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Combination of Baicalein and Temozolomide Eradicates Malignant Glioma through Cell Apoptosis

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Abstract: To study the synergistic effect of baicalein (BAI) combined with temozolomide (TMZ) on glioma cells,weselectedU251 cells as experimental cell. CCK-8 assay was used to detect the effect of baicalein、temozolomide and baicalein combined with temozolomide on the viability of U251 cells. Western blot was detected the expression of cleaved caspase-3 and Bcl-2 protein. DAPI staining was used to observe the changes of nuclei during apoptosis. The results show that the combination of baicalein and temozolomide was more effective than temozolomide and baicalein alone ($p < 0.000$), and the expression of cleaved caspase-3 and Bcl-2 protein was significantly increased in U251 cells treated with temozolomide and baicalein. DAPI staining showed that the nucleus disintegration was very obvious when the drugs were combined.

Keywords: glioma cells:temozolomide; baicalein; apoptosis

Introduction

Glioma (GBM) is one of the most common tumors with the highest incidence and difficulty in the treatment of brain tumors. The biological characteristics of glioma are highly invasive, with no significant boundaries around the normal brain tissue. Because of its location is very special, especially in the functional area of the tumor, surgery is difficult to complete resection, the prognosis is poor, the 5-year survival rate was only 9.8% [1].

Baicalein belongs to flavonoids, with a variety of pharmacological effects, it mainly used for clinical anti-inflammatory and antibacterial [2-3]. In recent years, the anti-cancer effect of Scutellaria has become a hot topic; scutellaria methanol extract can inhibit leukemia cell line THP-1 and human osteogenic sarcoma cell line HOS cell proliferation [4].The vitro experiments shows that injection of mouse bladder cancer cell line MBT-2 of C3H/HeN mice, Scutellaria had played an anti-tumor effect [5]. Scutellariabaicalensis extract, but also inhibit the growth of liver cancer cells HepG2[6].

Temozolomide (TMZ) is the first-line chemotherapeutic agent for the treatment of GBM. The median survival of patients with TMZ chemotherapy after radiotherapy and chemotherapy was increased from 12.1 months to 14.6 months and the two-year survival rate increased from 10.4% to 26.5%. However, due to endogenous and acquired resistance to the

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presence of factors, TMZ treatment of GBM is still not ideal [8], GBM recurrence is often unavoidable. Therefore, how to improve the efficacy of TMZ treatment of GBM is currently in the field of neurotumor research. This study was to investigate the effects of combination of baicalein and temozolomide on U251 glioma cells.

Materials and methods

Materials

The sources for the following reagents were: baicalcin(#456119),Sigma-Aldrich(St. Louis, MO, USA); temozolomide , National Institutes for Food and Drug Control(China); Cell Counting Kit(#027), Fanbo (Beijing, China); BCA(#PC0020), Solarbio (Beijing, China);anti-caspase 3(#9662),anti-Bcl2 (#2870P) Cell Signaling Technology(Danvers, MA, USA);anti-(β-actin) Bioss (Beijing , China);Secondary Antibody(#ASS1009), Abgent (SuZhou , China).

Cell culture

U251 Human Glioma Cell line came from Cellular and Molecular Lab of Pathogenic Biology Key Discipcine (Qindao, China)that were cultured in High Dulbecco's modified Eagle's medium (DMEM)supplemented with 10% fetal bovine serum at 37 °C in a 95% air and 5% CO_2 environment. All experiments were performed under low serum conditions (H-DMEM containing 5% fetal bovine serum), unless otherwise specified.

Cell Counting Kit-8

Cells were seeded in a 96-well plate at a density of 5×10^3 cells/well and were cultured with H-DMEM medium containing 10% FBS at 37°C for 24 hours to adhere in an incubator. Then, the medium in each well was replaced with fresh medium 100 µl containing a series of different test samples for another 12、24、36 or 48-hours culture. Subsequently, 10 µl of CCK-8 reagent was added to each well and the cells were incubated at 37°C for another 2 hours. Ultimately, the optical density (OD) values were measured at 450 nm, using a Synergy H1 Microplate Reader (Cell viability was calculated with the following formula:

$$\text{Cell viability } (\%) = \frac{\text{OD}_{\text{test}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}} \times 100$$

DIPA staining

The cells were fixed in the immunofluorescence plate at 2×10^5/ dish and treated with drugs for 36 hours. Then, The cells were plated in 4% paraformaldehyde-fixed cells at 4 ℃ for 30 min; the fixative was removed by washing; and a small amount of DAPI staining solution was addedat room temperature for 3-5 minutes; DAPI staining solution was aspirated and washed 2-3 times with PBS for 3-5 minutes, each time 3-5 minutes. Cells were observed under the confocal microscope.

Western Blotting

Cells were plated in 6cm plates containing 4mL plate^{-1} of H-DMEM supplemented with 5% fetal bovine serum and then treated with baicalein at 100 µM or TMZ 100 µM or BAI 100 µM and TMZ 100 µM for the indicated times. After treatment, cells were collected for total protein extraction and the protein content of the supernatants was quantified using the BCA protein assay [3P0010S; Beyotime ; Shanghai, China]. Lysates were boiled for 10 min in 5×SDS-PAGE loading buffer. Equal amounts of soluble proteins were separated by 12% Tris/glycine gels, and electrophoretically transferred to PVDF membranes. Membranes were blocked for 2 h in Tris-buffered saline containing 5% non-fat dry milk and incubated with the indicated primary antibodies at 4℃ with gentle shaking ,overnight. Then the membranes were incubated with primary antibodies at indoor temperature and were washed 10min, 3 times with TBST. After, the secondary antibody were incubated for 2 h at indoor temperature, and the membranes were washed 10min, 3 times with TBST. Finally, the immobile western chemiluminescent HRP substrate (Millipore) was added and the protein bands were exposure

Result

BAI and TMZ alone or combined treatment on

U251 glioma cell proliferation.

The results of CCK-8 assay showed that comparing with the blank control group, the growth of BAI combined with TMZ and TMZ alone tumor cells was significantly inhibited (P <0.000),and the inhibition gradually increased with the passage of time; while the inhibition of failure was not very obvious (P <0.000); Compared with the TMZ group, the survival rate of the combined treatment group was significantly different (P <0.000)（Figure 1）.

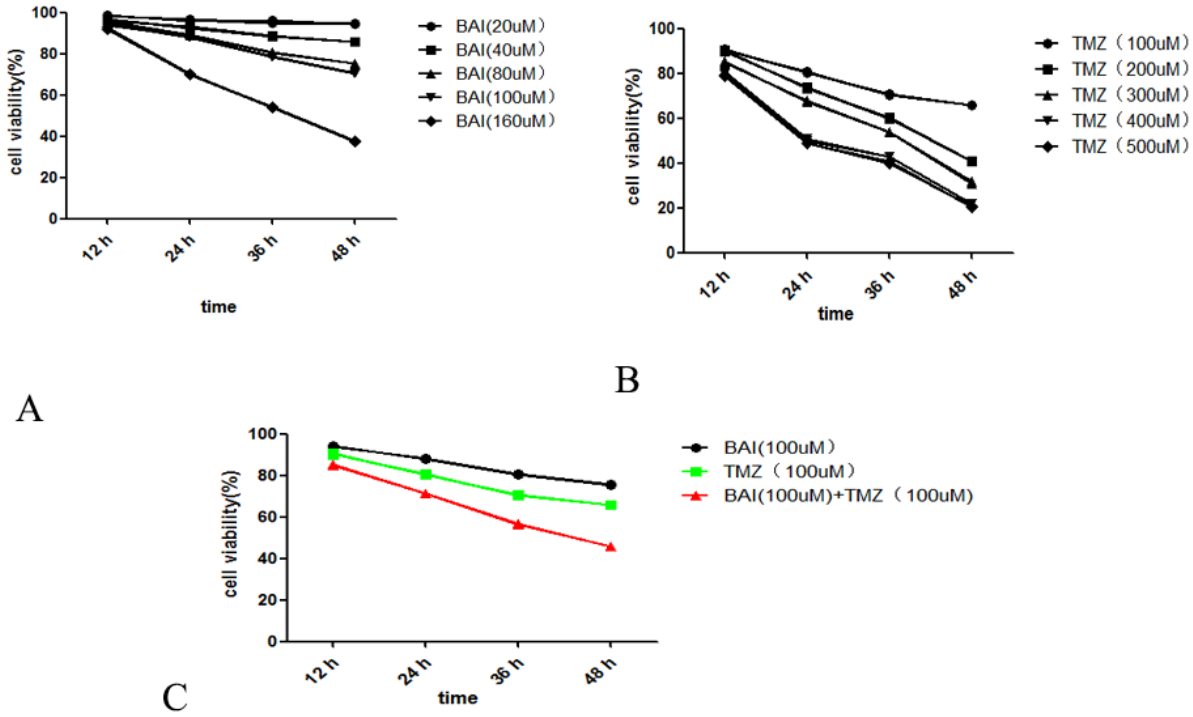

Figure 1 CCK-8 analysis of U251 cell survival

Expression of Apoptosis Related Proteins and Nuclear changes

The cleaved caspase-3 and Bcl-2 expression levels were up-regulated in drug com combination treated and TMZ-treated cells($p < 0.05$), while the cleaved caspase-3 and Bcl-2 in baicalein-treated cells were not significantly up-regulated（$p<0.05$）. The up-regulation of cleaved caspase-3 and Bcl-2 expression in U251 cells was significantly higher than that in BAI and TMZ alone groups（Figure 2）.

Four groups of experimental cells in the electron microscope observation found that the blank control group did not appear cell death. BAI group, TMZ group and combination of TMZ and BAI group showed different degrees of cell death, BAI and TMZ combined group was the most obvious. The nuclear fragmentation was not found in the blank control group and BAI group. The nuclear fragmentation of TMZ group and BAI + TMZ group was obvious, and BAI and TMZ combined group were the most obvious (figure 3).

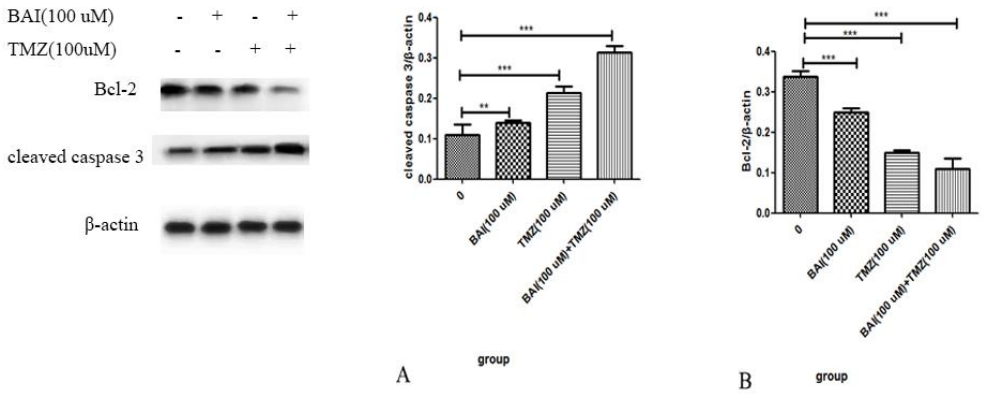

Figure 2 Western blotting was used to detect the expression of cleaved caspase 3 and Bcl-2 after treatment with different drugs in 36 hours.

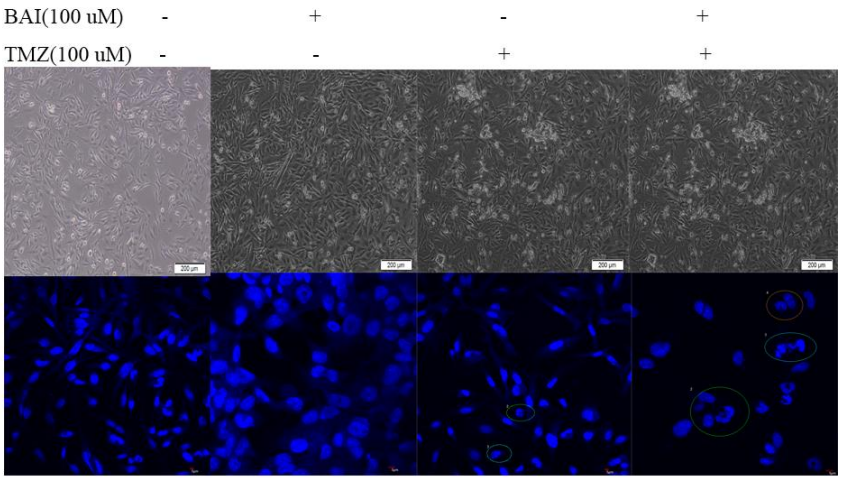

Figure 3 Electron microscopy and confocal microscopy were used to analyze the results of DIPA staining

Discussion

Because of the aggressive growth characteristics of malignant gliomas, it is difficult to remove them completely by surgery. Therefore, drug chemotherapy for the further killing of residual tumor cells play a very important role. Meta-analysis of 12 randomized controlled clinical trials showed that chemo-chemotherapy did extend the survival of glioma patients [9]. TMZ is an imidazole tetrazine derivative and a novel alkylating agent. Its cytotoxicity is mainly on the DNA chain methylation, causing DNA single or double strand breaks, blocking DNA replication, leading to tumor cell death [10-11]. And temozolomide is also a multi-center phase Ⅲ clinical trials confirmed that with radiotherapy alone radiotherapy to extend the survival of patients with glioma more effective [12]. However, the efficacy of drug chemotherapy for glioma is not very satisfactory; its efficiency is low, no significant effect on the survival of patients with prolonged effect. How to improve the efficacy of drug chemotherapy is an urgent problem to be solved, the consensus has been reached: the development of rational drug combination chemotherapy is to improve the efficacy of chemotherapy one of the ways [13].

Baicalein is a major active ingredient extracted from the plant Scutellariabaicalensis. It belongs to the plant flavonoids [14]. In vitro and in vivo studies have shown that baicalein has anti-tumor effect ， For example: baicalein has obvious inhibitory effect on cell proliferation and promotes the apoptosis of tumor cells on human pancreatic cancer HPAC cells and AsPC-1

cells and MiaPaCa-2 cells [15-16], mouse bladder cancer MBT-2 cells [17], skin cancer Cells [18], human hepatocellular carcinoma HepG2 and HepJ2 cells [19], human leukemia HL-60 cells [20], human non-small cell lung cancer H460 cells [21] and other tumor cells. Wang et al. [22] and Zhang et al. [23] showed that baicalein had a potent and time-dependent inhibitory effect on proliferation of human esophageal adenocarcinoma OE33 cells and human breast cancer MDA-MB-231 cells.

In this study, we tried to improve the effect of combination of baicalein and temozolomide on glioma. The effect of baicalein on malignant glioma cells cultured in vitro was not very obvious by CCK-8. But the combined effect of tumor cell activity decreased significantly.

Caspases family is a key element in the process of apoptosis, activation and abnormal expression can cause apoptosis, it is also known as the death of protease, it can interact with a number of protein factors in the regulation of apoptosis [24]. Among the 14 family members that have been identified, caspase-3 is one of the most important apoptosis promoters in the Caspases family as a converging point of multiple apoptotic stimuli signaling, the activation of caspase-3 is a marker of apoptosis in the irreversible stage. Caspase-3 can degrade the apoptosis-inhibiting protein Bcl‑2 through Fas-mediated death signal transduction pathway. Caspase-3 is activated by Caspases upstream and hydrolyzed into active enzyme forms a cascade reaction to catalyze the cleavage of poly (ADP‑ribose) polymerase (PARP) involved in DNA repair. Meanwhile, the extracellular matrix contact site DFF, CAD and other nucleic acid Enzyme activation, DNA can be cut in the nucleosome junction, resulting in times the size of 180 ~ 200 bp DNA fragment; and then the key structural proteins and housekeeping proteins, the cells lose their normal morphology and located in the cell and extracellular matrix contact sites on the VMEKK‑1, FAK and other protein kinase inactivation, loss of cell contact with the environment and further speed up the process of apoptosis [25-28]. Under normal conditions, the cytoplasm of caspase-3 inactive, in the form of procaspase‑3; when the cells receive apoptosis stimulation, it is activated by a series of reactions to form cleaved caspase 3, it is activated by a series of reactions, and induce cell apoptosis.

In this study, cleaved caspase 3 and Bcl-2 were used as the target proteins and Nuclear fragmentation to reflect the degree of apoptosis. The results showed that the combination of baicalein and temozolomide in U251 glioma cells induced the expression of cleaved caspase 3 protein and down-regulated the expression of Bcl-2 protein and promoted the apoptosis of tumor cells. Simultaneously, the nuclear fragmentation of the situation also confirmed the results. The cell viability and apoptosis-related proteins were detected by the above two methods, which indicated that baicalein combined with temozolomide could promote the apoptosis of glioma cells.

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