments were carried out in low-level light to minimize any influence of photo-activation.

For inhibitory experiment, 5, 7.5, 10 mM N-acetylcysteine (NAC), the ROS scavenger, was added to serum-free DMEM 1 h before loading Ce6. The inhibitor at the used concentration did not yield any significant cell damage to cultured cells.

2.4. Cytotoxicity

The cytotoxicity of PDT and SDT on MDA-MB-231 cells was analyzed by using the MTT assay. Briefly, cells after different treatment were added to 96 well culture plates, and viability was determined by adding 20 μl MTT solution (2.5 mg/ml in PBS) to each well and the mixture was incubated for additional 4 h at 37 °C in a CO2 incubator. After incubating, the mixture was removed and 150 μl pure DMSO was added per well. After shaking for 15 min at room temperature, the absorbance at 570 nm was recorded using a microplate reader (Bio-Tek ELX800, USA) against the reference value at 630 nm. Cytotoxicity was calculated using the following equation:

\[ \text{Cytotoxicity(\%)} = \frac{\text{OD}_{\text{control group}} - \text{OD}_{\text{treatment group}}}{\text{OD}_{\text{control group}}} \times 100\% \]

2.5. Determination of intracellular ROS

Intracellular ROS production was studied by measuring the fluorescence intensity of dichlorofluorescein (DCF) as described in our previous papers [3]. 2,7′-DCF-diaceitate (DCFH-DA), a non-fluorescent cell-permeant compound, is cleaved by endogenous esterases within the cell and the de-esterified product can be converted into the fluorescent compound DCF upon oxidation by intracellular ROS.

At 1 h after different treatment, cells were washed with PBS and incubated with serum-free DMEM containing 4 μM DCFH-DA at 37 °C in an incubator for 20 min with gentle shaking on a small shaking table. After incubation, samples were washed by PBS and harvested by trypsinization, then immediately detected by flow cytometry (Guava easyCyte BHT, Millipore, USA). Histograms were analyzed using FCS Express V3 software.

2.6. Determination of mitochondrial membrane potential

Rhodamine 123 (Rho 123) was used to evaluate perturbation in mitochondria membrane potential (MMP) as previously described [18]. At 1 h after different treatment, cells were harvested and washed with PBS then incubated at 37 °C with 1 μg/ml Rho 123 in serum-free DMEM for 20 min followed by washing with PBS. Then samples were immediately detected by flow cytometry.

2.7. Cell membrane integrity

To monitor membrane permeability after SDT and PDT, PI was added. PI can stain the nuclei by intercalating between the stacked bases of nucleic acid. PI can enter cells only if the cell membrane becomes permeable, so it is widely used to measure the integrity of the plasma membrane. Briefly, after PDT or SDT cells were harvested in the indicated times and washed three times with PBS then re-suspended in PBS containing 5 μg/ml PI for 5 min in the dark. The samples were then immediately detected by flow cytometry. Histograms were analyzed using FCS Express V3 software.
2.8. Statistical analysis

All values were expressed as means ± S.D. Differences among different groups were assessed with one-way ANOVA. Statistical significance was established at a value of $p < 0.05$. Every experiment was repeated for three times.

3. Results

3.1. The cytotoxicity of PDT or SDT on the growth of MDA-MB-231 cells

For PDT, cytotoxicity of cells was tested at 4 h and 24 h after treatment. Fig. 1A shows that at 4 h and 24 h after treatment, no inhibitory effect was observed in laser light alone (0.3–2.5 J/cm$^2$) groups. And in the presence of 1 μg/ml Ce6 (Fig. 2B), when the light dose was lower than 0.6 J/cm$^2$, there was also no obvious cell viability inhibition both at 4 h and 24 h after PDT treatment. The cell viability was significantly declined to 74.91% ($p < 0.05$) at 24 h post-PDT at a light dose of 1.2 J/cm$^2$. When the light dose was up to 2.5 J/cm$^2$, the cell viability loss was very significant both at 4 h and 24 h post-PDT treatment, and the cell viability was decreased from 75.62% ($p < 0.05$) to 13.45% ($p < 0.01$) when the incubation time increased from 4 h to 24 h.

For SDT, the cytotoxic effect was also detected 4 h and 24 h after ultrasound treatment at the intensity of 0.36 W/cm$^2$ and 0.72 W/cm$^2$. Fig. 2A showed that Ce6 alone did not exert cytotoxicity at the used concentration (1, 2, 5, 10 μg/ml). Both 4 h and 24 h after treatment, as Fig. 2B shows, 0.36 W/cm$^2$ ultrasound alone treatment were not cytotoxic to MDA-MB-231 cells. But when cells were treated with 0.36 W/cm$^2$ ultrasound combined with 1, 2, 5, and 10 μg/ml Ce6, the cell survival 4 h after treatment declined to 90.21% ($p > 0.05$), 82.24% ($p > 0.05$), 71.23% ($p < 0.05$) and 67.33% ($p < 0.01$), respectively. The cytotoxicity effect of 24 h after SDT treatment was similar to the results of 4 h and the viability was 91.36% ($p > 0.05$), 82.03% ($p > 0.05$), 70.01% ($p < 0.05$) and 67.29% ($p < 0.01$), respectively. When ultrasound intensity increased to 0.72 W/cm$^2$ (Fig. 2C), ultrasound alone treatment could result in 33.5% ($p < 0.01$) and 34.02% ($p < 0.01$) cell viability loss 4 h and 24 h after ultrasound irradiation. The addition of Ce6 enhanced the cytotoxicity induced by ultrasound, Fig. 2C revealed the result, at 4 h after treatment, 0.72 W/cm$^2$ ultrasound combined with 1, 2, 5 and 10 μg/ml Ce6 could decrease cell viability to 66.50% ($p < 0.01$), 58.85% ($p < 0.01$), 48.85% ($p < 0.01$) and 43.23% ($p < 0.01$), respectively. In addition, the detected results of 24 h post-SDT was 67.50% ($p < 0.01$), 54.82% ($p < 0.01$), 40.04% ($p < 0.01$), and 31.23 ($p < 0.01$), respectively.

3.2. The generation of ROS in PDT and SDT

To understand the role of ROS in PDT and SDT, it is necessary to measure the intracellular ROS formation. Fig. 3 showed that compared with control, in the presence of 1 μg/ml Ce6, there were
1.25%, 12.60% (p < 0.05), 34.15% (p < 0.01) and 50.55% (p < 0.01) of cells in 0.3, 0.6, 1.2 and 2.5 J/cm² laser light treatment groups showed high DCF fluorescence, respectively. Moreover, Fig. 4 revealed ROS level in cells treated with SDT, 0.72 W/cm² ultrasound alone showed no difference compared with control (p > 0.05), but when the Ce6 concentration was 1, 2, 5 and 10 µg/ml the ROS generation increased to 15.5% (p < 0.05), 21.45% (p < 0.05), 45.05% (p < 0.01) and 51.2% (p < 0.01), respectively.
In addition, cell survival was tested with the presence of ROS scavenger NAC both in PDT and SDT after 24 h incubation post-treatment. Fig. 5A displayed that 5 mM NAC can relieve the cytotoxicity, especially when the light dose was 2.5 J/cm², NAC could enhance the cell survival from 13.45% to 70.01%. Similar with PDT, 5 mM NAC could improve the survival of cells treated with SDT. To further confirm the role of ROS in PDT treatment, we increased the concentration of NAC to 7.5 mM and 10 mM. Result in the Fig. 5B showed that the decreased cell viability caused by PDT can totally rescued by 10 mM NAC. While, under the same condition even 10 mM NAC cannot absolutely rescue the cell viability loss caused by SDT treatment (Fig. 6B).

3.3. Mitochondrial dysfunction

The mitochondrial membrane potential (MMP) was determined with Rhod2 staining at 1 h post-PDT or SDT treatment and the decrease of Rhod2 fluorescence was used to reflect the loss in MMP. In PDT treatment, an increase in laser dose resulted in a significant decrease in MMP as seen in Fig. 7. The percentage of cells with MMP loss increased to 32.5% (p < 0.01) and 55.05% (p < 0.01) when the light dose was 1.2 J/cm² and 2.5 J/cm² in the presence of 1 μg/ml C66 respectively, indicating the collapse of MMP occurred early post-PDT in MDA-MB-231 cells. In SDT treatment, the MMP of cells treated with 0.72 W/cm² ultrasound alone showed no difference with control cells, while when we combined ultrasound with different C66 concentration, an obvious decline of MMP was occurred. Fig. 8 showed that the percentage of cells with MMP loss increased from 16.5% (p < 0.05) to 33.5% (p < 0.01) when C66 concentration increased from 1 μg/ml to 10 μg/ml combined with 0.72 W/cm² ultrasound treatment.

3.4. Cell membrane damage detection

PI staining combined with flow cytometry was used to evaluate PDT or SDT induced cell membrane damage. As we can see in Fig. 9, no matter immediately after PDT treatment (Fig. 9a) or 24 h after PDT treatment (Fig. 9b), MDA-MB-231 cells did not displayed higher PI fluorescence regardless of any light dose, suggesting the cell membrane was intact post-PDT. Fig. 10a indicates the result immediately after different intensity of ultrasound treatment in the presence of 1 μg/ml C66, we can see the percentage of cells with higher PI fluorescence gradually increased from 1.2% to 38.25% (p < 0.01) when cells were exposed to ultrasound dose range from 0.36 W/cm² to 0.9 W/cm² with 1 μg/ml C66. While after 24 h incubation post these treatments, the enhancement of cell membrane permeability could not be observed (Fig. 10b), there were no significance between the SDT treated groups with control.

4. Discussion

As a minimally invasive therapeutic approach, PDT has been widely used in the treatment of various tumors since the 1990s [4,12]. While sonodynamic therapy, in which sonosensitizers could be activated by ultrasound, has displayed anti-cancer activity and was considered potentially useful for future cancer treatment [12]. Because of the distinct physical properties of light and ultrasound, the mechanisms involved in PDT and SDT are different. Numerous researches have demonstrated that PDT relies on the generation of ROS [19–22], whereas the mechanisms of SDT are complex and unclear [23–25]. Thus, in this study we compared the effects of SDT and PDT on MDA-MB-231 cells under our experimental condition.
Both PDT and SDT revealed cytotoxic effect on MDA-MB-231 cells. The cell damage caused by SDT was instant \[11,26\] and serious damage occurred at early times after treatment. But the cell damage caused by PDT was a delayed effect, which was enhanced by the prolonged incubation time.

Several investigations supported the crucial role of ROS in PDT and SDT \[3,27,28\]. High-level ROS generation can induce significant oxidative damage to cellular bio-molecules, including lipids, proteins and nucleic acids \[29\]. Our data showed that the production of ROS in MDA-MB-231 cells was dependent on the
concentration of Ce6 in SDT, and on the light dose in PDT. Moreover, the results of cell survival in the presence of ROS scavenger NAC demonstrated that cell survival was obviously improved as the increasing NAC concentration, and 10 mM NAC could improve the cell survival from 13.45% to 99.04% in PDT. While under the same conditions, SDT-caused cell damage was partly remitted by NAC, and even 10 mM NAC could not rescue the damaged cells fully. These results indicated that PDT-induced cytotoxicity was totally due to the generation of ROS whereas ROS may be only one element among many in SDT-caused cell death.

**Fig. 9.** Effects of PDT on the cell membrane integrity of MDA-MB-231 cells. Cells were treated with PDT ((A) 1 μg/ml Ce6 alone; (B) 1 μg/ml Ce6 + 0.3 J/cm² laser light; (C) 1 μg/ml Ce6 + 0.6 J/cm² laser light; (D) 1 μg/ml Ce6 + 1.2 J/cm² laser light; (E) 1 μg/ml Ce6 + 2.5 J/cm² laser light), then stained with PI and analyzed by flow cytometry. (a) Immediately after PDT; (b) 24 h after PDT. Data shown are representative of three independent experiments.
The site of sensitizer localization is potentially important in either PDT or SDT, because of the short lifetime and diffusion distance of some radical products derived from the sensitizer produced during PDT and SDT treatment process [30]. Given that Ce6 mainly located in the mitochondria of MDA-MB-231 cells [31], so we subsequently determined the MMP of cells after PDT and SDT. Results in Figs. 7 and 8 indicated that both PDT and SDT led to MMP loss at a very early stage post-treatment, suggesting mitochondria damage may be the initial damage target in both PDT and SDT. Mitochondria play vital roles in many stimuli induced apoptosis [32,33], the impaired mitochondria may subsequently trigger apoptotic responses in Ce6 mediated PDT and SDT.

**Fig. 10.** Effects of SDT on the cell membrane integrity of MDA-MB-231 cells. Cells were treated with SDT ((A) 1 μg/ml Ce6 alone; (B) 0.36 W/cm² + 1 μg/ml Ce6; (C) 0.54 W/cm² + 1 μg/ml Ce6; (D) 0.72 W/cm² + 1 μg/ml Ce6; (E) 0.9 W/cm² + 1 μg/ml Ce6), then stained with PI and analyzed by flow cytometry. (a) Immediately after SDT; (b) 24 h after SDT. Data shown are representative of three independent experiments.
Hirao et al. [12] suggested that the mechanism of action of ultrasound was primarily due to certain mechanical stress, such as augmentation of physical disruption of cellular membrane in the close vicinity of cells by sensitizers, and/or cavitation bubbles. In addition, many researchers reported that ultrasound can change the permeability of the cell membrane which is benefit to transmit macromolecule substances, such as specific drugs, into cells [33,34]. Here, we used PI to evaluate the cell membrane permeability and integrity after different treatment. The data showed that the membrane integrity could not be affected by PDT at 0 h and 24 h post-treatment. Whereas immediately after SDT treatment with increasing ultrasound intensity, the fluorescence of PI was rapidly increased, indicating serious damage occurred in cell membrane, and this phenomenon disappeared at 24 h after treatment. The undetected cell membrane damage may be due to (1) the recovery of enhanced cell membrane permeability, (2) the disappearance of necrotic cells. Previous researches indicated that ultrasound could affect the permeability of cell membrane [35,36], high intensity ultrasound can kill cells immediately with the irreversible cell membrane broken, whereas ultrasound at low intensity would not cause serious cytotoxicity but can affect the membrane permeability significantly [31]. Moreover, the damage of cell membrane caused by low intensity would recover in a few minutes or hours post-ultrasound irradiation [37].

Based on the above-mentioned results, we hypothesize that both PDT and SDT can cause mitochondrial damage, because C6 mainly located in the mitochondria of MDA-MB-231 cells. In both PDT and SDT, ROS played a vital role in cytotoxicity and may be the main factor to in mitochondrial damage, and then the impaired mitochondria may initiate the secondary cell death. Moreover, except for the oxidative stress, SDT-induced mechanical damage also made a contribution to the cell killing effect on MDA-MBA-231 cells.

5. Conclusion

In summary, this study evaluated the cytotoxicity induced by SDT and PDT on MDA-MB-231 cells and intended to compare the different mechanisms between the two therapies. Our findings illustrated that both PDT and PDT could cause MDA-MB-231 cells viability loss and the synergistic damage may be due to the targeting of mitochondrial damage, excessive intracellular ROS generation and the loss of MMP. Besides, the cell membrane may be another important target in SDT, but not in PDT.

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