

# Mechanism of As<sub>2</sub>O<sub>3</sub> induces apoptosis of glioma U87 cells

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**Abstract.** – **OBJECTIVE:** To investigate the mechanism of arsenic trioxide (As<sub>2</sub>O<sub>3</sub>)-induced apoptosis of glioma cells.

**MATERIALS AND METHODS:** U87 cells were treated by different concentrations of As<sub>2</sub>O<sub>3</sub> (8 μmol/L, 6 μmol/L, 4 μmol/L, 2 μmol/L, 1 μmol/L and 0.5 μmol/L) for 24 h, 48 h and 72 h, respectively. Cell viability was detected by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the appropriate dosage and time were screened. Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) was used to stain cells, followed by an investigation on the apoptosis of cells. In the study of molecular mechanism, the expression of p53 in the cells was determined by immunofluorescence, and then apoptosis-related factors, Fas, FasL and Bax, were tested using Real-time polymerase chain reaction (RT-PCR). Finally, the effect of As<sub>2</sub>O<sub>3</sub> on apoptosis-related proteins, caspase-3 and caspase-9, was investigated by Western blotting.

**RESULTS:** As<sub>2</sub>O<sub>3</sub> could significantly inhibit proliferation of U87 cells, and the result of TUNEL staining displayed As<sub>2</sub>O<sub>3</sub> had the function of inducing apoptosis. Immunofluorescence results demonstrated that p53 was highly expressed in glioma cells, which was reduced after drug administration. The results of detection of apoptosis factors using RT-PCR revealed that mRNA expressions of Fas, FasL and Bax in the glioma cells were distinctly higher than those in the As<sub>2</sub>O<sub>3</sub> group. The result of Western blotting indicated that caspase-3 and caspase-9 proteins were highly expressed in glioma cells. Analysis of variance showed that the difference between the control group and the As<sub>2</sub>O<sub>3</sub> group was statistically significant ( $p < 0.01$ ).

**CONCLUSIONS:** As<sub>2</sub>O<sub>3</sub> can inhibit proliferation of glioma cells and induce its apoptosis, which may be correlated with down-regulation of expressions of apoptosis-related factors, Fas, FasL and Bax, and apoptosis-related proteins, p53, caspase-3 and caspase-9.

Key Words:

As<sub>2</sub>O<sub>3</sub>, Glioma cells, Apoptosis.

## Introduction

Glioma is a common intracranial malignant tumor with high incidence and rapid growth<sup>1</sup>. Currently, glioma is mainly treated by surgical resection, with radiotherapy and chemotherapy as auxiliary therapies. However, the patient's survival rate is less than 50% after 1 years of treatment, and the two-year survival rate is even less than 10%<sup>2</sup>. The disease seriously affects human life and health and brings heavy burden to the families of patients and society. In recent years, with the rapid development of comprehensive therapies, such as neural microsurgery, radiotherapy, chemotherapy and biotherapy, the treatment of glioma has been improved. The prognosis is still poor<sup>3,4</sup>, which is mainly associated with high invasiveness, high drug-resistance and low sensitivity to chemotherapy of tumor and it is unable to be completely removed<sup>5</sup>. Recently, there are many chemotherapeutic agents for glioma after operation, but the side effects and prognosis have not been significantly ameliorated. Thus, it is of great significance to search for a new type of chemotherapeutic drug.

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>), which was once a poison in China, is one of the earliest drugs used in the treatment of cancer, which has multiple pharmacological effects although it has large toxic effects<sup>6</sup>. In recent years, the study on anti-tumor effect of As<sub>2</sub>O<sub>3</sub> has become a hot spot. As<sub>2</sub>O<sub>3</sub> displays unique advantages in the treatment of refractory and relapsed acute promyelocytic leukemia<sup>7</sup>. The study indicates that As<sub>2</sub>O<sub>3</sub> plays an important role in the treatment of hematological malignancy mainly through inhibiting tumor cell growth and inducing tumor cell apoptosis<sup>8</sup>. Additionally, As<sub>2</sub>O<sub>3</sub> not only can treat blood tumors, but also acts on other solid tumors<sup>9,10</sup>. However, there are still few studies on its effect on central nervous system tumors. This work focused on the

mechanism of As<sub>2</sub>O<sub>3</sub>-induced glioma (U87) cell apoptosis, so as to provide a basis for As<sub>2</sub>O<sub>3</sub> used for the further treatment of glioma.

## Materials and Methods

### Cell line and Main Regents

U87 cell line was purchased from American Type Culture Collection (ATCC). Bicinchoninic acid (BCA) Protein Quantification Kit (Beyotime, Shanghai, China); TRIzol total RNA extraction kit (Tiangen, Shanghai, China); Reverse transcription kit (Tiangen, Beijing, China); Transferrase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) kit (Roche, Basel, Switzerland); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-p53, caspase-3 and caspase-9 monoclonal antibodies, secondary antibodies and fluorescent secondary antibodies (CST, Danvers, MA, USA).

### Cell Culture

U87 cells were cultured in the Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were cultured in the incubator (37°C, 5%CO<sub>2</sub>). The nutrient solution was changed every three days.

### Toxicity of As<sub>2</sub>O<sub>3</sub> Detected by MTT Assay

U87 cells were inoculated on the 96-well plate with the density of 5×10<sup>4</sup>/mL, 100 μL in each well. After culture for 24 h, different concentrations of As<sub>2</sub>O<sub>3</sub> (8 μmol/L, 6 μmol/L, 4 μmol/L, 2 μmol/L, 1 μmol/L and 0.5 μmol/L) were added. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the inhibitory rate of As<sub>2</sub>O<sub>3</sub> on cell growth.

### Cell Proliferation Explored by MTT Assay

U87 cells were inoculated on the 96-well plate with the density of 5×10<sup>4</sup>/mL, 100 μL in each well. After culture for 24 h, different concentrations of As<sub>2</sub>O<sub>3</sub> (8 μmol/L, 6 μmol/L and 4 μmol/L) were added for 24 h, 48 h and 72 h, respectively. Subsequently, MTT assay was applied to detect cell proliferation.

### Cell Apoptosis by TUNEL Staining

U87 cells were inoculated on the 6-well plate with the density of 1×10<sup>5</sup>/mL, 1 mL in each well. After culture for 24 h, different concentrations of As<sub>2</sub>O<sub>3</sub> (8 μmol/L, 6 μmol/L and 4 μmol/L) were added for 72 h. Subsequently, TUNEL kit was utilized to stain cells and determine the cell apoptosis.

### Real-time PCR

U87 cells were extracted according to the steps of operation in RNAiso Plus Kit (TaKaRa, Otsu, Shiga, Japan). The purity and content of the extracted RNA sample were calculated. The samples were packed separately and stored at -80°C for reservation. Reverse transcription reaction fluid was prepared according to the proportion on the instruction of PrimeScript<sup>®</sup> RT reagent Kit with gDNA Eraser Kit (Beijing, China). The corresponding RNA sample was added followed by reverse transcription to obtain cDNA. The mRNA level was detected in accordance with the instructions of SYBR<sup>®</sup>Premix Ex Taq<sup>™</sup> II (TliRNaseH Plus) Kit (TaKaRa, Otsu, Shiga, Japan). The corresponding RNA primer sequences are shown in Table I.

### Immunofluorescence Staining of p53

U87 cells were inoculated on the 6-well plate with the density of 1×10<sup>5</sup>/mL, 1 mL in each well.

**Table I.** Analysis on primer sequences of related gene using Real-time PCR.

Gene name	Primer sequence
Fas	5'-3' ACATGGACAAGAACCATTATGCTGA 3'-5' CTGGTTTGCACTTGCACTTGGTA
FasL	5'-3' CATGCAGCAGCCCATGAATTAC 3'-5' CTCTAGGCCCCACAAGATGGACAG
Bax	5'-3' CAGGATGCGTCCACCAAGAA 3'-5' CGTGTCCACGTCAGCAATCA
β-actin	5'-3' GAGCCGGGAAATCGTGCGT 3'-5' GGAAGGAAGGCTGGAAGATG

After culture for 24 h, different concentrations of As<sub>2</sub>O<sub>3</sub> (8 μmol/L, 6 μmol/L and 4 μmol/L) were added for 72 h. Subsequently, the sample was fixed by 10% formaldehyde and sealed using 5% evaporated milk, followed by incubation at 37°C for 1 h. The sample was added with p53 primary antibody (diluted at proportion of 1:100) and incubated at 4°C overnight. One day later, the sample was rinsed by phosphate buffer solution (PBS) for three times and added with fluorescent secondary antibody (avoiding the light), followed by being incubated at 37°C for 1 h and rinsed using PBS. The fluorescence microscope was utilized to take photographs, so as to observe the expression of the protein.

### Western Blotting

The operation was performed according to the whole protein extraction kit, and lysate was added. The sample was centrifuged at 4°C at 12000 g for 10 min, and then supernatant was collected, namely total protein. The protein concentration was detected by bicinchoninic acid assay (BCA) protein concentration kit and samples were packed separately and stored at -80°C for reservation. The whole protein extraction fluid was mixed uniformly with 2 × loading buffer (100 μL + 4 μL β-mercaptoethanol) according to the volume of 1:1, followed by boiling water bath for 5 min. Then, sample was treated by natural temperature reduction, and stored at 4°C for reservation. The proper proportion of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation gel was prepared according to the molecular weight of the target protein, followed by solidification for about 1 h, and then 5% SDS-PAGE concentrated gel was prepared, followed by solidification for about 30 min. The electrophoretic buffer was added, and the denatured protein samples were added to the loading sample well. The loading was conducted in accordance with protein concentration to make the equal content of total protein content in each well. The sample was treated by electrophoresis under constant pressure of 220 V, until the bromophenol blue to bottom of gel, the electrophoresis was stopped. According to the molecular amount of target protein, the gel was cut and placed into transfer buffer. A layer of polyvinylidene difluoride (PVDF) membrane and 6 layers of filter paper were cut in accordance with the size of gel. PVDF membrane was firstly soaked in carbinol for 10 s, and then PVDF membrane and filter paper were placed into transfer buffer. Next, it was

placed in the transfer apparatus with attention to edge alignment to prevent blistering. Transmembrane was conducted for 2 h under constant pressure of 110V. PVDF membrane with protein was placed in 5% skimmed milk powder and sealed on the table concentrator at room temperature for 3 h. The sealed membrane was rinsed by tween 20/tris-buffered salt solution (TTBS) for 5 min and placed into primary antibody with corresponding proportion, followed by incubation at 4°C overnight. The membrane was rinsed by TTBS for three times, 10 min each, followed by placement into the corresponding secondary antibody. Then, it was incubated on the table concentrator at room temperature for 3 h and rinsed by TTBS for three times, 10 min each. The gel imager was started up to preheat for 30 min. The reagents of A, B in the enhanced chemiluminescence (ECL) reagent box were mixed uniformly as the equal volume, followed by being drop on the PVDF membrane for full contact and developing avoiding light for 1 min. By adopting filter paper, the excess liquid around the membrane was sucked, and the membrane was placed in the gel imager. The photograph was taken using dynamic integral model, and the results were observed. Image analysis was completed by Lab Works 4.6 professional image analysis software.

### Statistical Analysis

Experimental data were expressed as mean ± standard deviation (Mean ± SD). Experimental results were statistically analyzed by Statistical Product and Service Solutions (SPSS Inc., Chicago, IL, USA) 17.0 software. The *t*-test was used to compare means between the two groups; one-way analysis of variance (One-Way ANOVA) was adopted to compare mean samples among multiple groups; *t* test was utilized for pair wise comparison. *p*<0.05 suggested that the difference was statistically significant.

## Results

### As<sub>2</sub>O<sub>3</sub> Inhibited Proliferation of U87 Cells

The effect of As<sub>2</sub>O<sub>3</sub> on proliferation of U87 cells was investigated in this study. The results are shown in Figure 1, which indicated that As<sub>2</sub>O<sub>3</sub> significantly inhibited proliferation of U87 cells. For U87 cells, apoptosis occurred when concentration of As<sub>2</sub>O<sub>3</sub> reached 8 μmol/L, 6 μmol/L, 4 μmol/L and 2 μmol/L. Thus, 8 μmol/L, 6 μmol/L and 4 μmol/L were selected as the drug concen-

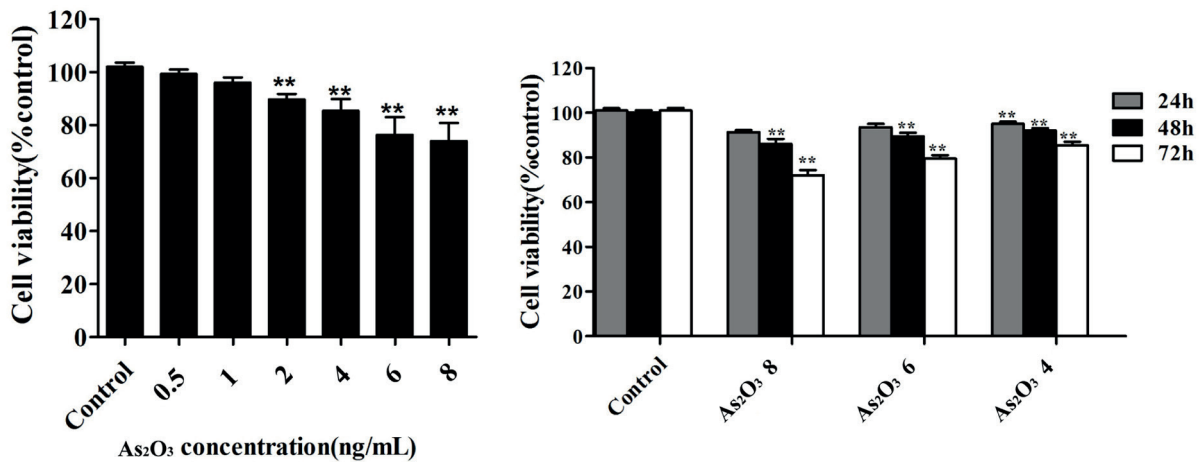


Figure 1. Results of proliferation of U87 cells inhibited by As<sub>2</sub>O<sub>3</sub> using MTT. Compared with control group, \**p*<0.05, \*\**p*<0.01 (n=3).

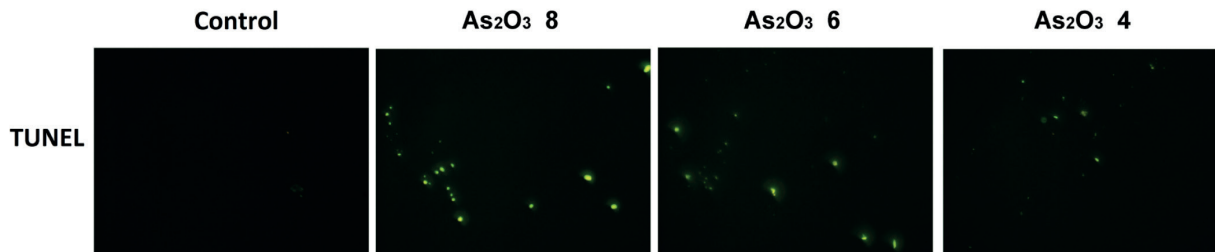


Figure 2. TUNEL staining results of Control group and As<sub>2</sub>O<sub>3</sub> treatment group.

trations for U87 cells. The cells were treated for 24 h, 48 h and 72 h, and finally, the inhibitory effect of action for 72 h was the best.

### As<sub>2</sub>O<sub>3</sub> Induced Significant Cell Apoptosis

As shown in Figure 2, there were almost no apoptotic cells in the control group. After drug administration of As<sub>2</sub>O<sub>3</sub>, cell apoptosis occurred distinctly, which was in a dose-dependent manner. The total RNA was respectively extracted from the cell samples in control group and As<sub>2</sub>O<sub>3</sub> group. Through RT-PCR, as shown in Figure 3, the expressions of Fas, FasL and Bax were remarkably lower than those in the control group after administration of As<sub>2</sub>O<sub>3</sub>. From the figure, it could be seen that apoptosis-related factors were over-expressed in glioma cells, and As<sub>2</sub>O<sub>3</sub> could induce apoptosis in glioma cells (Figure 3).

### Immunofluorescence Staining Results of p53

The expressions of p53 in cell samples of control group and As<sub>2</sub>O<sub>3</sub> group were detected,

as shown in Figure 4. It could be seen that the expression level of p53 in the control group was significantly higher than that in the As<sub>2</sub>O<sub>3</sub> group, which was in a dose-dependent manner.

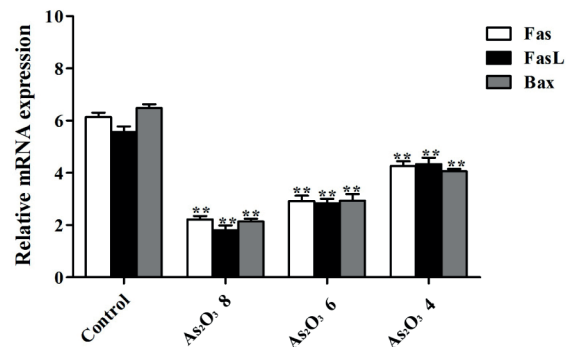


Figure 3. Expressions of Fas, FasL and Bax in control group and As<sub>2</sub>O<sub>3</sub> group. Compared with control group, \**p*<0.05, \*\**p*<0.01 (n=3).

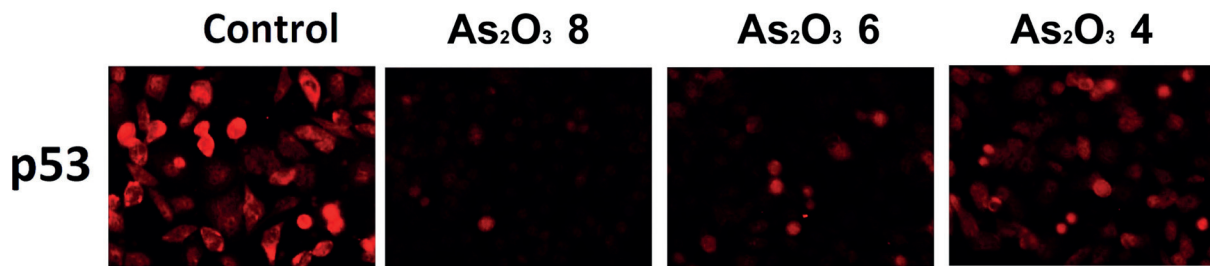


Figure 4. Fluorescence expressions of p53 in control group and  $\text{As}_2\text{O}_3$  group.

#### **Expressions of Caspase-3 and Caspase-9 Proteins in Control Group and $\text{As}_2\text{O}_3$ Group**

The results of Western blotting displayed the expressions of caspase-3 and caspase-9 proteins in tissues of control group and  $\text{As}_2\text{O}_3$  group. As shown in Figure 5, caspase-3 and caspase-9 proteins were highly expressed in cell samples of control group, and its expression levels were much higher than those in  $\text{As}_2\text{O}_3$  group.

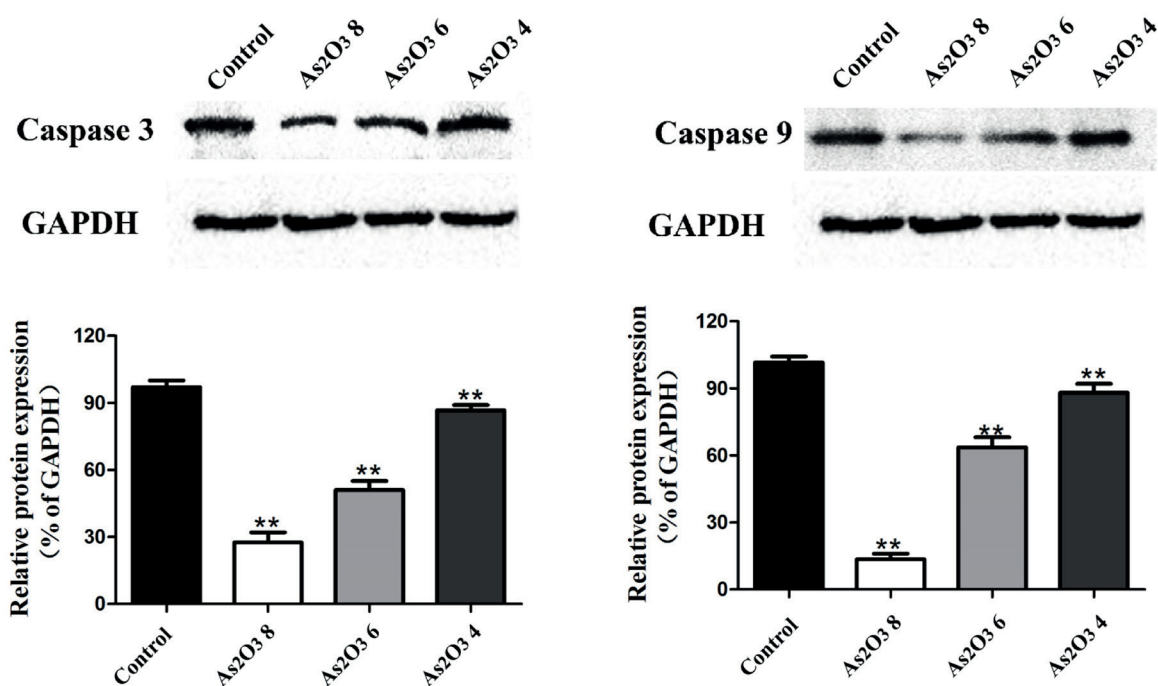
#### **Discussion**

For many years, the drug-resistance and side effects of chemotherapeutic drugs have been the two difficult problems in cancer treatment. It has become the research direction to find new and effective anti-tumor drugs with small side effects<sup>11</sup>.  $\text{As}_2\text{O}_3$  is the first chemical drug used clinically in the treatment of leukemia and has been continuously applied for more than 30 years<sup>6</sup>. Recent studies have found that  $\text{As}_2\text{O}_3$  is not only effective against acute promyelocytic leukemia, but also for other types of leukemia and many other malignant solid tumors, such as gastric cancer, lung cancer, liver cancer, prostate cancer, breast cancer, etc.<sup>12-14</sup>.

Apoptosis is a complex process that is the autonomous and ordered cell death controlled by gene<sup>15</sup>. Apoptosis is an active process that involves the activation, expression and regulation of a range of different genes<sup>16-18</sup>. Apoptosis is a basic biological phenomenon that exists and plays a necessary role in multicellular organisms removing unnecessary or abnormal cells<sup>19</sup>. Apoptosis is of great significance in many systems, including the evolution of organisms, the stability of internal environment and the development of multiple systems. Apoptosis is a special type of cell death, which has important biological significance and complex molecular biological

mechanism<sup>20</sup>. Apoptosis is a tightly controlled process of multiple genes, such as Bcl-2 family, caspase family, oncogenes and tumor-suppressor gene p53, all of which are involved in the apoptosis of cells<sup>21</sup>. With the development of molecular biology technology, a great deal of understanding has been obtained about the process of multiple apoptosis. However, the exact molecular mechanism of apoptosis is still unclear<sup>22</sup>. The disorder of apoptosis process may be directly or indirectly related to the occurrence of many diseases, such as tumor, autoimmune diseases, etc.<sup>23</sup>. Modern studies have suggested that the occurrence and development of tumor is not only associated with the proliferation and differentiation abnormality of tumor cells, but also related to the abnormal regulation of apoptosis<sup>24, 25</sup>. Therefore, it has become a new direction to search for an effective method to induce tumor cell apoptosis in the treatment of cancer.

In this study, glioma cell U87 was selected as the object of study. The concentration and time of drug were screened by MTT assay. At last, 8  $\mu\text{mol/L}$ , 6  $\mu\text{mol/L}$  and 4  $\mu\text{mol/L}$  were selected as the concentrations of administration, and the time of administration was 72 h. Cells were divided into control group and  $\text{As}_2\text{O}_3$  group. Apoptosis in control group and  $\text{As}_2\text{O}_3$  group were detected by TUNEL apoptosis kit. The expressions of apoptosis-related factors, Fas, FasL and Bax mRNA in cells of different groups were determined by reverse transcription-polymerase chain reaction (RT-PCR). Subsequently, the expression intensity of p53 in cells of different groups was tested by immunofluorescence. The expressions of caspase-3 and caspase-9 in control group and  $\text{As}_2\text{O}_3$  group were detected by Western blotting. The experimental results displayed that  $\text{As}_2\text{O}_3$  can significantly inhibit proliferation of tumor cells and induce apoptosis of glioma cells. These results strongly confirmed that  $\text{As}_2\text{O}_3$  is a potential drug with an anti-glioma effect.



**Figure 5.** Expressions of caspase-3 and caspase-9 proteins in control group and As<sub>2</sub>O<sub>3</sub> group. Compared with control group, \* $p < 0.05$ , \*\* $p < 0.01$  ( $n = 3$ ).

## Conclusions

As<sub>2</sub>O<sub>3</sub> can inhibit proliferation of glioma cells and induce its apoptosis, which may be correlated with down-regulation of expressions of apoptosis-related factors, Fas, FasL and Bax, and apoptosis-related proteins, p53, caspase-3 and caspase-9.

## Conflict of interest

The authors declare no conflicts of interest.

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