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Inhibition of Glioblastoma Cell Growth In Vitro and In Vivo by Brucine, a Component of Chinese Medicine

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Glioblastoma multiforme (GBM) is one of the most common glial cell tumors and has drawn more and more attention in the clinic in recent years. Brucine has been reported to significantly suppress gastric cancer, lung cancer, and prostate cancer growth in vivo by inducing cell apoptosis. Here, the effects of brucine on U251 human glioma cell growth were investigated in vitro by cell proliferation assay, FACs, and qPCR in a xenograft tumor model. Treatment with brucine reduced the expression of BCL-2 and cyclooxygenase-2 (COX-2), while upregulated BAX expression in U251 human glioma cells resulted in reduced glioma cell survival rate and inhibited the growth of xenograft tumors. We concluded that brucine has a suppressive effect on U251 human glioma cells in vitro and in vivo, which could help in understanding the role of brucine in glioma cells and guiding drug use in the clinic.

Key words: Brucine; Glioma; Apoptosis; Glioblastoma multiforme (GBM)

INTRODUCTION

Glioblastoma multiforme (GBM) is the most general and vital primary malignant brain tumor, affecting approximately 25,000 patients per year (1). Currently, the treatment for this disease consists of surgical resection and radiotherapy (RT) with medicine therapy after surgery. However, overall survival of patients remains poor with reported median and 2-year survival rates of 14.6 months and 26.5%, respectively (2). Two major bionomics of glioma cells, microvascular proliferation and diffuse infiltration, contribute to its poor prognosis (3). Moreover, the surgical removal of tumor cells has been challenged by the invasion of normal brain cells infiltrating tumor cells, which may underline therapeutic failures (4). At present, no specific treatment has been developed targeting this lethal tumor cell (5–7). Furthermore, for patients who cannot undergo surgery or bear a high risk of recurrence and metastasis postsurgery, drug treatments that can control tumor growth to some extent should be considered. Therefore, it is essential to develop a new drug with strong antitumor effects and lower systemic toxicity for GBM treatment and the prevention of recurrence and metastasis.

Brucine is an effective component of traditional Chinese medicine and is a white odorless crystal. It has been reported that brucine has antitumor effects on hepatocellular carcinoma (8), and brucine represses hepatocellular carcinoma cell migration and metastasis (9). Brucine inhibits the growth rate of human hepatoma cells in vitro (10,11). The effects of brucine on glioblastoma have not been reported. Therefore, in this research, we investigate the effects of brucine on glioblastoma by adding brucine to cultured cells in vitro or by injection of brucine into xenograft tumors formed by glioblastoma cells in nude mice, which may provide some fundamental guidelines for clinical treatment.

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MATERIALS AND METHODS

Cell Strain and Reagents

Brucine (cat lot # S843768) and all the other reagents and chemicals used in this study were obtained from Sigma-Aldrich Corporation, unless otherwise indicated. U251 human glioma cells were purchased from the Chinese Academy of Sciences Shanghai Institute. The basal medium for culturing U251 human glioma cells consisted of RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS; Gibco, certified, US origin) and 100 U/ml pen strep (penicillin and streptomycin; Gibco, US origin). Trypsin and DPBS were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Rabbit polyclonal anti-COX-2 antibody was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit polyclonal anti-BAX and BCL-2 were purchased from Abcam (Cambridge, UK). Monoclonal mouse anti-actin antibody and relative horseradish peroxidase-conjugated second antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell Culture

The human U251 glioma cell line was purchased from the Chinese Academy of Sciences, Shanghai Institute of Biochemistry and Cell Biology. U251 was maintained in RPMI-1640 containing 10% FBS, 100 U/ml penicillin, and 100 g/ml streptomycin. Cells were maintained in a series II water jacketed CO_2 incubator at 37°C with 5%.

Cell Proliferation Assay

U251 cells were digested in logarithmic growth phase with 0.25% trypsin and the concentration of cells adjusted to 5×10^4 cells/ml, adding 100 µl cell suspensions to each hole of a sterile 96-well culture plate. Culture medium was changed with medium with a different concentration of brucine (0, 0.15, 0.3, 0. 6, 1.2, 2.0 mmol/L) for 12, 24, and 48 h before cell viabilities were determined.

For proliferation assays, cell viabilities were determined at the indicated time with the Cell Counting Kit-8 (Dojindo Laboratories, Gaithersburg, MD, USA), per manufacturer's instructions. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium], monosodium salt is reduced by the mitochondrial enzyme NAD-dependent succinate dehydrogenase to form a colored formazan product, which is soluble in the culture medium. The amount of formazan dye generated by the activity of the dehydrogenases in cells is known to be directly proportional to the number of living cells. To measure the proliferative activity of cells in 96-well microplates, 10 µl of the cell counting kit solution was added into each well, followed by incubation of the microplates at 37°C in 5% CO_2 95% air for 4 h. Absorbance at 450 nm was measured using a microplate reader (Bio-tek Instruments).

Fluorescence Activated Cell Sorting (FACS)

Trypsinized U251 cells were counted in log phase and adjusted to a density of 5×10^4 cells/well. They were then seeded in a six-well plate, the cells cultured in 37°C with 5% CO_2. The media was replaced with condition media with 0.15, 0.6, 1.2 mmol/L brucine for the experimental group and with the same amount of basal media for the control group when the cells became adherent. After 24-h culture, cells were trypsinized and washed three times with DPBS. FITC-annexin V was added and cultured for 20 min on the ice. PI (1 g/ml) was immediately added and apoptosis detected with FACS Calibur (Becton Dickinson, USA). Cells that are annexin V and PI double negative are normal cell, those that are annexin V positive and PI negative are cells during early apoptosis, and those that are annexin V and PI double positive cells late apoptosis.

Extraction of RNA

For real-time RT-PCR analysis, RNA was prepared from collected glioma cells. Briefly, in each treatment, glioma cells were snap frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using the mRNA Direct Kit (Qiagen, Germany) according to the manufacturer's instructions. Samples were lysed in 500 µl lysis buffer and swirled at room temperature for 1 min to lyse the cells. The lysate was transferred to reaction tubes and centrifuged at 12,000 rpm for 1 min to make lysate combine with the membrane of the reaction tube. The RNA bound to the membrane was washed twice using washing buffer. Total RNAs were eluted from membranes with 27 µl double-distilled DEPC-treated water.

Real-Time RT-PCR Quantification

Total RNAs were incubated with RNase-free DNase I to remove any remaining genomic contamination. For amplification of the targets, RT and PCR were run in two separate steps. RNA was reverse transcribed with PrimeScript® RT reagent Kit (TaKaRa, Inc., Dalian, China) following the manufacturer's directions and performed at 37°C for 15 min for reverse transcription and then 85°C for 5 s to inactivate the reverse transcriptase. The system of RT-PCR reverse transcription consisted of 2 µl 5× PrimeScript® Buffer, 0.5 µl PrimeScript® RT Enzyme Mix I, 0.5 µl Oligo dT Primer (50 µM), 0.5 µl Random 6 mers (100 µM), and 6.5 µl total RNA.

Quantification of BAX, COX-2, and BCL-2 mRNA in glioma cells was performed with a real-time polymerase chain reaction (qPCR) method. Relative levels of BAX, COX-2, and BCL-2 mRNA were quantified using SYBR® Premix Ex Taq™ II (TaKaRa, Inc.) and a DNA

Engine Opticon2 Two-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). The primers used in this study were chosen to avoid the amplification of genomic DNA and were directed toward a selected region spanning the junction of exons of the gene encoding BAX, COX-2, and BCL-2. The PCR reaction mixture (20 µl) contained 10 µl 2× SYBR green mix and 0.4 µl (0.5 mM) each of forward- and reverse-specific primers, 2 µl of cDNA, and 7.2 µl H_2O. The qPCR protocol included an initial step of 94°C for 3 min, followed by 40 cycles of 94°C for 10 s, 60°C for 25 s, and 72°C for 20 s. GAPDH was used as a control for reaction efficiency and variations in concentration of mRNA in the original reverse transcription. The comparative CT method was used to quantify relative expression levels. According to the comparative CT method, the ΔCT value was determined by subtracting the target gene CT value for each sample from the CT value of the sample for each gene. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$ (12). The qPCR primers were synthesized by Invitrogen Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

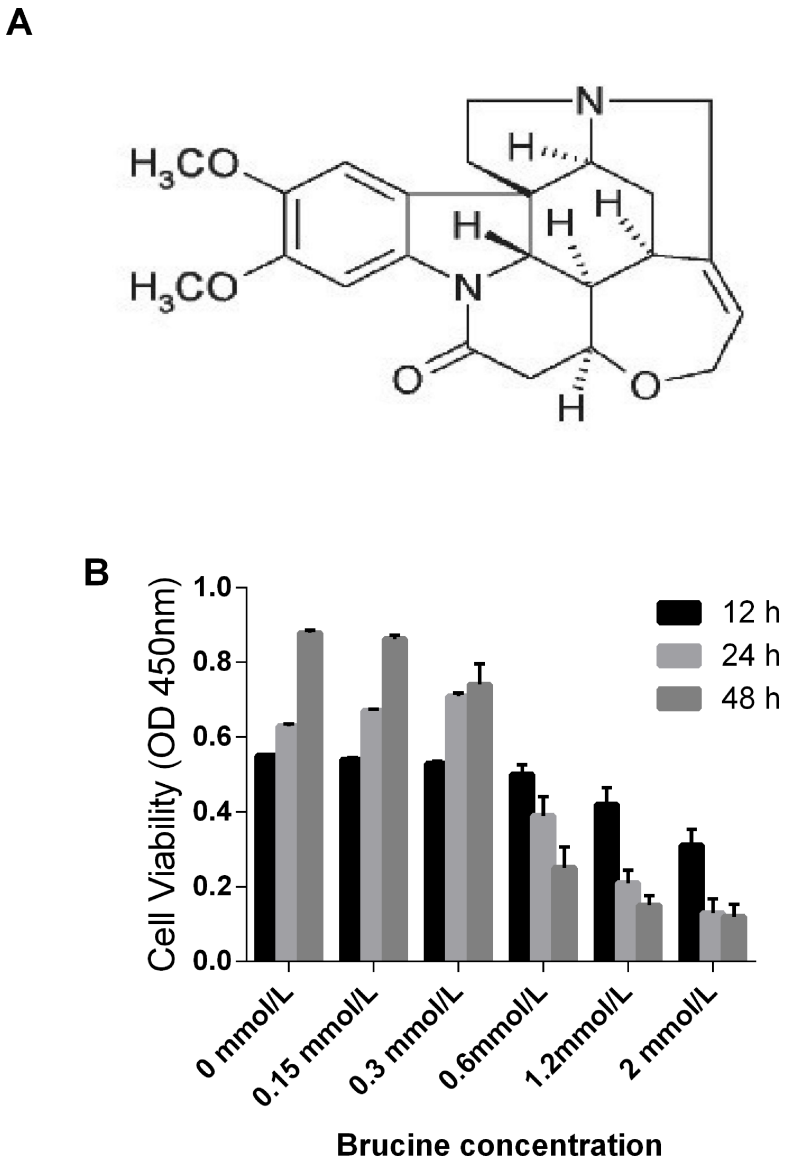

Figure 1. Brucine effect on cell viability. (A) Chemical structure of brucine is shown. (B) Cells were seeded in a 96-well plate and treated with different concentrations of brucine for the indicated time; then cell viability was detected with MTT assay described elsewhere. Data are presented as mean ± SD and represent three independent repeats.

PCR production in different culture times was identified by amplification curve and single melting curve peak of real-time PCR, and the identity of amplified production was confirmed by ethidium bromide staining and 2% agarose gel electrophoresis (100 v, 15 min). Sequences were confirmed by Invitrogen.

Tumor Xenograft Model in Nude Mice

About 3×10^6 glioma cells were suspended in 100 µl serum-free RPMI-1640 culture medium and subcutaneously injected into the flank of 6-week-old female nude mice. After the formation of a palpable tumor, mice were separated into two groups; one group was treated with brucine by intravenous injection at a concentration of 1.2 mM every other day, and the other was treated with the respective vehicle for the indicated days before sacrifice. Tumor size was measured every other day, beginning at day 13. The tumor volume was calculated as follows: length $\times$ width^2 $\times$ 1/2. All mice were killed 25 days after implantation. The tumors were isolated from the mice and stored at −80°C. All studies were performed under the China Association for the Accreditation of Laboratory Animal Care guidelines for humane treatment of animals and adhered to national and international standards.

Statistical Analysis

In all experiments, the data were confirmed from at least three independent replicates and the values were given as mean ± SD. Statistical analyses of all data were done by one-way ANOVA followed by Tukey's Multiple Comparison test (GraphPad Prism; GraphPad Software Inc., San Diego, CA, USA). All percentage data were subjected to arcsine transformation before statistical analysis. A value of $p<0.05$ was considered to indicate a significant difference.

RESULTS

Brucine Reduces the Viability of U251 Cells

To investigate the effects of brucine on U251 human glioma cells, the cells were treated with different concentrations of brucine (0, 0.15, 0.3, 0. 6, 1.2, 2.0 mmol/L) for 12, 24, or 48 h. The abilities of the cells were measured

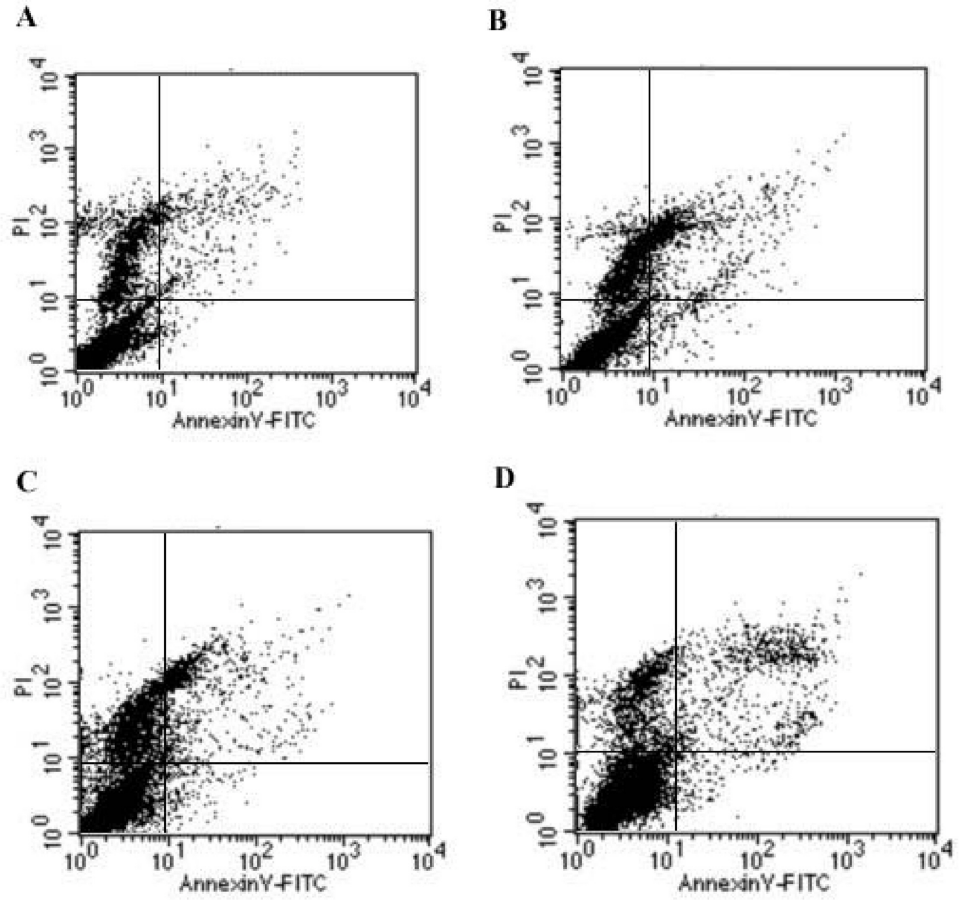

Figure 2. Effects of brucine on apoptosis of U251 cells. Cells were cultured in a six-well culture plate, and treated with 0 mmol/L (A), 0.15 mmol/L (B), 0.6 mmol/L (C), 1.2 mmol/L (D) brucine for 24 h before harvesting. The harvested cells were treated with cell death kit per instruction of the manufacturer and detected with FACS (Becton Dickinson, USA).

using a CCK-8 assay, and as shown in Figure 1, the viabilities of the cell were gradually reduced in a time- and dose-dependent manner. Brucine with the concentration of 0.6, 1.2, 2.0 mmol/L exhibited a significant inhibitory role on U251 human glioma cell growth compared with control (0 mmol/L), indicating that brucine reduces the survival of glioma cell in vitro.

Brucine Induces Apoptosis in U251 Cells

To find out the underlying mechanism responsible for the reduction of cell viability of U251 cells treated with brucine, we performed FACS to test the effects of brucine on apoptosis of U251 cells. As presented in Figure 2, annexin V positive and annexin V-PI double staining cells were increased in a brucine concentration-dependent manner, indicating that brucine induced apoptosis in U251 glioma cells.

The Effect of Brucine in U251 Cells

The effect of most antitumor drugs is to induce mitochondria-mediated apoptosis in cancer cells. To investigate the possible mechanisms of brucine in regulating human glioma cells, the glioma cells were treated with brucine (0, 0.15, 0.3, 0.6, 1.2 mmol/L), and then total RNAs were extracted for detecting the expression of BCL-2, COX-2, and BAX mRNA (Fig. 3).

The results of real-time PCR indicated that brucine can significantly reduce the level of BCL-2 (Fig. 3A) and COX-2 mRNA (Fig. 3B), two vital antiapoptotic factors, when compared with control group ($p<0.05$). However,

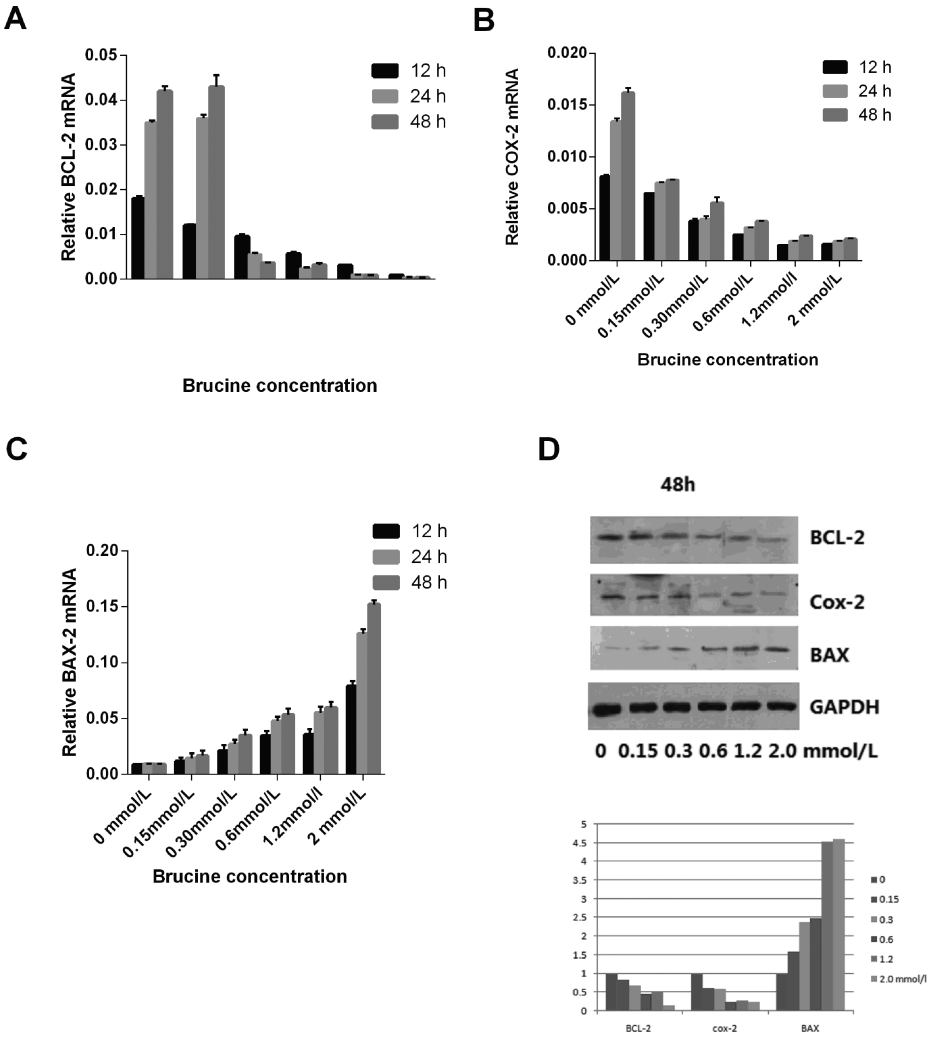

Figure 3. Effect of brucine on BCL-2, COX-2, and BAX mRNA and protein. Cells were treated with indicated concentrations of brucine for 24 h before RNA was extracted or 48 h before proteins were prepared. The expression level of BCL-2 mRNA (A), COX-2 mRNA (B), and BAX mRNA (C) was detected by qRT-PCR, and the protein level was measured by Western blot (D). U6 small RNA and GAPDH were used as inner control for qRT-PCR and Western blot, respectively, and the results are presented as mean ± SD.

the expression of proapoptotic BAX mRNA showed significant increase (Fig. 3C).

To further validate the results of qRT-PCR, we detected the effect of brucine on protein level of BCL2, COX-2, and BAX by Western blot. As shown in Figure 3D, the protein level of BCL2 and COX-2 was reduced along with increasing concentration of brucine, while the level of BAX was increased, validating the results of qRT-PCR.

Brucine Reduced the Growth of Xenograft Tumors In Vivo

Brucine increased the apoptosis rate of U251 cells in vitro. To answer the question of whether this effect of brucine also affects the growth of tumors in vivo, we performed a xenograft tumor assay. We first injected an equal number of U251 cells into the flanks of null mice. After tumor formation, the mice were separated into two groups; one group was treated with brucine by intravenous injection at a concentration of 1.2 mM every other day, and the other was treated with the respective vehicle for the indicated number of days before being sarificed. The tumor sizes were measured at day 13 postinjection. As indicated in Figure 4A and B, treatment with brucine significantly reduced the size of the tumor. Furthermore, the apoptosis of tumor cells in the xenograft tumor was measured by tunnel assay. Brucine significantly increased apoptosis in the xenograft tumor (Fig. 4C), which may explain the reduced tumor size in this group.

DISCUSSION

Glioma is one of the most common malignancies in the central nervous system, and chemotherapy plays an essential role in comprehensive treatment. Although the treatment with chemotherapy has greatly advanced, and the breakthrough of the blood–brain barrier and intra-arterial chemotherapy, the problem of resistance to drugs has not been resolved well, which makes clinical therapy to gliomas a breakthrough progress. Therefore, to understand the sensitivity of glioma to different medical treatments before chemotherapy has important guiding significance, which drafts individualized treatment programs for every patient, avoiding ineffective chemotherapy, and reducing unnecessary adverse reactions.

In this study, U251 human glioma cells were cultured with different concentrations of brucine in vitro, and significantly reduced cell viabilities were observed. Brucine

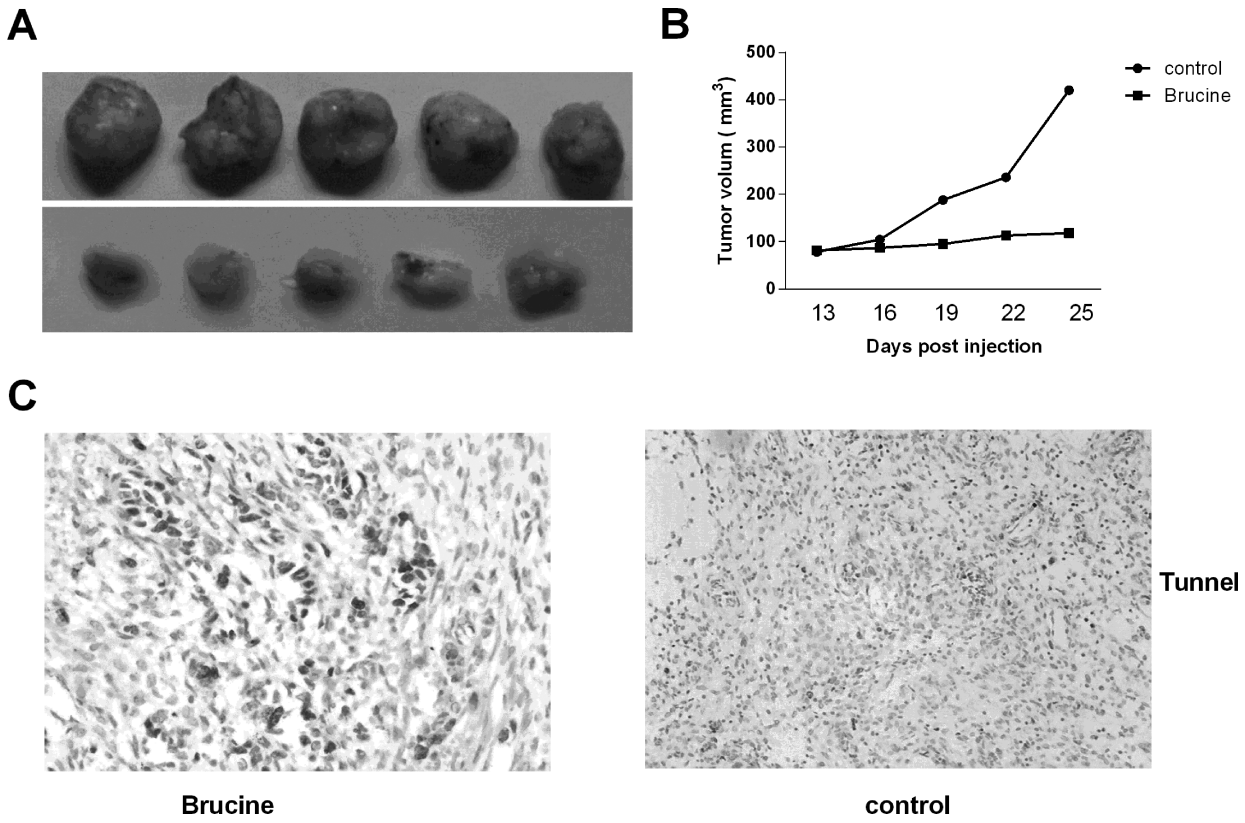

Figure 4. The effect of brucine on xenograft tumor. An equal number of U251 cells were injected into the flank of null mice, and the mice were separated into two groups while the tumor formed, and then treated with brucine or vehicle, respectively. At the indicated time, the size of the tumor was measured (B). The mice were sacrificed at the end of the experiment, forming tumors shown in (A). The apoptosis of the cell was detected by TUNNEL assay (C).

is an alkaloid with indole structure extracted from the Chinese medicine strychnos and is one of the main components of strychnos. Furthermore, the antitumor activity of brucine has gradually become the focus of treating cancer. It has been reported that brucine could prominently inhibit liver cancer, lung cancer, prostate cancer, and other tumor cell growth (8,10). Here, our results demonstrated that brucine has an inhibiting effect on glioma cell growth. Brucine suppressed the survival rate of U251 human glioma cells by inducing apoptosis. This result was consistent with the previous research, which found that brucine induced SHG44 human glioma cell apoptosis, and brucine inhibited cell growth (13,14).

To further confirm the inhibiting effect of brucine on U251 human glioma cell growth, brucine was added to the culture media to assess its effect on the expression of COX-2, BCL-2, and BAX genes in vitro. In our results, it was found that brucine significantly reduced BCL-2 mRNA and COX-2 mRNA levels, while significantly increasing the BAX mRNA level. In accordance with the results of qRT-PCR, treatment with brucine significantly reduced the protein level of BCL-2 and COX-2, while increasing the protein level of BAX. Previous researchers found that brucine-promoted human hepatoma cells apoptosis is mediated via BCL-2 and Ca^{2+} signaling pathways (15). Expression of COX-2 was elevated in many tumor tissues, including lung cancer, and not expressed in most normal tissues (14,15). The studies indicated that COX-2 was the factor that stimulated cell proliferation and inhibited cell apoptosis, promoted tumor angiogenesis, enhanced tumor cell invasion and metastasis, and suppressed immune function (16,17). However, the underlying molecular mechanisms by which brucine stimulates human glioma cell apoptosis are not completely clear at present. Thus, it could be assumed that brucine could activate the related pathway of cell proliferation or apoptosis, such as BAX protein expression increase and BCL-2 expression downregulation.

To explore whether the effects of brucine on U251 cells in vitro applies to in vivo situations, a xenograft tumor treatment model was performed. As is the case in in vitro situations, brucine significantly reduced the size of xenograft tumors compared with the control group. Tunnel assay demonstrated that brucine induced apoptosis in vivo. Thus, brucine may be considered as a clinical benefit antitumor drug for glioma.

In conclusion, the present study demonstrated: (1) brucine distinctly inhibited the U251 human glioma cell survival rate; (2) brucine promoted or inhibited the expression of apoptosis genes or proliferative genes, which provided a theoretical basis for the clinical treatment of brucine; and (3) brucine reduced the growth of xenograft tumors by inducing apoptosis in vivo. Further studies are necessary to explore the antiproliferation mechanism of brucine.

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