


# Ultrasound-Induced Cell Death of Nasopharyngeal Carcinoma Cells in the Presence of Curcumin

Integrative Cancer Therapies  
10(1) 70–76  
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DOI: 10.1177/1534735410377197  
<http://ict.sagepub.com>  


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## Abstract

**Objectives.** Curcumin, a natural pigment from a traditional Chinese herb, has been attracting extensive attention. The present study aims to investigate cell death of nasopharyngeal carcinoma (NPC) cells induced by ultrasound sonication in the presence of curcumin *in vitro*. **Methods.** The NPC cell line CNE2 was chosen as a tumor model, and curcumin concentration was kept constant at 10  $\mu$ M while the cells were subjected to ultrasound exposure for 8 s at an intensity of 0.46 W/cm<sup>2</sup>. Cell death was evaluated using flow cytometry with annexinV-FITC and propidium iodine staining, and nuclear staining with Hoechst 33258. Mitochondrial membrane potential and intracellular reactive oxygen species (ROS) were analyzed using flow cytometry with rhodamine 123 and dichlorodihydrofluorecein diacetate staining. **Results.** Flow cytometry showed that the combination of ultrasound and curcumin significantly increased the necrotic or late apoptotic rate by up to 31.37% compared with the controls. Nuclear condensation was observed in the nuclear staining, and collapse of  $\Delta\Psi_m$  and ROS increase were found in the CNE2 cells after the treatment with curcumin and ultrasound. **Conclusions.** The findings demonstrate that the presence of curcumin significantly enhances the ultrasound-induced cell death and ROS level, and induces the collapse of  $\Delta\Psi_m$ , suggesting that ultrasound sonication can increase the cell death of NPC cells in the presence of curcumin and that the treatment using curcumin and ultrasound together is a potential therapeutic modality in the management of malignant tumors.

## Keywords

curcumin, ultrasound sonication, cell death, nasopharyngeal carcinoma

## Introduction

Nasopharyngeal carcinoma (NPC) remains one of the most common cancers in Southern China, especially Hong Kong. Although recent therapeutic modalities for NPC, such as surgery, radiotherapy, and chemotherapy, are initially successful in the majority of cases, the 5-year survival rate is about 25% in NPC patients.<sup>1</sup> Therefore, novel and more efficient therapeutic strategies and drugs are currently being pursued in the management of NPC.

Hematoporphyrin derivative (HpD)-mediated photodynamic therapy (PDT) has been introduced as an alternative for NPC, but prolonged skin photosensitivity and poor penetration in biological tissues limit the clinical application of HpD-PDT.<sup>2-4</sup> To overcome the shortcomings of HpD, new and efficient photosensitizers are being explored. Our previous studies have also investigated the effect of PDT with the use of some second-generation photosensitizers on NPC. Our findings suggested that photosensitization of MPPa and temoporfin (mTHPC) could effectively kill NPC cells.<sup>4,5</sup> In

view of their relatively nontoxic and inexpensive characteristics, natural products or herbs have been attracting extensive interest for screening as novel and efficient sensitizers. Recently, curcumin, a natural pigment of perylquinone derivatives (Figure 1), has been found to be a naturally occurring photosensitizer, and light irradiation significantly enhanced curcumin-induced cell death of CNE2 cells.<sup>6,7</sup> However, the poor penetration of light in biological tissues is still a limitation on effective activation of curcumin in solid tumor tissues.

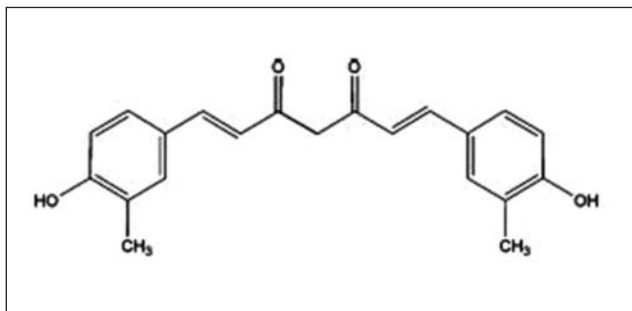
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**Figure 1.** Chemical structure of curcumin

Recent studies showed that ultrasound could efficiently activate some sensitizers to produce cytotoxic reactive oxygen, directly or indirectly killing tumor cells.<sup>8-10</sup> The fact that ultrasound is a more efficient activating source because it has a better penetration power than light has drawn our interest to this area of study. In our previous studies, we have investigated the combined effect of ultrasound and several sensitizers on tumor cells. Our findings showed that hematoporphyrin monomethyl ether and hypocrellin B could enhance the “sonokilling” action of ultrasound in tumor cells.<sup>11-13</sup> In the present study, we have explored whether curcumin could enhance the effect of ultrasound on the cell death of CNE2 cells *in vitro* in order to provide a novel technology for treating malignancies.

## Materials and Methods

### Sensitizer

Curcumin was purchased from Sigma (USA). A stock solution was made in dimethyl sulfoxide at a concentration of 100 mM and kept in the dark at  $-20^{\circ}\text{C}$ .

### Cell Culture

NPC cell line CNE2 was provided by the Shanghai Biology Institute under approval from the ethics committee of Chongqing Medical University. The cells were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum (Gibco), 50  $\mu\text{g}/\text{mL}$  penicillin, 50  $\mu\text{g}/\text{mL}$  streptomycin, and 10  $\mu\text{g}/\text{mL}$  neomycin. The cells were incubated at  $37^{\circ}\text{C}$  in a humidified  $\text{CO}_2$  (5%) incubator.

### Ultrasound Treatment

The CNE2 cells were exposed to ultrasound 1 hour after the incubation with curcumin (10  $\mu\text{M}$ ). A 1-cm diameter transducer with a central frequency of 1.7 MHz and intensity of  $0.46 \text{ W}/\text{cm}^2$  was used to generate continuous ultrasound energy at 8 s. The plane transducer was placed in a water

tank filled with degassed water, and the temperature was kept at  $37^{\circ}\text{C}$  during ultrasonic exposure.<sup>11,12</sup> All experiments were randomly divided into 4 groups: Curcumin–ultrasound treatment, ultrasound sonication alone, and the controls, including curcumin treatment alone and sham sonication. For the curcumin–ultrasound treatment, the cells were pretreated with curcumin incubation before the ultrasound exposure. The cells in the ultrasound sonication alone group were only exposed to ultrasound but not incubated with curcumin. Those in the curcumin treatment alone group were only pretreated with curcumin incubation with no ultrasound sonication. The cells in the sham sonication group were not treated with ultrasound or curcumin.

### Cell Death Detection

After ultrasound treatment, the CNE2 cells were incubated in culture flasks at  $37^{\circ}\text{C}$  for 6 hours. Cell death was analyzed by flow cytometry with annexin V-FITC Apoptosis Detection Kit (Beyotime, Jiangshu, China). Briefly, the cells from each sample were suspended in 195  $\mu\text{L}$  of  $1\times$  annexin V-FITC binding buffer and 5  $\mu\text{L}$  annexin V-FITC. The cells were incubated at room temperature for 10 minutes. Then, each sample was centrifuged at 1000 g for 5 minutes, suspended again in 190  $\mu\text{L}$  of binding buffer, to which was added 10  $\mu\text{L}$  of propidium iodide (PI) working solution. Then, the samples were analyzed by the flow cytometric method (FCM). The population was separated into 3 groups: live cells with a low level of fluorescence, apoptotic cells in the earlier period with green fluorescence, and necrotic and advanced-stage apoptotic cells with both red and green fluorescence.

### Nuclear Staining

The CNE2 cells ( $2 \times 10^5$  cells/well) were incubated in a 24-well microplate at  $37^{\circ}\text{C}$  for 6 hours after ultrasonically induced cell damage in the presence and absence of curcumin. The cells were washed with phosphate buffered saline (PBS), and nuclear staining was performed. Briefly, the treated and the control cells were stained with Hoechst 33258 (5  $\mu\text{g}/\text{mL}$ ; stock solution 1 mg/ml in sterile water) for 5 minutes at  $37^{\circ}\text{C}$ . Stained cells were washed twice with PBS and then observed immediately under a fluorescence microscope. A filter set of Ex/Em of BP330-380/LP420 nm was used, and the images were recorded by a color charge-coupled device camera.

### Mitochondrial Membrane Potential ( $\Delta\Psi\text{m}$ ) Measurement

Mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ) was monitored using FCM with rhodamine 123 staining. Briefly, the CNE2 cells were sensitized with curcumin (10  $\mu\text{M}$ ) for 1 hour. The

cells were then sonicated by ultrasound at an intensity of  $0.46 \text{ W/cm}^2$  for an exposure of 8 s and further incubated for 3 hours. The rhodamine 123 ( $5 \mu\text{g/mL}$ ) was added 30 minutes before cell harvesting. The washed cells were resuspended in PBS and analyzed using a flow cytometer FACSVantage (SE, Becton Dickinson) with the excitation setting at 488 nm, and signals were acquired at the FL-2 channel. At least 10 000 cells per sample were acquired in histograms, and data were analyzed using CellQuest software.

### Intracellular Reactive Oxygen Species (ROS)

Intracellular ROS were detected using the FCM with 2,7-dichlorodihydrofluorecein diacetate (DCFH-DA) staining. The DCFH-DA was supplied by Sigma and dissolved in ethanol to produce a 1 mmol/L stock solution. The DCFH-DA ( $10 \mu\text{M}$ ) was added 3 hours after cells were treated by ultrasound. The washed cells were resuspended in PBS and analyzed using a flow cytometer FACSVantage (SE, Becton Dickinson) with the excitation setting at 488 nm, and signals were acquired at the FL-2 channel. At least 10 000 cells per sample were acquired in histograms, and data were analyzed by CellQuest software.

### Statistical Analysis

All data were processed using analysis of variance (ANOVA). A *P* value of less than .05 was considered a significant difference.

## Results

### Cell Death of the CNE2 Cells Induced by Curcumin and Ultrasound

Flow cytometry with annexin V (annexin V-FITC) and PI staining can identify early and late apoptotic cells by annexin V-FITC binding to PS exposed on the outer leaflet in early apoptotic cells. This can also distinguish early apoptosis from late apoptosis or necrosis because PI is impermeable to living cells and early apoptotic cells are impermeable to PI.<sup>14</sup> The results from our studies showed that ultrasound exposure in the presence of curcumin increased the necrotic or late apoptotic rates of the CNE2 cells. Figure 2A shows that the early apoptotic and necrotic (late apoptotic) rates of the CNE2 cells 6 hours after treatment with sham sonication were 0.96% and 3.18%, respectively. Figure 2B shows that the early apoptotic and necrotic (late apoptotic) rates of the CNE2 cells after treatment with curcumin alone were 2.12% and 4.79%. Figure 2C shows that the early apoptotic and necrotic (late apoptotic) rates of the CNE2 cells after the treatment with ultrasound sonication in the absence of curcumin were 1.57% and 4.55%. Figure 2D shows that the apoptotic and necrotic (late apoptotic) rates after ultrasound

sonication and curcumin together significantly increased to 2.91% and 31.37%, respectively, under the intensity of  $0.46 \text{ W/cm}^2$  for 8 s. These findings suggest that ultrasound sonication and curcumin together could obviously enhance necrosis or late apoptosis of the CNE2 cells. To further verify curcumin-sonodynamic therapy induced cell death of the CNE2 cells, the cells were stained using Hoechst 33258 after the treatment with ultrasound and curcumin together. The CNE2 cells in the sham control, curcumin treatment alone, and ultrasound sonication alone groups displayed a weak fluorescence, whereas nuclear condensation was found 6 hours after ultrasound sonication in the presence of curcumin (Figure 3).

### Mitochondrial Membrane Potential ( $\Delta\Psi\text{m}$ )

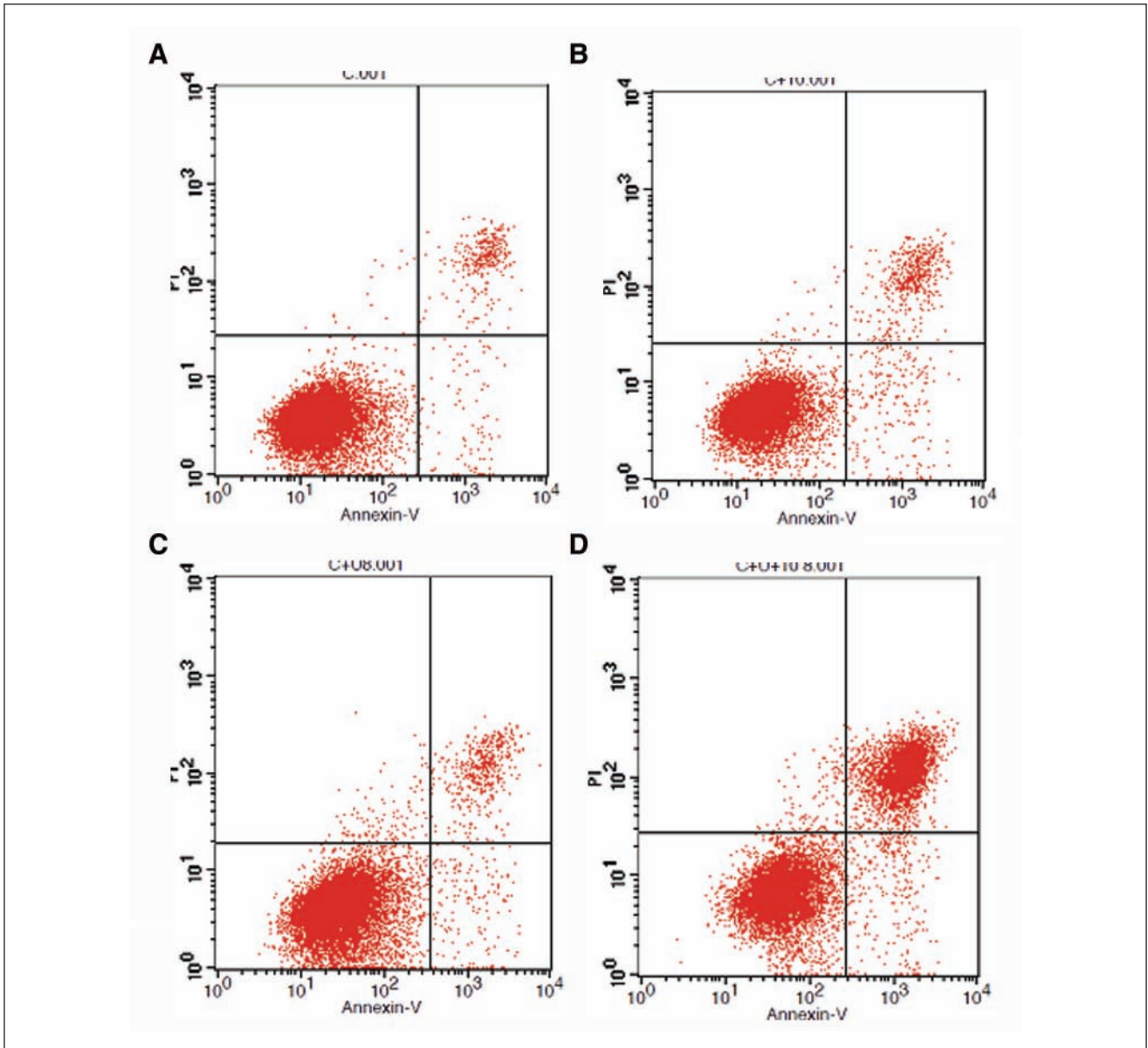
The fluorescent intensity value of rhodamine 123 reflects the mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ) and the functioning of mitochondria. FCM with rhodamine 123 staining was used to measure the change of mean fluorescent intensity in  $\Psi\text{m}$ . CNE2 cells were sensitized with curcumin ( $10 \mu\text{M}$ ) and then treated with ultrasound ( $1.7 \text{ M}$ ) at an intensity of  $0.46 \text{ W/cm}^2$  for an exposure time of 8 s. Figure 4 shows the significant changes in mean fluorescent intensity in  $\Delta\Psi\text{m}$  in the CNE2 cells after ultrasound sonication in the presence of curcumin. A collapse of  $\Delta\Psi\text{m}$  was observed in the CNE2 cells treated with ultrasound and curcumin together.

### Intracellular Reactive Oxygen Species

The fluorescent intensity of cells was detected by flow cytometry with DCFH-DA staining, showing that the spectral shift of the fluorescence curve to the right after the combination of sonication and curcumin was more significant than that after ultrasound treatment alone and curcumin treatment alone (Figure 5). This indicated that ultrasound sonication in the presence of curcumin significantly enhanced the intracellular ROS in the CNE2 cells.

## Discussion

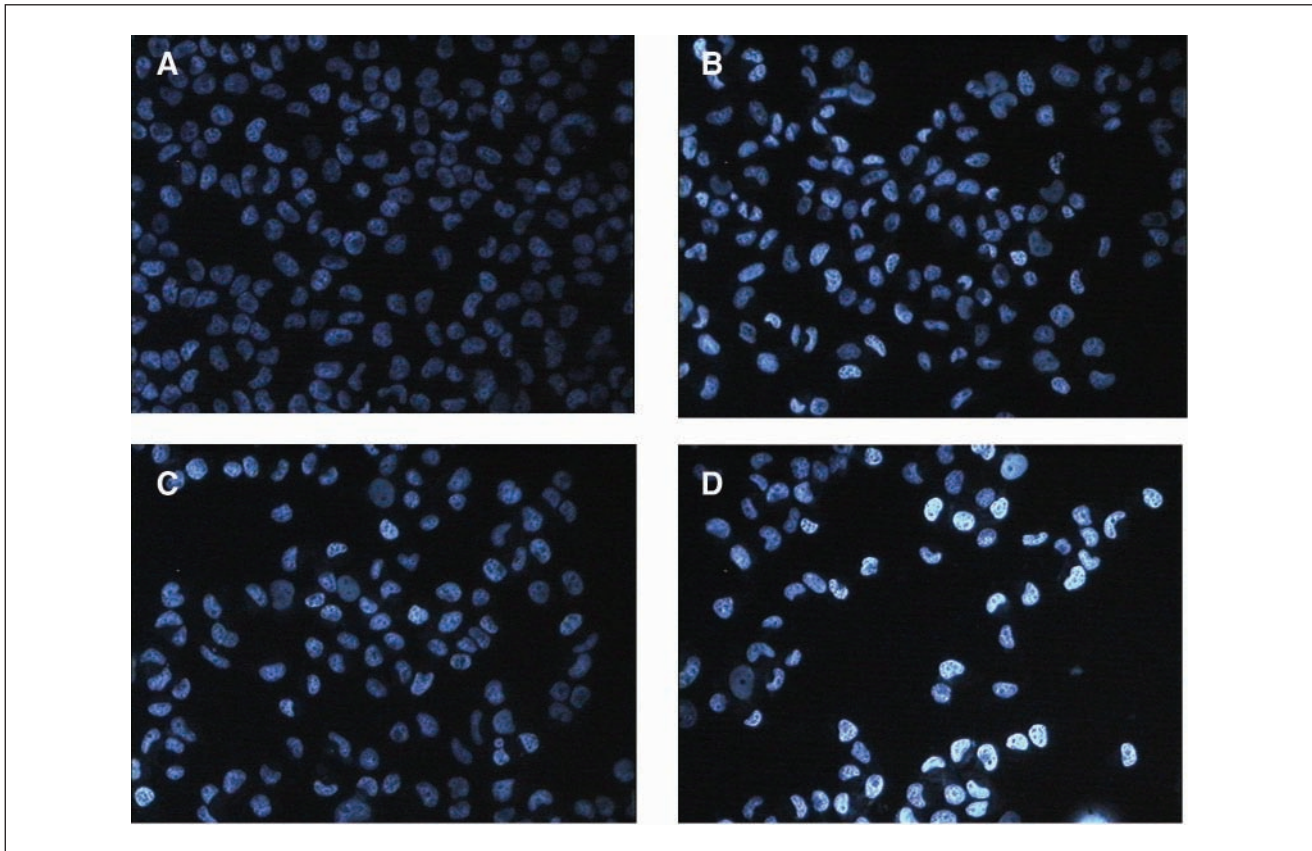
Curcumin, a natural pigment of diferuloylmethane, is obtained from the rhizome of the plant *Curcuma longa*.<sup>1,15</sup> In India and China, curcumin has been consumed as a dietary supplement and extensively used to treat body aches, rheumatic disease, skin diseases, urinary diseases, and digestive and inflammatory disorders.<sup>16</sup> In view of its various important pharmacological properties, including antioxidative, antiviral, anti-inflammatory, and antitumoral effects, curcumin has been extensively studied as an important chemopreventive and chemotherapeutic drugs.<sup>15,17-19</sup> Recent evidence showed that curcumin was a potent photosensitive drug and that visible light could increase its cytotoxicity in cancer cells.<sup>6,7</sup> However, its absorption wavelength is



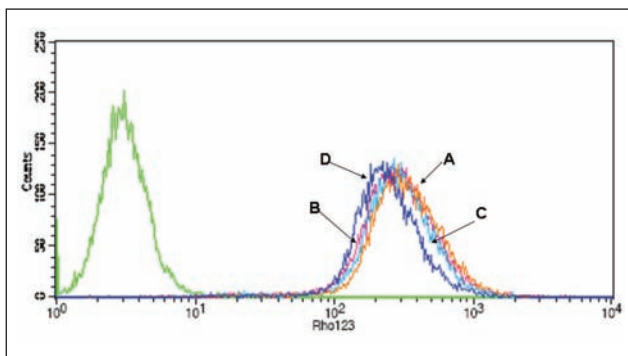
**Figure 2.** Cell death of the CNE2 cells was analyzed 6 hours after ultrasound sonication in the presence of curcumin (10  $\mu$ M) under ultrasound sonication (1.7 MHz) with an intensity of 0.46 W/cm<sup>2</sup> for 8 s. Flow cytometry with annexin V-FITC and propidium iodide (PI) staining: annexin V-FITC in conjunction with PI staining could distinguish early apoptotic (annexin V-FITC positive, PI negative; bottom right quadrant of each panel) from late apoptotic or necrotic (annexin V positive, PI positive; top right quadrant of each panel) cells. Fluorescence intensity for annexin V-FITC is plotted on the x axis, and PI is plotted on the y axis. A. Sham sonication. B. Curcumin alone. C. Ultrasound sonication alone. D. ultrasound sonication with curcumin

located in the range of 450 nm to 550 nm. Its low penetration in biological tissues limits the clinical application of light-activated curcumin. Because of the ability of ultrasound to noninvasively target specific regions of interest at depth without affecting the intermediate tissue,<sup>20</sup> ultrasound-activated curcumin may be developed as a novel noninvasive approach for combating malignancies. To investigate the effect of curcumin on ultrasound-induced

cell death, the poorly differentiated CNE2 cells were chosen as a tumor model for cell death testing in the present study. Cells die through 2 major modes—namely, necrosis and apoptosis. Necrosis is a passive event usually ensuing from rigorous inflammation, directly resulting in cell death. Apoptosis is a programmed cell death with an active self-destruction of a single cell characterized by specific nuclear and cytoplasmic features.<sup>21,22</sup> In this

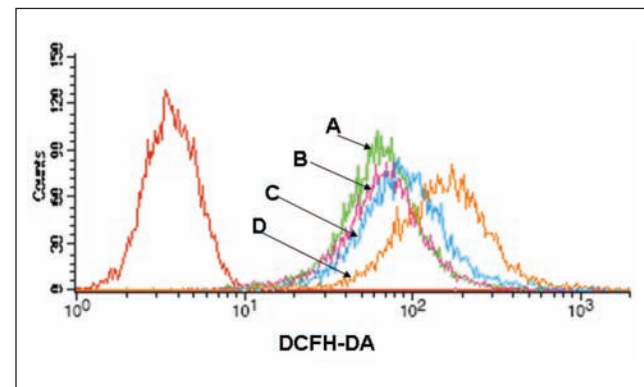


**Figure 3.** Observation of nuclear features in the CNE2 cells 6 hours after ultrasound sonication in the presence of curcumin (10  $\mu$ M) was analyzed by the Hoechst33258 staining method. A: Sham sonication. B: Curcumin alone. C: Ultrasound sonication alone. D: Ultrasound sonication with curcumin



**Figure 4.** Collapse of  $\Delta\Psi_m$  in the CNE2 cells was analyzed using flow cytometry with rhodamine 123 staining 3 hours after ultrasound sonication in the presence of curcumin (10  $\mu$ M). A: Sham sonication. B: Curcumin alone. C: Ultrasound sonication alone. D: Ultrasound sonication with curcumin

study, the apoptosis and necrosis of the CNE2 cells induced by ultrasound sonication and curcumin together (10  $\mu$ M) were analyzed 6 hours after ultrasound exposure using nuclear staining and flow cytometry with annexin V and PI staining. Nuclear staining indicated that the treatment



**Figure 5.** Measurement of reactive oxygen species level in the CNE2 cells using flow cytometry with 2,7-dichlorodihydrofluorecein diacetate staining 3 hours after ultrasound sonication in the presence of curcumin (10  $\mu$ M). A: Sham sonication. B: Curcumin alone. C: Ultrasound sonication alone. D: Ultrasound sonication with curcumin

with curcumin and ultrasound sonication together significantly induced nuclear condensation in the CNE2 cells. Flow cytometry with annexin V and PI staining showed

that the necrotic or late apoptotic rate of the CNE2 cells after ultrasound sonication significantly increased to 31.37% and the early apoptotic rate only up to 2.91% in the presence of curcumin. Our findings suggest that necrosis or late apoptosis might be a main mode of cell death induced by the combined treatment with curcumin and ultrasound.

Mitochondria are known to be a regulatory center in the process of cell death. The alterations of mitochondrial structure and function directly or indirectly interfere with cellular respiration and energy metabolism; the permeability transition pore opens and mitochondria membrane potential (MMP/ $\Delta\Psi_m$ ) collapses, causing cell death.<sup>23,24</sup> Thus, mitochondrial integrity and function is a decisive factor in cellular fate between life and death.<sup>23</sup> Our present study shows that after ultrasound sonication alone,  $\Delta\Psi_m$  began to decline but significantly decreased to its lowest after the combined treatment of curcumin and ultrasound sonication, demonstrating that ultrasound sonication in the presence of curcumin significantly resulted in the collapse of  $\Delta\Psi_m$ . The collapse of  $\Delta\Psi_m$  is a standard indicator of mitochondrial damage.<sup>25</sup> Our results demonstrated that the combined effect of curcumin and ultrasound sonication seriously damaged the mitochondria of the CNE2 cells. When  $\Delta\Psi_m$  collapses, the mitochondria generate morphological and functional changes, resulting in cell death. Therefore, combined treatment of curcumin and ultrasound sonication induced cell death probably through the mitochondrial pathway.

Oxidative stress is an important event in ultrasound-induced cell damage. Ultrasound sonication can increase the level of ROS in ultrasound-treated cells.<sup>26</sup> The highly reactive oxygen can initiate further oxidative modification of lipid and protein molecules causing damage to the target cells/tissues.<sup>27</sup> Our flow cytometry results also showed that ROS was significantly retained in the CNE2 cells after ultrasound sonication in the presence of curcumin. Previous studies reported that curcumin possessed an inhibitory effect on the generation of ROS. Our findings demonstrate that the production of ROS induced by ultrasound sonication in the presence of curcumin exceed the inhibitory effect of curcumin treatment alone, causing the excessive accumulation of ROS in the CNE2 cells. The high level of ROS resulted in the mitochondrial damage and cell death of the CNE2 cells. It is well known that ROS are a group of reactive, short-lived species, including free radicals and singlet oxygen ( $^1O_2$ ). However, it was unclear whether the combined treatment of ultrasound sonication and curcumin increased cell death of the CNE2 cells mainly through producing free radicals or through producing singlet oxygen. The exact mechanisms should be determined by further investigations.

In summary, the present study manifested that ultrasound sonication could effectively enhance the CNE2 cell death in the presence of curcumin. Our results highlight the importance

of ROS production and MMP collapse in the process of cell death induced by combined treatment of curcumin and ultrasound sonication. Considering that photosensitizers have the feature of preferential retention in tumor tissues,<sup>28</sup> and ultrasound has the advantage of better tissue penetration and targeted focus, our present results indicate that ultrasound sonication in the presence of curcumin might be a potential modality for the treatment of cancer. As there may be differences between in vitro and in vivo studies, further investigation will be focused on the efficiency in vivo.

### Acknowledgments

We express our sincere thanks to Professor Faqi Li, Mr. Wah Lap Cheung, Mr. Jianyong Wu, Mr. Dejiang Chao, Ms Qinglin Li, and Mr Yong Tang for their helpful assistance.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

### Funding

The author(s) disclosed receipt of the following financial support for the research and/or authorship of this article: this work was funded with support from Hi-Tech Research and Development Program of China (Dr Chuanshan Xu; 2006AA02Z4F0) and Direct Grants from The Chinese University of Hong Kong (Professor Albert Wingnang Leung; 2030391 and 2030408).

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