Apoptosis of SAS Cells Induced by Sonodynamic Therapy Using 5-Aminolevulinic Acid Sonosensitizer

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Abstract. *Background: 5-Aminolevulinic acid (ALA) has been used as a photodynamic sensitizer for cancer treatment using photodynamic therapy. However, the light has markedly limited penetration depth. It was found that ALA also responds to low energy ultrasound, which has the capability to penetrate deep into tissues. Therefore, sonodynamic therapy (SDT) is a promising method for noninvasive treatment of tumors embedded deep in the tissue. It is desirable to kill the cancer cells via apoptosis rather than necrosis, and therefore, it is necessary to gain a better understanding of the mechanisms of treating cancer using SDT. Materials and Methods: The apoptosis of SAS cells induced by pulsed 1.05MHz ultrasound in combination with ALA was investigated in vitro. Results: The cells exposed to SDT with 10 μg/ml ALA displayed significantly higher apoptosis than cells treated by ultrasound alone. There was notably increased reactive oxygen species (ROS) production in the cells treated by SDT with ALA than by ultrasound alone, resulting in higher lipid peroxidation (LPO) level and more cells losing their mitochondrial membrane potential (MMP). Conclusion: ALA-mediated SDT produced strong apoptotic effects on SAS cells, which were mainly related to the excessive intracellular ROS production followed by LPO increase and MMP decrease.*

Sonodynamic therapy (SDT) based on the synergistic effects of ultrasound and certain sonosensitizers has been used in cancer cells *in vitro* (1-4) and in animals bearing malignant tumors (5-

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8). Ultrasound is able to penetrate deep tissues noninvasively due to its relatively low tissue-attenuation coefficient. The energy of an ultrasound beam is able to be focused on sites of malignancy and, thus, locally activate sonosensitizers that have preferentially accumulated in tumor cells, causing desired cell death with minimal damage to peripheral healthy tissue. In addition, normal cells are found to be relatively more resistant to ultrasonic irradiation than malignant cells (9, 10). To increase treatment efficiency and reduce undesired responses, there have been many studies on the sonodynamic effects of sonosensitizers (1-8). Tumor-localizing porphyrins, which are traditionally used as photodynamic sensitizers, displayed sonodynamic cytotoxicity in cancer cells and malignant tumors (1-8, 11-14). The generation of reactive free oxygen radicals from the ultrasonic activation of sonosensitizers leads to an attack on the cell biomembrane and inactivation of key antioxidant enzymes (12, 15), which is considered responsible for the sonodynamic damage of cancer cells and, eventually, tumor growth inhibition.

Among these porphyrins, protoporphyrin IX (PpIX) shows enhanced cytotoxic activity and antitumor effects when irradiated by ultrasound, while it is nontoxic without sonication (11-14). In addition to the exogenous supply of PpIX itself, the accumulation of PpIX in tumors or cells may be produced by the administration of its precursors, such as 5-aminolevulinic acid (ALA) or ALA derivatives (16-21). The PpIX content in malignant tumors or cells after exposure to ALA is notably higher than in healthy tissues or cells due to the low activity of ferrochelatase in cancer cells (22, 23). The pharmacokinetics of ALA-induced porphyrin in tumor-bearing mice demonstrated that the maximum contrast of porphyrin concentration between the tumor and the nonmalignant organs may reach a value of 30 at 4-6 h post-injection (18), whereas 24 h or more is required for the direct administration of PpIX (5). It has been proven that the application of ALA or its derivatives in photodynamic therapy (PDT) induced the photoinactivation of various carcinoma cells (16, 17, 20-22). Moreover, ALA-mediated

PDT presented better photodynamic efficiency than the exogenous administration of PpIX (16, 20). One reason for this involves the different intracellular PpIX monomer concentration from endogenous and exogenous PpIX. More importantly, the subcellular localization pattern of PpIX induced by ALA is distinct from that of the exogenous supply, *i.e.* ALA-derived PpIX is selectively localized in the mitochondria, while exogenous PpIX accumulates mainly in cell membranes (16, 20). Although the PDT effects on cancer cells have been proven, to date there are few studies on the effects of endogenous PpIX in SDT. This work describes an investigation of the sonodynamic effects of ALA-induced PpIX on the SAS human tongue carcinoma cell line *in vitro*. The potential ALA-mediated SDT mechanism of cell apoptosis was investigated in detail by examining the intracellular reactive oxygen species (ROS) production, lipid peroxidation (LPO) level and mitochondrial membrane potential (MMP) loss.

Materials and Methods

Cells and cell culture. The OSCC-derived cell line SAS (Human Science Research Resources Bank, Osaka, Japan) was grown in RPMI-1640 medium with 10% fetal bovine serum and cultured in a humidified incubator (Heraeus, Hanau, Germany) at 37°C and 5% CO_2. Before conducting the experiments, cell viability was always more than 97%.

Reagents. ALA (Aldrich, St. Louis, MO, USA) of chromatographic grade was dissolved in phosphate-buffered saline (PBS), sterilized and stored in the dark at 4.0°C. Fluorescein isothiocyanate (FITC)-labeled annexin V apoptosis detection kit (Nanjing KeyGen Biotech Co. Ltd., Nanjing, P.R. China), ROS assay kit (Applygen Technologies Co. Ltd., Beijing, P.R. China), malonaldehyde (MDA) detection kit (Beyotime Co. Ltd., Nantong, P.R. China) and rhodamine123 (Sigma, St. Louis, MO, USA) were used.

Ultrasonic device and intensity measurement. The ultrasonic setup is shown in Figure 1A. A 15 μm thick polyethylene film with low ultrasound attenuation and rapid heat dissipation was made into a small bag (dimensions, 16 mm×17 mm) in order to contain the cell suspension. This cell suspension bag was immersed in degassed water and directly faced the transducer surface of an ultrasonic device (resonance frequency: 1.05 MHz, duty factor: 60%, repetition frequency: 100 Hz, focal length: 7.0 cm). The internal surface of the acrylic sonication tank was padded with ultrasound-absorbing foam to minimize standing wave formation. The cell suspension was 8 cm away from the transducer surface. Ultrasonic intensities were measured inside the tank using a PT-0907110 needle-type hydrophone (active element size: 2 mm, bandwidth: 1 M–10 MHz; Institute of Acoustics, Chinese Academy of Sciences, Beijing, P.R. China), three PSA150-11-X translation stages (Zolix, Beijing, P.R. China) and a DPO4014 digital oscilloscope (Tektronix, Beaverton, OR, USA). The peak acoustic pressure was 0.314 MPa and the temporally averaged intensity distribution is shown in Figure 1B. A thermocouple showed that the temperature in the cell suspensions was raised less than 2°C in these experiments.
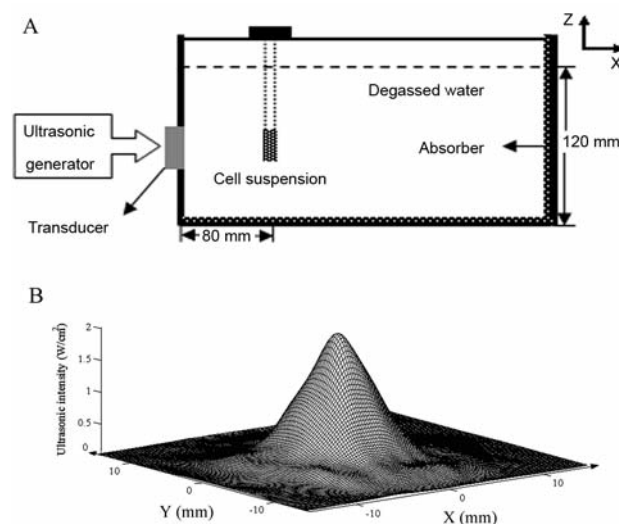

Figure 1. *A: Schematic diagram of the sonication setup. B: The intensity distribution of pulsed ultrasound. The measured plane in the sonication tank was 8 cm away from the transducer, where the cell suspension bags were located.*

Methods. There were four groups of cells in the experiment: control (C), ALA alone (A), sonication alone (U) and ultrasound plus ALA (UA). For the A and UA groups, ALA solutions were added to the cell suspension to reach different concentrations, and then re-incubated for 4 h in the dark. The cells in the C and U groups were put into an equivalent PBS solution instead of the ALA solution. After 4 h incubation, the cells were collected, washed twice with cold PBS and re-suspended in 1 ml medium with about 1.2×10^6 cells. The cell suspensions in the U and UA groups were insonicated at 37°C in the dark.

Cell viability and growth. A trypan blue exclusion test was conducted by mixing 200 μl cell suspension with an equal amount of 0.4% trypan blue solution to measure cell viability. After a 3 min reaction at room temperature, the cells were counted by an ECLIPSE TS100 optical microscope (Nikon, Tokyo, Japan) with a hemocytometer. The cell viability was quantified by the cell survival rate, defined as the percent ratio of the number of intact cells to the number of total cells. After 2, 6, 12 and 24 h incubation, the effects of ALA at 0, 1, 10, and 50 μg/ml concentrations on cell growth were studied by MTT assay with a BioPhotometer Plus (Eppendorf, Hamburg, Germany) at a wavelength of 600 nm.

Cell apoptosis detection. Flow cytometry was performed with FITC-labeled annexin V and propidium iodide (PI) to detect cell apoptosis. The cells were collected and washed with cold PBS after 6 h incubation. According to the manufacturer's instructions, the cells were resuspended in 500 μl binding buffer, 5 μl annexin V and 5 μl PI. After being incubated at room temperature for 10 min in the dark, the cells were analyzed with a flow cytometer (FACSCount, NY, USA).

ROS measurement. Intracellular ROS content was determined by measuring the fluorescence of 2,7-dichlorofluorescein (DCF). The
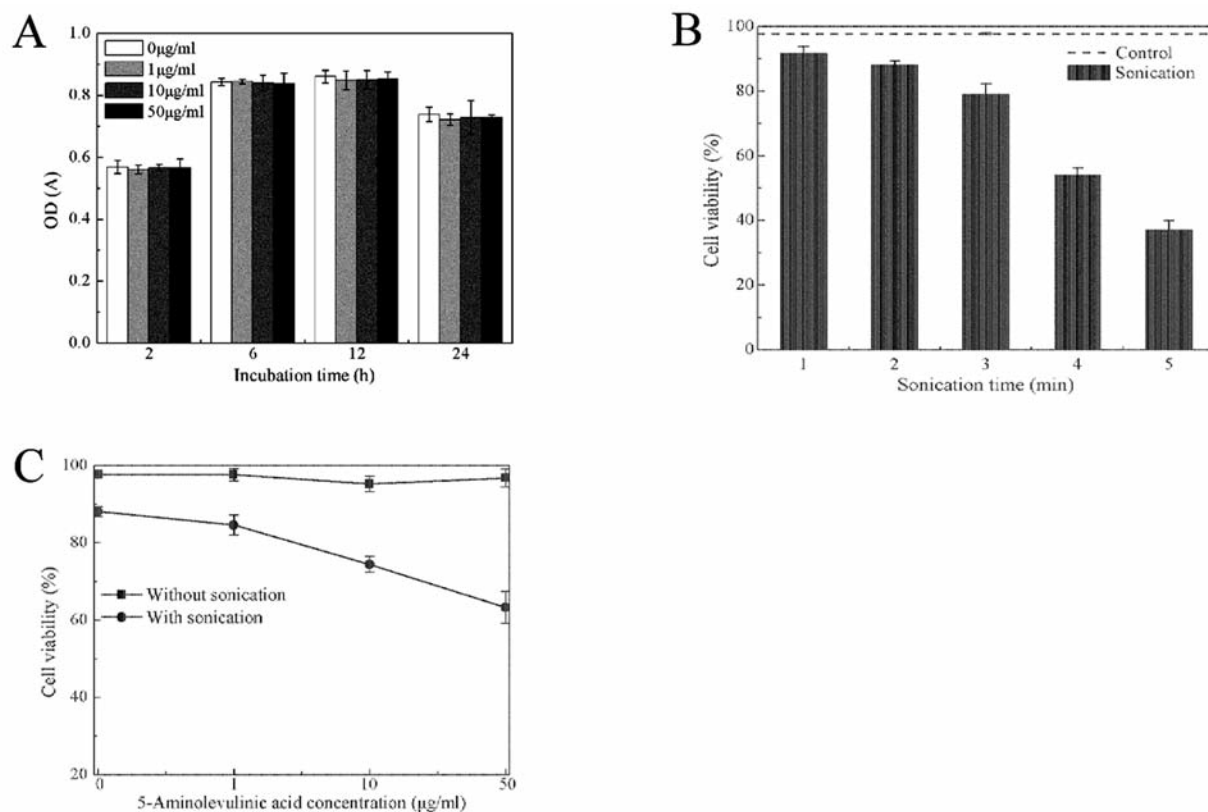
Figure 2. *A: The effects of ALA solution of different concentrations on SAS cells growth after different incubation periods (n=3). B: Survival rates of SAS cells after sonication alone for 1, 2, 3, 4 and 5 min (n=6). C: Survival rates of SAS cells after 2 min sonication plus ALA at 0, 1, 10 and 50 μg/ml (n=6). Data are presented as the mean±standard deviation.*

cells were suspended in 1 ml serum-free DCFH-DA of 10 μM, incubated at 37˚C for 20 min in the dark and then washed carefully with PBS following the manufacturer's instructions. Immediately after the treatment, a total of 1×10^6 cells were collected, resuspended in serum-free medium and measured using a Fluoromax-4 fluorescence spectrophotometer (Horiba Jobin Yvon, Paris, France) at 488 nm excitation and 525 nm emission wavelengths. The intracellular ROS production was expressed as the fluorescence intensity (10^6 cps) of 1×10^6 cells.

LPO determination. MDA, a stable endproduct of fatty acid peroxidation, can react with thibabituric acid (TBA) at acidic conditions to form MDA-TBA adduct with a maximum absorbance at 532 nm. A total of 1×10^6 cells were collected and lysed after the treatment. Following the instructions of the MDA detection kit, the LPO level was determined using a U-4100 UV-Visible-NIR Spectrophotometer (Hitachi, Tokyo, Japan).

MMP detection. The MMP decrease was determined using a flow cytometer with Rho123 staining. After the treatment, Rho123 solution was added into the cell suspension to attain a 2 μg/ml final concentration, and cells were further incubated at 37˚C for 30 min in the dark and washed twice with PBS. Fluorescence was measured at 488 nm excitation and 530 nm emission wavelengths.

Statistical analysis. All data were reported as mean value±standard deviation. Differences between groups were assessed according to the Student *t*-test. Statistical evaluation was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Differences with $p < 0.05$ were considered statistically significant.

Results

Cell viability and growth. The MTT assay showed that the cell growth treated by ALA solution alone was similar to that of the control group (Figure 2A). The trypan blue exclusion test displayed no obvious cell death under ALA treatment alone (Figure 2C). The survival rates of the SAS cells exposed to ultrasound displayed an obvious decrease as sonication time increased from 1 to 5 min (Figure 2B). Ultrasonic treatment of 2 min or longer increased cell killing rates compared to the control group. When the cells were treated by ultrasound in the presence of ALA at different concentrations, the cell viability decreased with the increase of ALA concentration (Figure 2C). The cells exposed to ultrasonic irradiation plus 10 μg/ml ALA or more displayed notably lower viabilities than those subjected to sonication alone.
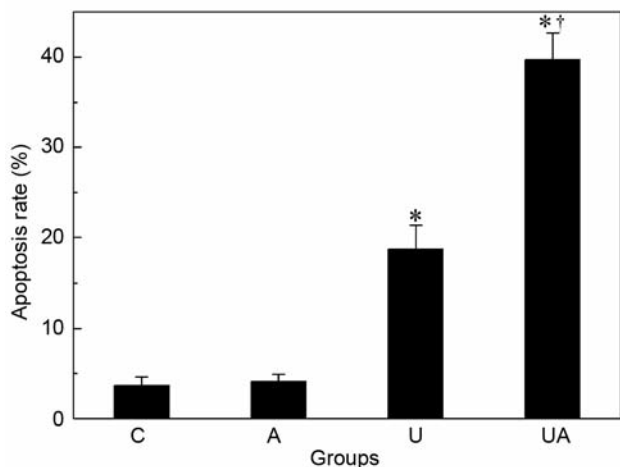
Figure 3. *Apoptosis rates of SAS cells after treatment (n=6). Data are presented as the mean±standard deviation. C: Control group without treatment; A: 10 µg/ml ALA alone; U: 2 min sonication alone; UA: 2 min sonication plus 10 µg/ml ALA; *p<0.05 compared to C; †p<0.05 compared to U.*
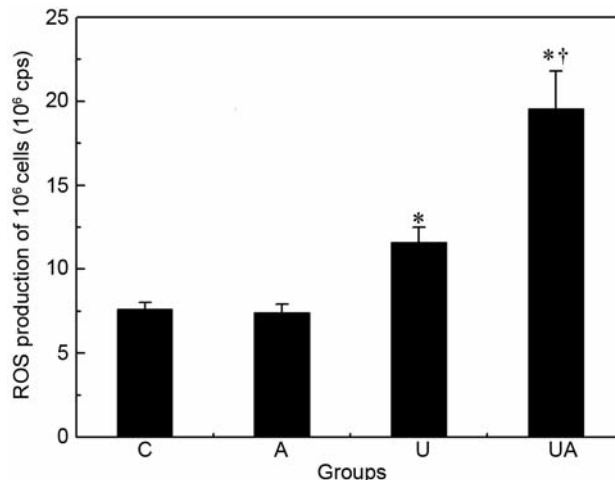

Figure 4. *Intracellular reactive oxygen species (ROS) production in SAS cells (n=6). Data are presented as the mean±standard deviation. C: Control group without treatment; A: 10 µg/ml ALA alone; U: 2 min sonication alone; UA: 2 min sonication plus 10 µg/ml ALA; *p<0.05 compared to C; †p <0.05 compared to U.*

Cell apoptosis. After 6 h incubation, the apoptosis rates of SAS cells in all four groups were analyzed using flow cytometry with double staining of annexin V/PI. There were nearly equal cell apoptosis rates in the ALA treatment alone and the control groups (Figure 3). However, the cells incubated in 10 µg/ml ALA for 4 h and subsequently sonicated for 2 min displayed a much higher apoptosis rate than the other three groups ($p<0.05$). Specifically, there was a 20.91% increase in apoptosis rate compared to the group subjected sonication alone without the ALA.

ROS production, LPO level and MMP loss. ALA treatment alone caused no obvious difference from the control group in terms of ROS production, LPO level and MMP loss (Figures 4, 5 and 6). Sonication alone without sonosensitizer increased the ROS production and LPO level compared to the control group ($p<0.05$). However, the intracellular ROS production, LPO level and MMP loss of the SAS cells treated by ultrasound plus ALA achieved the highest level among these groups ($p<0.05$).

Figure 5. *Lipid peroxidation levels of SAS cells after treatment (n=6). Data are presented as the mean±standard deviation. C: Control group without treatment; A: 10 µg/ml ALA alone; U: 2 min sonication alone; UA: 2 min sonication plus 10 µg/ml ALA; *p<0.05 compared to C; †p<0.05 compared to U.*

Discussion

Recently, ALA has been widely investigated as a photodynamic sensitizer (16-23). Since ultrasound has much lower attenuation than light in biological tissues, SDT is potentially more promising for the noninvasive treatment of deep tumors. In this work, the sonodynamic effects of ALA-derived PpIX on SAS cells were investigated *in vitro*. ALA treatment alone had no growth inhibition or direct cytotoxicity (Figures 2A and 2C). Although the SAS cell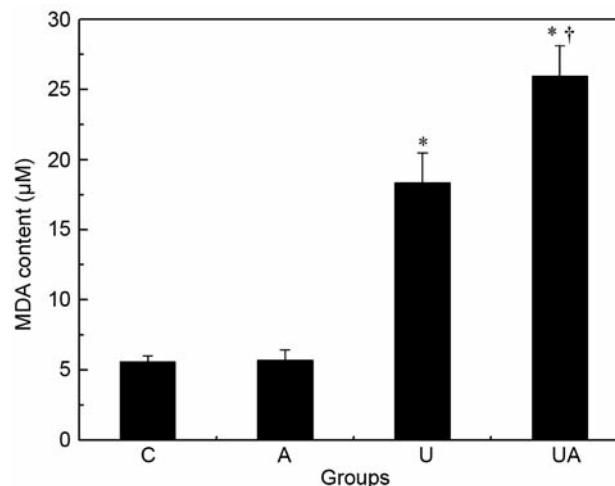s were killed directly by either ultrasound alone (Figure 2B) or ALA-mediated sonodynamic treatment (Figure 2C), the rapid cell death in cancer therapy always produces undesirable immune reactions *in vivo* (24). However, the instant cell killing produced by the present ultrasonic condition may be less for the *in vivo* case due to the self-repair of some cells and structural configurations of cells within the body (2). Thus, 2 min sonication and 10 µg/ml ALA were considered appropriate in the present study for diminishing undesirable immune response *in vivo*.
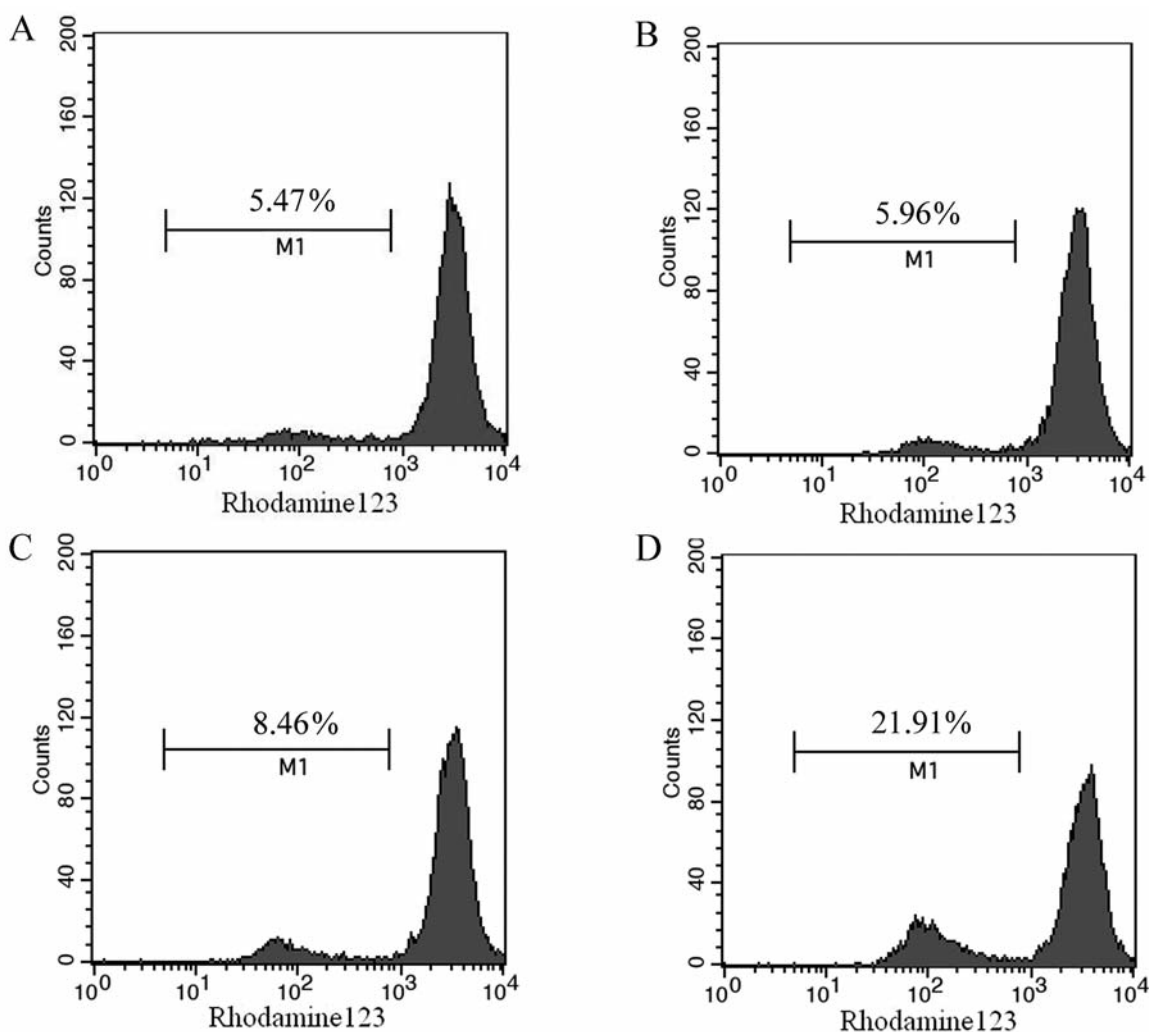
Figure 6. *MMP decrease of SAS cells after treatment. The histograms representing cell counts versus fluorescence intensity of rhodamine123 are representative of four independent experiments. M1: Cells with loss of MMP. A: control group without treatment; B: 10 μg/ml ALA alone; C: 2 min sonication alone; D: 2 min sonication plus 10 μg/ml ALA.*

Apoptosis, which does not trigger immune reactions, is considered a better way to kill cancer cells than instant cell death and necrosis (24, 25). This study revealed that the apoptotic effect of ultrasonic irradiation on SAS cells is significantly enhanced in the presence of ALA. It has been reported that ultrasound in combination with exogenous PpIX effectively increased cell damage and apoptosis on sarcoma-180 cells and hepatoma-22 cells compared to sonication alone (12, 13, 15). In the present study, the cellular PpIX was synthesized in mitochondria by the administration of ALA (26). There are differences in pharmacokinetics (5, 18), PpIX monomer concentration (20) and subcellular localization (16) between the direct supply of PpIX and the ALA-converted PpIX. The present results demonstrated that ALA-mediated SDT is an efficient method to induce SAS cell apoptosis.

Based on the theory of ultrasonic cavitation, the high temperature and high pressure produced by the burst of cavitational bubbles may excite the sensitizers to a higher energy state; then, the sensitizers may interact with oxygen molecules and water to form hydrogen peroxide, superoxide radicals, alkoxyl radicals *etc*. (6). In addition, sonoluminescence caused by ultrasonic cavitation may also excite the sensitizers directly, generating highly reactive singlet oxygen. These ROS may react with proteins, nucleic acids, lipids and other molecules, leading to the change of their structures and, thus, causing damage in cells and tissues. It should be noted that there are numerous defensive mechanisms in the cells based on the antioxidative enzymes and action of low molecular antioxidants, which are able to eliminate excessive active oxygen and inhibit free-radical

reactions (27). However, the defensive system may be destroyed by the formation of excessive free radicals, resulting in irreversible modifications of biologically fundamental macromolecules. ALA treatment alone caused similar ROS production and LPO level with the control group, which also did not produce noticeable cell killing (Figure 2C), growth inhibition (Figure 2A) or cell apoptosis (Figure 3). However, SDT with ALA induced much more ROS contents in SAS cells than other groups, which is consistent with previous studies of SDT of S180 cancer cells in the presence of PpIX (15). The joint effect of the ultrasound and the sonosensitizers was responsible for the observed significant increase of cell damage and apoptosis of SAS cells. The generation of oxygen free radicals by the ultrasonic activation of ALA-derived cellular PpIX may decrease the activities of key antioxidant enzymes in cells and may further attack polyunsaturated fatty acids in the membrane lipids to produce numerous oxygenated compounds, including MDA. A much higher MDA content was observed in the group combining ultrasound with ALA (Figure 5), which corresponded well with the result of the highest intracellular ROS production in the group treated by ALA-mediated SDT. The integrity of the cell membrane plays an important role in the exchange of substances in or out of the cell. The occurrence of LPO due to oxidative stress may alter the structure of biological membranes, modify the physiological function of cell membrane and, finally, lead to cell death.

The treatment efficiency of cancer cells is proven to be dependent on subcellular localization patterns of sonosensitizers, which may be related to the fact that some cell organelles are more sensitive to cancer therapy than others (16, 20). Previous investigations suggested that mitochondrial dysfunction participates in the induction of cell apoptosis and even plays a central role in the apoptotic pathway (28). The present study indicated that the cells treated by either ALA alone or sonication alone displayed similar MMP loss when compared with the control group, whereas the highest percentage of SAS cells losing MMP was observed after ALA-mediated sonodynamic treatment (Figure 6). When the cells are exposed to ALA solution, the cellular PpIX, an effective sensitizer for SDT, is synthesized in mitochondria (26). Since the hydroxyl radicals and singlet oxygen have very short lifetime and limited migration range (6, 29), the intracellular oxygen free radicals from the ultrasonic activation of cellular PpIX are mostly accumulated in the mitochondria. Therefore, the mitochondrion may be an important target among the cellular organelles in ALA-mediated SDT and may be destroyed by the intracellular ROS, leading to a loss of MMP. The MMP loss has been found to reduce the function of mitochondrial adenosine triphosphate synthesis (16). Additionally, the release of cytochrome *c*, Smac/Diablo and AIF from intermembrane space into the cytosol is observed due to the decreased MMP and the changed mitochondrial permeability, which then activates caspase-9 and caspase-3 (10, 12, 28). These results imply that the enhanced apoptosis of SAS cells in sonodynamic treatment with ALA is potentially related to the mitochondrial pathway.

In conclusion, ALA-mediated SDT exhibited a synergistic damage and apoptosis of SAS cells *in vitro*, implying that ALA is an effective sonosensitizer for cancer treatment using SDT. The excessive intracellular ROS production followed by the LPO increase and the MMP decrease was mainly responsible for the significantly higher cell killing and apoptosis rates induced by SDT in the presence of ALA.

Acknowledgements

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