

# The effects of ultrasound and arsenic trioxide on neurogliocytoma cells and secondary activation of macrophages

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## ABSTRACT

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**Aims and background.** As a new technique for clinical therapeutics, ultrasound has synergistic effects on traditional chemotherapy. Arsenic trioxide (AS<sub>2</sub>O<sub>3</sub>), an apoptosis-inducing drug, has successfully been used in the treatment of some tumor types in recent years. Macrophages have both positive and negative effects on the occurrence and development of tumors. The aim of this study was to observe the effects of ultrasound and AS<sub>2</sub>O<sub>3</sub> on a glioma cell line and the secondary activation of macrophages by cell death, in order to provide a theoretical basis for the clinical application of AS<sub>2</sub>O<sub>3</sub> and ultrasound in glioma treatment.

**Methods.** Different AS<sub>2</sub>O<sub>3</sub> concentrations were used solely or combined with ultrasound in rat glioma C6 cells to induce cell death. The degree of C6 cell death was determined by Annexin V-FITC and PI double staining. The intracellular arsenium concentration and the release of lactate dehydrogenase (LDH) from C6 cells were also measured. The supernatant of C6 cells was then used to stimulate macrophages. Finally, the activation of NF-κB and the secretion of TNF-α and TGF-β1 by macrophages were determined.

**Results.** The cell death increase in the group where ultrasound was used together with AS<sub>2</sub>O<sub>3</sub> was significantly higher than that obtained by either ultrasound or AS<sub>2</sub>O<sub>3</sub>. The increase was also significantly higher than the sum of the increases in the ultrasound and the AS<sub>2</sub>O<sub>3</sub> only groups. At the same AS<sub>2</sub>O<sub>3</sub> concentration, additional treatment with ultrasound can significantly increase the intracellular arsenium concentration. The release of LDH from C6 cells showed a close, direct correlation with late apoptosis and necrosis, but did not exhibit an obvious correlation with early apoptosis. The activation of NF-κB and the secretion of TNF-α and TGF-β1 in macrophages also showed a close direct correlation with late apoptosis and necrosis.

**Conclusions.** This *in vitro* study demonstrates that ultrasound may synergistically enhance the cell-killing effect by promoting AS<sub>2</sub>O<sub>3</sub> to enter the C6 cells. Macrophages may be activated by killed C6 cells, especially by necrotic C6 cells.

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## Introduction

Cerebral glioma shows the highest incidence rate among malignant intracranial tumors, the therapeutic efficacy of surgery is not satisfactory, and tumors show a tendency to recur after radiotherapy and chemotherapy<sup>1</sup>. Various biological behaviors, such as uncontrolled cell proliferation, tumor cell invasion and angiogenesis, constitute challenging problems in the therapeutic approach to neurogliocytomas<sup>2</sup>.

Even though ultrasonic therapy is a new multidisciplinary technology, it has been widely used in many applications since its appearance more than a decade ago, particularly in the treatment of tumors<sup>3</sup>. Some experiments show that ultrasound has

**Key words:** neurogliocytoma, macrophage, cell death, ultrasound.

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synergistic effects on traditional chemotherapy<sup>4</sup>. Many drugs can exert their anticancer actions only after entering the cells, but some drug-resistant tumor cells can prevent the drugs from penetrating and thus avoid being destroyed<sup>5</sup>. Ultrasound can increase the intracellular bioaccumulation of drugs by increasing the membrane permeability in tumor cells and thus reducing the threshold values of cell death<sup>6</sup>.

Arsenic trioxide (AS<sub>2</sub>O<sub>3</sub>) has shown significant therapeutic effects and has been gradually accepted internationally since its first application in the treatment of acute promyelocytic leukemia by Harbin Medical University of China in 1992<sup>7,8</sup>. It has been approved by the US FDA for the treatment of recurrent or refractory acute promyelocytic leukemia. Subsequently, AS<sub>2</sub>O<sub>3</sub> has been used for clinical treatment of other types of leukemia and has shown good therapeutic effects<sup>9</sup>. Its effectiveness in other malignant diseases such as liver cancer<sup>10</sup> and stomach cancer<sup>11</sup> was also experimentally observed. However, the research into the effects of AS<sub>2</sub>O<sub>3</sub> on cerebral glioma is still in its infancy<sup>12</sup>. Most researchers believe that AS<sub>2</sub>O<sub>3</sub> can treat cancer by inducing cell death, but less attention is being given to the secondary reactions of the organism after cell death, such as the reactions of the immune cells.

Currently, inducing tumor cell death is considered the endpoint in most nonsurgical therapies, but there is increasing evidence that different death modes of tumor cells have different effects on the functions of immune cells, particularly macrophages, which play important roles in the occurrence and development of tumors<sup>13</sup>.

Using the rat glioma cell line C6, we studied the effects of ultrasound on the cell death induced by different AS<sub>2</sub>O<sub>3</sub> concentrations and the secondary activation of macrophages, in order to provide helpful guidance for the combined employment of ultrasound and AS<sub>2</sub>O<sub>3</sub> in treating neuroglioma.

## Materials and methods

### *Collection of macrophages from rats*

Male Wistar rats (250 ± 20g of body weight) were provided by the Center of Experimental Animals, Harbin Medical University (Harbin, China). All animals were treated in accordance with the protocols approved by the local Animal Use and Care Committee and executed according to the National Animal Welfare Law.

One percent sodium thiopental at a dose of 1 mL/100 g was used for anesthesia by hypodermic injection in rats. The abdominal wall of the rat was cut open to the peritoneum, and 30 mL phosphate-buffered saline (PBS) was injected into the abdominal cavity by puncturing. The peritoneum was cut open after shaking and the rinsing solution of the abdominal cavity was removed. After this solution was centrifuged and rinsed

twice with PBS, cells were suspended in RPMI 1640 medium containing 10% calf serum and cultured for 2 hours in an incubator with 5% CO<sub>2</sub> at 37 °C. The supernatant was discarded and RPMI 1640 medium without phenol red was used to wash out the cells that did not adhere to the wall. Purified macrophages of rats were thus obtained.

### *Cell culture*

The rat C6 neuroglioma cell line (which was provided by the Institute of Neurosurgery, Harbin Medical University) was incubated in IMDM medium containing 10% fetal bovine serum while the rat macrophages were inoculated in 6-well plates with RPMI 1640 medium containing 10% calf serum. They were kept in the incubator with 5% CO<sub>2</sub> at 37 °C.

### *Experimental procedure*

Rat C6 cells were inoculated in 6-well plates and incubated for 24 hours, and they were subjected or not subjected to ultrasound exposure. During ultrasound exposure the bottom of the 6-well plates was kept in a water bath of 37 °C, and the plane ultrasound transducer was immersed in the water at 1.0 cm distance from the cells in the 6-well plate. Frequency, sound intensity and exposure time were 1.0 MHz, 0.5 W/cm<sup>2</sup> and 120 seconds, respectively. Different concentrations (0 μmol/L, 0.5 μmol/L, 1.0 μmol/L, 2.0 μmol/L, 4.0 μmol/L, 8.0 μmol/L and 16.0 μmol/L) of AS<sub>2</sub>O<sub>3</sub> (from Yida, Harbin, China) were added to the corresponding wells for 24 hours. IMDM medium was employed to rinse the plate and the cells were incubated for another 6 hours in media without drugs. The cells were collected and the mode of cell death was measured by Annexin V-FITC and PI double staining. The intracellular arsenium concentration was determined by the atomic fluorescence method. One milliliter of the supernatant of the culture solution for C6 cells was collected to measure the release of lactate dehydrogenase (LDH), while another 1 mL was added to the culture solution for macrophages for 6 hours. Finally the macrophages were collected to measure the level of NF-κB activation, and the supernatant of the culture solution for macrophages was collected to detect the secretion of TNF-α and TGF-β1.

Annexin V-FITC and PI double staining to measure the death mode of neuroglioma cells

Cell death measurements were carried out according to the instructions of the ApoAlert Annexin V-FITC kit (BD Company, Franklin Lakes, NJ, USA). The C6 cells were digested by trypsin without EDTA, then rinsed twice with PBS. 5 × 10<sup>5</sup> cells were collected and suspended by adding 500 μL binding buffer, and afterwards were fully mixed with 5 μL Annexin V-FITC and 5 μL PI. The reaction was carried out in the dark at room tem-

perature for 15 minutes. The experimental results were measured by a confocal laser microscope (LSM 510 Meta, Carl Zeiss, Germany) and flow cytometer (FACS Aria, BD Corporation, USA).

#### Determination of intracellular arsenium concentration in neurogliocytoma cells

$1 \times 10^5$  C6 cells were processed with strong nitric acid and perchloric acid and deoxidized by ascorbate and thiourea. By reading out the fluorescence intensity values (If) on an atomic fluorophotometer (AFS-9130, Beijing, China), calculations on the concentration of arsenium were conducted using the working curve method (Linear equation:  $If = 150.4319C + 13.1095$  with a correlation coefficient of  $r = 0.9995$  and a sensitivity of  $1 \mu\text{g/L}$ ); the recovery rate of intracellular arsenium obtained by this method was 99.5%.

#### Detection of LDH release in neurogliocytoma cells

The supernatant of the culture solution of C6 cells was collected and the levels of LDH release were measured using a colorimetric method. Procedures were performed strictly following the instructions for the LDH detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China)

#### Detection of NF- $\kappa$ B activation in macrophages by flow cytometry

$1 \times 10^6$  macrophages were collected and single-cell nucleus suspensions were prepared according to the instruction manual of the nuclear extraction kit (KGA826, Keygentec, Nanjing, China). The concentration of cell nuclei was adjusted to  $1 \times 10^6/\text{mL}$ . Forty microliters of NF- $\kappa$ B p65 McAb (Santa Cruz Company, Santa Cruz, CA, USA) and the cell nuclei were incubated for 20 minutes, then  $1 \mu\text{L}$  FITC-labeled second antibody (Jackson ImmunoResearch, West Grove, PA, USA) was added and the cell nuclei were incubated for another 20 minutes. Finally,  $20 \mu\text{L}$  PI was added and the cell nuclei were incubated for a further 30 minutes. The reaction system was detected by flow cytometry (FACS Aria, BD Corporation, USA).

#### Detection of TNF- $\alpha$ and TGF- $\beta$ 1 secreted by macrophages using enzyme-linked immunosorbent assay (ELISA)

The supernatant of the culture solution of macrophages was collected and the levels of TNF- $\alpha$  and TGF- $\beta$ 1 secreted by macrophages to the supernatant of the culture solution were determined utilizing a double-antibody sandwich ELISA. The procedures were carried out according to the instructions of the kit (Biosource International, Camarillo, CA, USA). The absorbance of the samples was measured with a Microplate Reader (Model 680, Bio-Rad Laboratories, Inc., USA) at  $450\text{nm}$  ( $A_{450}$ ). A standard curve graph was plotted according to

the standard preparations, and linear regression analysis was carried out to obtain the linear regression equation. The measured  $A_{450}$  values of different samples were put into the linear regression equation, and the levels of TNF- $\alpha$  and TGF- $\beta$ 1 were obtained.

#### Statistical analysis

SPSS 11.5 software was employed for statistical analysis. Each group of experiments was repeated 5 times, and the results were reported as means  $\pm$  SD. The differences between groups treated with  $\text{As}_2\text{O}_3$  alone and combined with ultrasound were analyzed using Student's *t*-test. The differences between groups with different  $\text{As}_2\text{O}_3$  concentrations were subjected to single-factor analysis of variance (ANOVA), and the Student-Newman-Keuls test was used for comparison of any 2 from the groups. The correlation between 2 variables was subjected to the Pearson correlation analysis.  $P < 0.05$  was considered statistically significant.

## Results

#### Experimental results of death modes of C6 cells

In Figure 4 it can be seen that early apoptosis rose with the increase in  $\text{As}_2\text{O}_3$  concentration, reaching its peak when the  $\text{As}_2\text{O}_3$  concentration reached  $8.0 \mu\text{mol/L}$  and decreasing with the further increase in the  $\text{As}_2\text{O}_3$  concentration. Late apoptosis and necrosis increased steadily following the increase in the  $\text{As}_2\text{O}_3$  concentration.

The experiment shows that ultrasound exposure can only induce C6 cells to achieve certain levels of cell death. The increase was significant when ultrasound and different  $\text{As}_2\text{O}_3$  concentrations were jointly used, and the increase was significantly higher than the sum of the increases of the ultrasound and  $\text{As}_2\text{O}_3$  only groups. The early apoptosis peak appeared at  $4.0 \mu\text{mol/L}$   $\text{As}_2\text{O}_3$ . This demonstrates that ultrasound has a synergistic enhancing effect with  $\text{As}_2\text{O}_3$  on inducing cell death. When combined with ultrasound, relatively low  $\text{As}_2\text{O}_3$  concentrations can obtain the same effects of cell death induction (Figures 1-4).

#### Determination of intracellular arsenium concentration

The arsenium concentration in C6 cells increased with the increase in the  $\text{As}_2\text{O}_3$  concentration in the culture system. With the same  $\text{As}_2\text{O}_3$  concentration, ultrasound treatment can increase the intracellular arsenium concentration (Figure 5).

#### Detection of LDH release in C6 cells

The release of LDH in  $\text{As}_2\text{O}_3$  (-) ultrasound (-) groups was relatively low, and the levels of LDH release in the  $\text{As}_2\text{O}_3$  (+) ultrasound (-) groups increased with the increase in  $\text{As}_2\text{O}_3$  concentration; combined use of ultra-

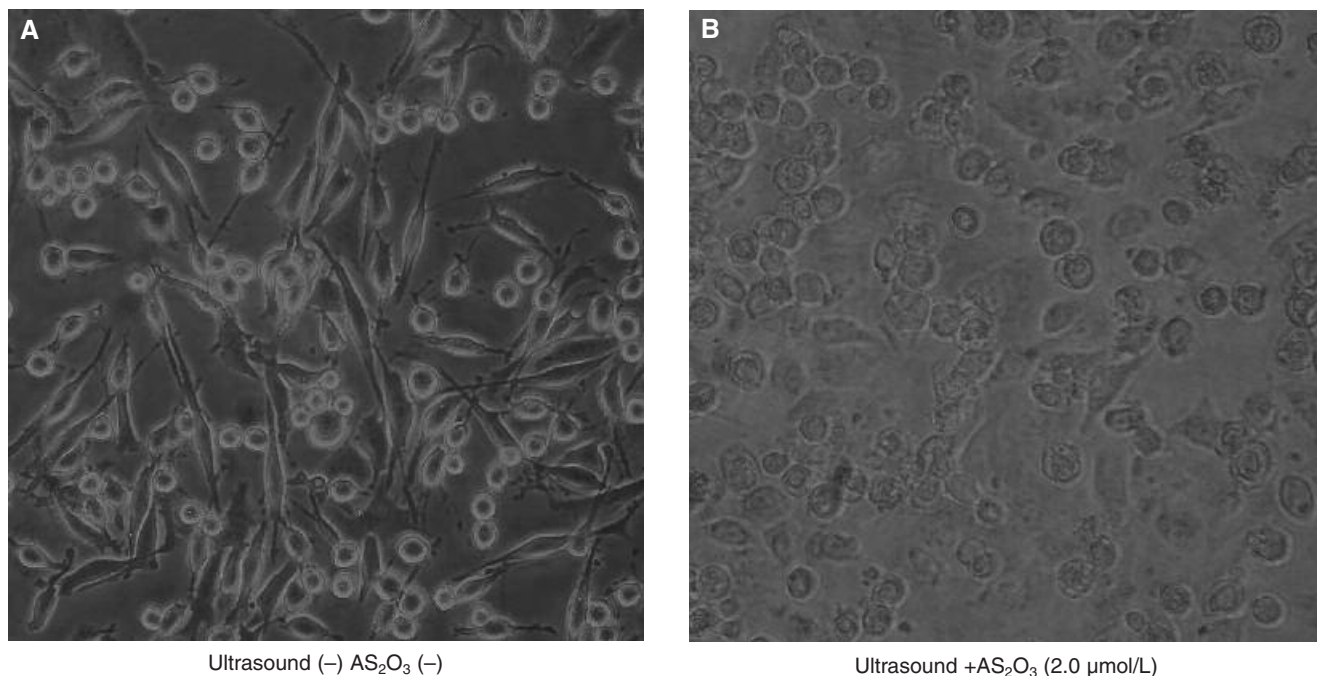


Figure 1 - Morphological features of C6 cells observed under inversed phase contrast microscopy (original magnification ×400).

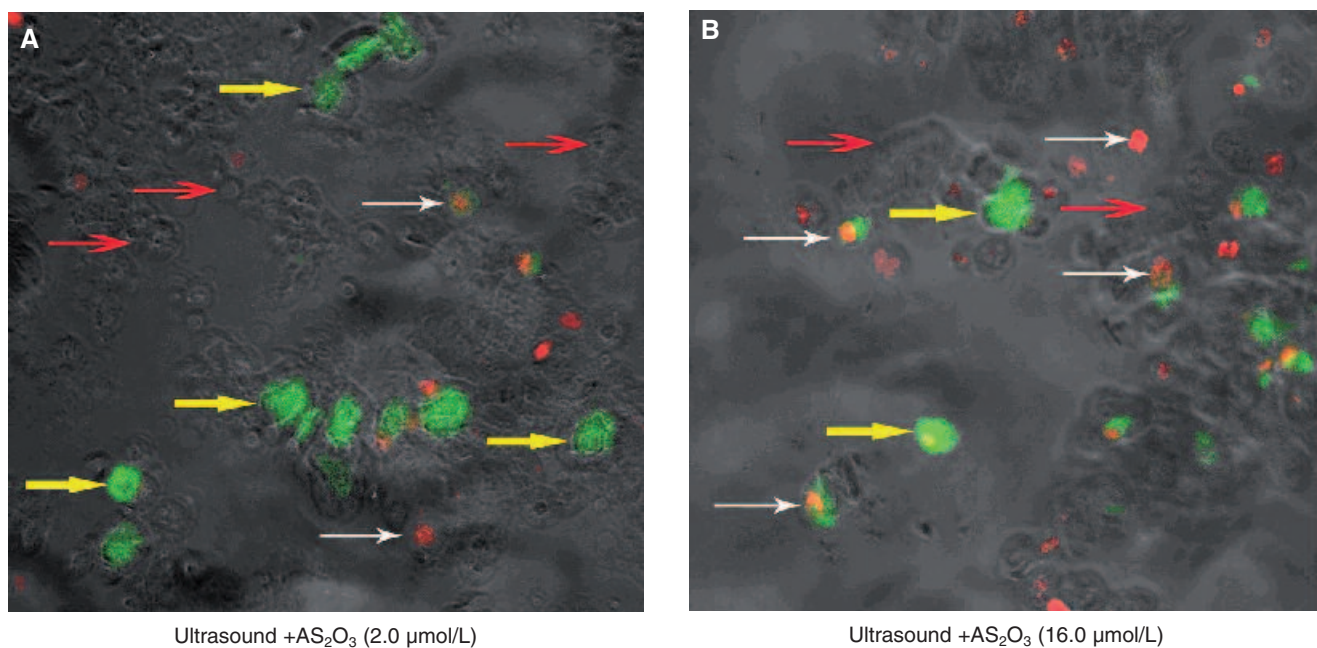


Figure 2 - The results of confocal laser microscope detection by Annexin V-FITC and PI double staining of C6 cells (original magnification ×400). White arrows indicate late apoptotic and necrotic cells, yellow arrows represent early apoptotic cells, and red arrows signify normal cells. The cell membrane of the apoptotic cells can be labeled by Annexin V-FITC with green fluorescence. As the cell membrane of normal and early apoptotic cells remains intact, it can resist the staining of nucleic acid dye PI, whereas the cell membrane of late apoptotic and necrotic cells is breached, and therefore PI can enter the cells and stain the nucleus with red fluorescence.

sound and  $AS_2O_3$  further promoted the release of LDH (Figure 6).

Correlation analysis for the level of LDH release and different death modes of C6 cells revealed that the LDH

level showed a close direct correlation with late apoptosis and necrosis ( $r = 0.503, P < 0.01$ ), but did not exhibit an obvious correlation with early apoptosis ( $r = 0.252, P > 0.05$ ).

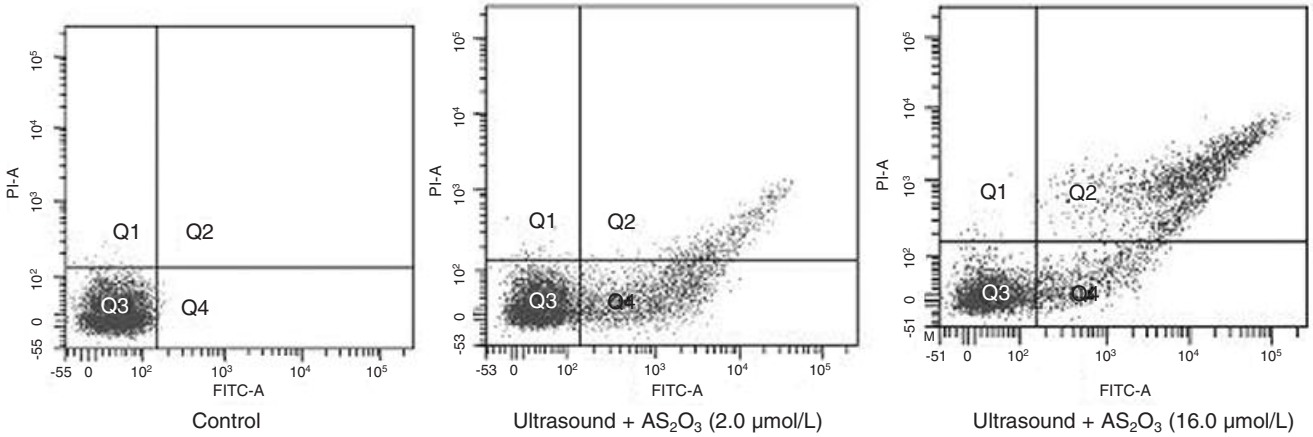


Figure 3 - The results of flow cytometry assay for Annexin V-FITC and PI double staining of C6 cells. Q3 (AnnexinV<sup>-</sup>/PI<sup>-</sup>) represents normal cells, Q4 (AnnexinV<sup>+</sup>/PI<sup>-</sup>) represents early apoptotic cells, and Q2 (AnnexinV<sup>+</sup>/PI<sup>+</sup>) represents late apoptotic and necrotic cells.

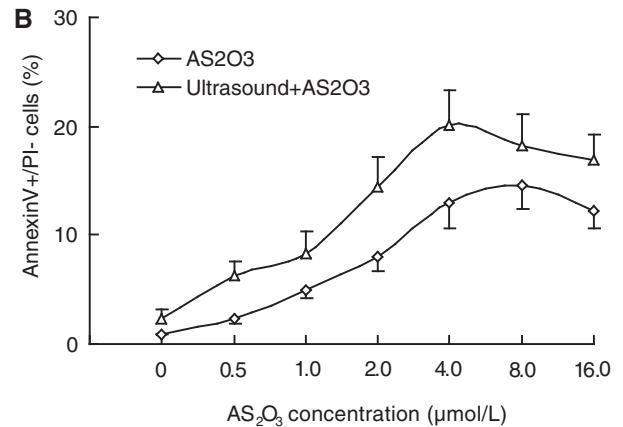
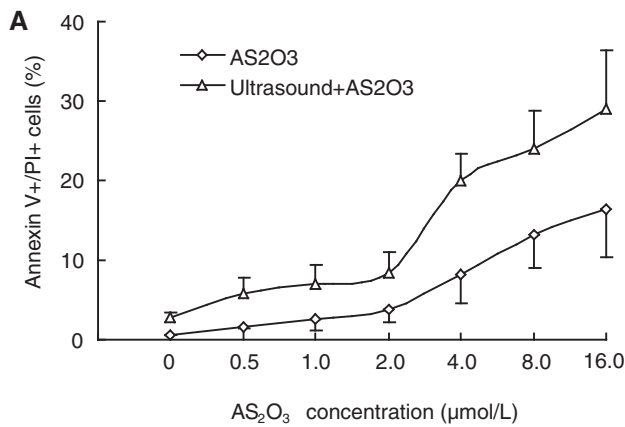


Figure 4 - The detection of C6 cell death by Annexin V-FITC and PI double staining. A) Comparison of percentage of Annexin V/PI double-positive cells (late apoptotic and necrotic cells) after treatment with different AS<sub>2</sub>O<sub>3</sub> concentrations alone or together with ultrasound. B) Comparison of percentage of Annexin V single-positive cells (early apoptotic cells) after treatment with different AS<sub>2</sub>O<sub>3</sub> concentrations alone or combined with ultrasound. Results represent the mean ± SD of 5 independent experiments.

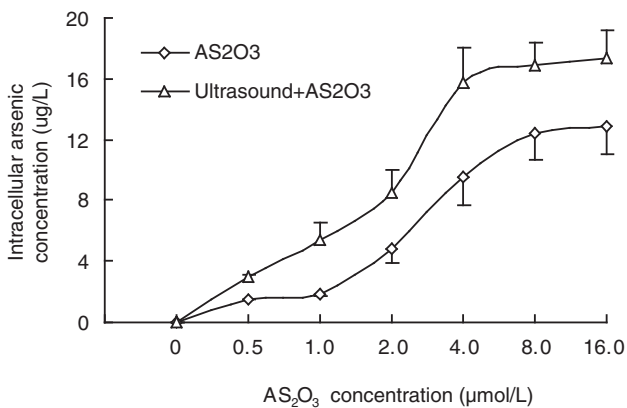


Figure 5 - Results of intracellular arsenic concentrations of C6 cells after treatment with different AS<sub>2</sub>O<sub>3</sub> concentrations alone or together with ultrasound. Results represent the mean ± SD of 5 independent experiments.

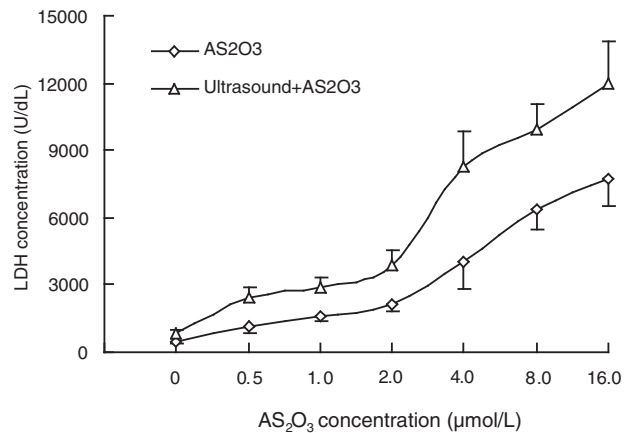


Figure 6 - The release of LDH from C6 cells after treatment with different AS<sub>2</sub>O<sub>3</sub> concentrations alone or together with ultrasound. Results represent the mean ± SD of 5 independent experiments.

*Detection of NF-κB activation in macrophages*

The results demonstrate that the level of NF-κB activation in the AS<sub>2</sub>O<sub>3</sub> (-) ultrasound (-) group was relatively low, while the level increased in AS<sub>2</sub>O<sub>3</sub> (+) ultrasound (-) groups in an AS<sub>2</sub>O<sub>3</sub> concentration-dependent manner. The joint use of ultrasound and AS<sub>2</sub>O<sub>3</sub> further promoted the activation of NF-κB (Figure 7).

Correlation analysis for the activation level of NF-κB and different cell death modes revealed that the level of NF-κB activation showed a significant direct correlation with late apoptosis and necrosis ( $r = 0.437, P < 0.01$ ), but did not show an obvious correlation with early apoptosis ( $r = 0.319, P > 0.05$ ).

*Detection of TNF-α and TGF-β1 secreted by macrophages*

The secretory levels of TNF-α and TGF-β1 in the AS<sub>2</sub>O<sub>3</sub> (-) ultrasound (-) group were relatively low, and the levels increased with the increase in AS<sub>2</sub>O<sub>3</sub> concentration in different groups. The combined effects of ultrasound and AS<sub>2</sub>O<sub>3</sub> further increased their secretion (Figure 8).

In the further correlation analysis, the secretory levels of TNF-α ( $r = 0.382, P < 0.05$ ) and TGF-β1 ( $r = 0.425, P < 0.05$ ) showed a significant direct correlation with late apoptosis and necrosis, but did not show an obvious correlation with early apoptosis ( $r = 0.283$  and  $0.304$ , respectively,  $P > 0.05$ ).

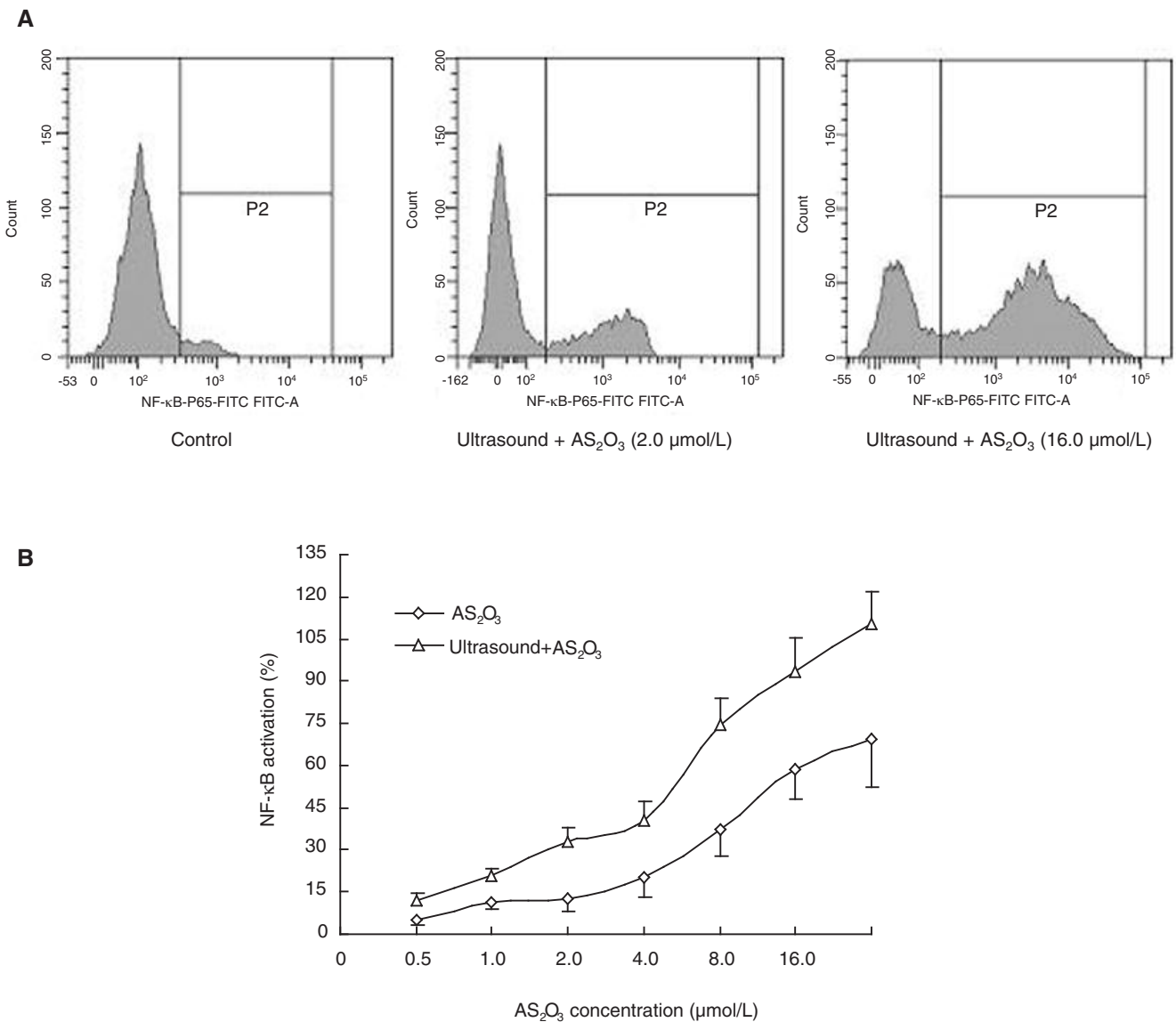


Figure 7 - A) NF-κB activation of macrophages detected by flow cytometry. B) Comparison of NF-κB activation of macrophages in the AS<sub>2</sub>O<sub>3</sub> groups and ultrasound + AS<sub>2</sub>O<sub>3</sub> groups. Results represent the mean ± SD of 5 independent experiments.

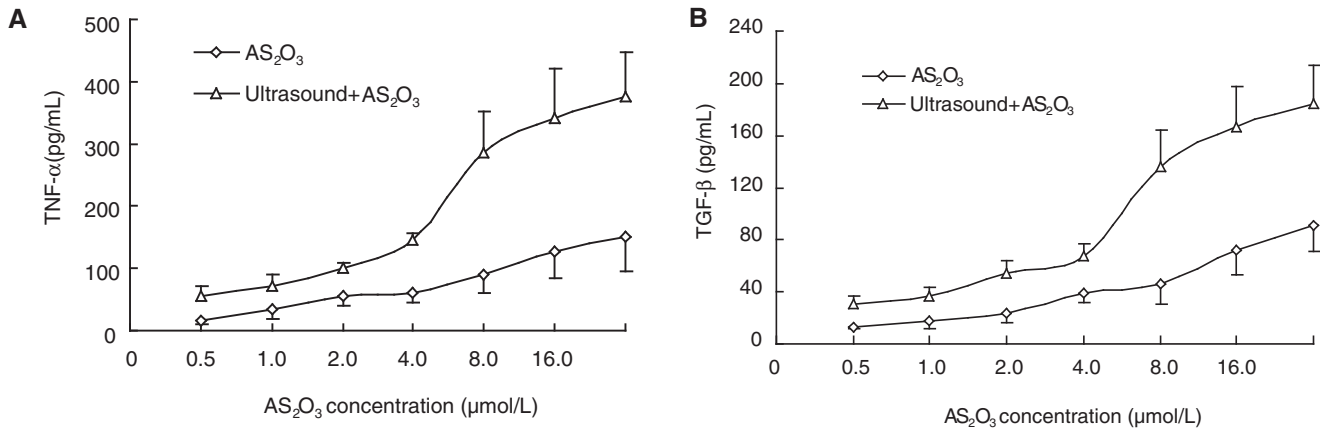


Figure 8 - A) The level of TNF- $\alpha$  released from macrophages in the AS<sub>2</sub>O<sub>3</sub> groups and ultrasound + AS<sub>2</sub>O<sub>3</sub> groups. B) Comparison of the level of TGF- $\beta$  released by macrophages in the AS<sub>2</sub>O<sub>3</sub> groups and ultrasound + AS<sub>2</sub>O<sub>3</sub> groups. Results represent the mean  $\pm$  SD of 5 independent experiments.

## Discussion

Siu *et al.*<sup>14</sup> reported that low-intensity ultrasound could reinforce the cytotoxic effects of doxorubicin on PC3 prostate carcinoma cells. Yu *et al.*<sup>15</sup> pointed out that the combination of ultrasound with adriamycin could induce apoptosis of ovarian cancer cells and directly kill the tumor cells. The results of the present study indicate that ultrasound exposure had synergistic enhancing effects on the death of rat glioma C6 cells induced by AS<sub>2</sub>O<sub>3</sub>. These results are similar to those of the above-mentioned reports.

Ultrasound can penetrate deeply into tissues and can be directly targeted to tumors. Ultrasound itself can induce certain biological reactions, for example denaturing the proteins in tumor cells and affecting their enzymatic activities, destroying the intactness and configuration of nucleic acid molecules, destroying lipids and cell membranes, etc. When combined with some chemotherapeutics, the main synergetic effect is to increase the membrane permeability as well as the intracellular drug accumulation<sup>16</sup>. Considering the direct correlation between intracellular arsenium concentration and cell death in the present study, we inferred that arsenium could kill C6 cell after entering cell. By further observing the relationship between intracellular arsenium concentration and ultrasound exposure, it was found that ultrasound exposure could promote arsenium entering the C6 cells, thereby inducing cell death. Clinically, if doctors continuously increase the AS<sub>2</sub>O<sub>3</sub> dose in order to improve the therapeutic effects, the toxic damage from AS<sub>2</sub>O<sub>3</sub> to normal cells and other organs will increase accordingly, whereas the introduction of ultrasound applied directly to tumors can lead to the same therapeutic efficacy with a lower level of AS<sub>2</sub>O<sub>3</sub> intake, which will enhance the sensitivity of tumors to AS<sub>2</sub>O<sub>3</sub> treatment.

Attention should not only be paid to the total amount of dead cells when inducing tumor cell death, because different death modes have different meanings. Cell death modes can be mainly divided into apoptosis and necrosis<sup>17</sup>, but their roles in the onset and development of malignant tumors remains unclear.

Changes in the death modes of C6 cells induced by different conditions were observed in the present study. Different from the late apoptosis and necrosis that is induced by AS<sub>2</sub>O<sub>3</sub> in a dose-dependent manner, early apoptosis will reduce after reaching its peak following the increase in AS<sub>2</sub>O<sub>3</sub> concentration. It is notable that ultrasound exposure can reduce the AS<sub>2</sub>O<sub>3</sub> concentration where the cell apoptosis reaches its peak value.

Annexin V-PI double staining cannot distinguish necrosis from late apoptosis. Here the LDH release assay was used to further quantify necrosis. LDH normally only exists intracellularly and thus is always used as a marker for the release of cell contents<sup>18</sup>; accordingly, it indirectly represents the degree of necrosis. The finding that the release of LDH has a significant direct correlation with late apoptosis and necrosis but does not exhibit an obvious correlation with early apoptosis indicates that the two measurements, LDH release and percentage of late apoptosis and necrosis, can both reflect the extent of necrosis.

Macrophages have both positive and negative effects on the immune response to tumors<sup>19-21</sup>. Several papers have reported that macrophages in the tumor microenvironment can promote the occurrence and development of breast cancer, prostatic cancer, endometrial cancer, bladder cancer and other cancers<sup>22-24</sup>. Reducing the inflammatory reactions in experimental conditions can reduce the occurrence of tumors<sup>25</sup>. Therefore, it is important to study the functions of macrophages in the progress of tumors, which will most possibly become a new target in tumor treatments<sup>26</sup>.

NF- $\kappa$ B is a crucial factor regulating the expression of numerous genes related to inflammatory reactions<sup>27,28</sup>. TNF- $\alpha$  is a cytokine that is produced and secreted by many kinds of immune cells. It has multiple biological functions such as mediating inflammation, immunological adjustment, and resistance to tumors. It was discovered recently that TNF- $\alpha$  can promote the formation of blood vessels in tumor<sup>29-31</sup>. TGF- $\beta$  exerts important functions in the regulation of the growth and development of embryos and in cell differentiation, proliferation and apoptosis, and it is closely related to the occurrence of tumors and to the infiltration and metastasis of tumor cells<sup>32,33</sup>.

In this study the finding of the activation of NF- $\kappa$ B in macrophages and the release of TNF- $\alpha$  and TGF- $\beta$ 1 from macrophages reveal that certain cell contents released by necrotic C6 cells can activate macrophages. Ultrasound treatment alone can also activate macrophages, so special attention should be paid to this aspect when it is used clinically.

In summary, this *in vitro* study demonstrates that ultrasound can synergistically enhance the killing effect of arsenium on the rat glioma C6 cell. Macrophages can be activated by killed C6 cells, particularly necrotic C6 cells. Studies like ours provide a theoretical basis for the clinical application of AS<sub>2</sub>O<sub>3</sub> and ultrasound in glioma treatment.

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