Caffeine inhibits the growth of glioblastomas through activating the caspase-3 signaling pathway *in vitro*

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Abstract. – OBJECTIVE: To study the effects and associated mechanisms of caffeine on cell viability, cycle dynamics, proliferation and apoptosis both in glioblastoma C6 and U87MG cells.

MATERIALS AND METHODS: Cell livability in presence or absence of caffeine was detected by the methyl thiazolyl tetrazolium (MTT) colorimetric assay. Flow cytometric analysis was conducted to investigate the cell cycle dynamics and Cell Counting Kit-8 (CCK-8) was used to further study the proliferation of C6 and U87MG glioblastoma cells after treated with caffeine or DMSO. To study the influence of caffeine on apoptosis of glioblastoma C6 and U87MG cells, the value of apoptosis ratio (AR) was calculated by flow cytometry detection. Western blot analysis was used to detect the expression of apoptosis-related factors, including Caspase-3, Cyt-C, Bax and Bcl-2.

RESULTS: Caffeine at 1 mM reduced the cell viability of the both rat C6 and human U87MG glioblastoma cells to less than 70%. Flow cytometry detection found that caffeine remarkably arrested the C6 and U87MG cells in G0/G1 phase (C6, U87MG: $p<0.01$, $p<0.05$). Nevertheless, the percentage of cells in S phase obviously decreased in the caffeine-treated group, when comparing to that of the normal control (C6, U87MG: $p<0.01$, $p<0.01$). CCK-8 assay demonstrated that significant decreases in the number of glioblastoma cells were observed in caffeine treatment group, when comparing to that of the normal control (C6, U87MG: $p<0.01$, $p<0.05$). Flow cytometric analysis also found that the application of caffeine induced much higher apoptosis of glioblastoma cells, compared with the normal control (C6, U87MG: $p<0.01$, $p<0.05$). Furthermore, caffeine markedly reduced the expression of Bcl-2 (C6, U87MG: $p<0.01$, $p<0.01$), and promoted the expression of Cyt-C (C6, U87MG: $p<0.05$, $p<0.01$) and Caspase-3 (C6, U87MG: $p<0.01$, $p<0.01$), comparing to the normal control.

CONCLUSIONS: Caffeine inhibits proliferation and induces apoptosis of C6 and U87MG cells, leading to an imbalance in the ratio of proliferation and apoptosis. The apoptosis might be promoted by the motivation of the caspase-3 signaling pathway, which is induced by the release of Cyt-C as well as the elevated rate of Bax/Bcl-2.

Key Words:
Caffeine, Glioblastoma, Proliferation, Apoptosis, Caspase-3.

Abbreviations

PBS = phosphate buffered saline; DMSO = dimethyl sulfoxide; Cyt-C = Cytochrome C; TBST = tris buffered saline and Tween 20; SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis; GAPDH = glyceraldheyde 3-phosphate dehydrogenase.

Introduction

Gliomas, with the overall incidence in excess of 40% among brain tumors, are one of the most frequent primary neoplasms in human central nervous system (CNS)[1]. The invasive impacts of glioma on other brain tissue make this kind of disease difficult to cure, and despite surgery and chemotherapy, the mean overall survival rate for glioma patients is usually less than one year[2]. According the histological properties, gliomas are classified into three types: oligodendroglioma, oligoastrocytoma and astrocytoma. Meanwhile, in accordance with malignancy grades, they are also sorted into four

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grades: Grade I, Grade II, Grade III and Grade IV[3,4]. Grade IV astrocytomas, also named glioblastomas or glioblastomas multiforme, are one of the most common malignant primary neoplasms in the adult CNS[5]. In spite of positive multimodal therapy with maximal surgery resection followed by chemotherapy and radiation therapy, the prognosis of glioblastomas is still unsatisfactory, with a 3-year survival rate of only 10%[6,7]. These bleak situations need a great many of efforts to search for novel antiglioma agents and therapies.

Caffeine, a methylxanthine, is a common component of many different kinds of drinks, such as tea, coffee, and soft drinks and is the widely ingested neuroactive substance in the world[8]. Evidence has demonstrated that caffeine can easily pass through the blood-brain barrier (BBB), which normally restricts chemotherapeutic agents or drugs[9]. So, caffeine has a wide range of pharmacological effects on the CNS. Previously, caffeine and its analogs have shown the diverse influence on pain, Alzheimer's disease, asthma, diabetes mellitus, and schizophrenia[10]. Recently, several studies have further reported the anti-cancer effects of caffeine through inducing the apoptosis and inhibiting the proliferation of cells[11] in several cancer types, such as neuroblastoma[12], lung adenocarcinoma[13], and skin cancer[14]. Recent studies have also shown that caffeine induces p53-independent G1 phase arrest in lung adenocarcinoma cells[15]. Furthermore, in recent studies, it has been reported that caffeine can restrain metastasis in the mouse mammary tumor model, protect the lung adenoma from turning into adenocarcinoma and inhibit the cell transformation induced by epidermal growth factor in JB6 cells[16-18].

Although the relationship between caffeine consumption and the risk of gliomas was reported by several studies[19, 20], the effects of caffeine on glioblastomas have not been fully elucidated. Therefore, in this article, we plan to study the effects and associated mechanisms of caffeine on cell viability, cycle dynamics, proliferation and apoptosis both in glioblastoma C6 and U87MG cells, which might provide the scientific evidence and experimental bases for the pharmacological treatment of glioblastomas in clinical.

Materials and Methods

Cell Culture

The glioblastoma rat C6 and human U87MG cell lines, obtained from Fengshou Company (Shanghai Fengshou Company, Shanghai, China), were cultured in Dulbecco's Modified Eagle Medium (DMEM, Boster Biology Co., Wuhan, China) plus 10% fetal bovine serum (FBS, Boster Biology Co., Wuhan, China). All the C6 and U87MG cells were cultured at the density of 50000/well at 37 °C in a 5% CO_2 atmosphere. Caffeine (Sigma Chemical Co., St. Louis, MO, USA) stock solution was prepared in dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA) and then deliquated in the culture medium. The cells, treated with caffeine, were regarded as the experimental group. Meanwhile, the cells, treated with the equal amount of DMSO, were regarded as the normal control group.

Cell ViabilityAssay

According to previous studies[21], the effects of caffeine on glioblastoma cells growth were determined using the methyl thiazolyl tetrazolium (MTT) colorimetric assay. The C6 and U87MG glioblastoma cells were cultured at 5000 cells in the 96-well plates, and were incubated at 37 °C overnight. Then, the cells were treated with caffeine at different doses of concentrations (Figure 1) for 24 h. Cell growth was measured by adding 20 ml of 5 mg/ml MTT (Boster Biology Co., Wuhan, China) to each well, and the plates were incubated at 37 °C for 4 h. The supernatant was removed afterwards. Then, the absorbance at 570 nm was determined using the multiwell spectrophotometer (Bio-Rad, Hercules, CA, USA). Cell viability = (experimental group absorbance value/control group absorbance value) * 100%. Each experiment was done at least quintic.

Cell Cycle Analysis

To study the cell cycle dynamics of C6 and U87MG cells, flow cytometric analysis was carried out to observe the number of cells at different cell cycle phases. After treated with caffeine or DMSO for 24 h, rat C6 and human U87MG glioblastoma cells were collected and digested with 0.25% trypsin (Sigma Chemical Co., St. Louis, MO, USA) at 37°C for 15 minutes. Following 100-μm mesh sieve screening, the prepared cell suspension was treated with 75% ethanol at 4°C for fixation. The cell suspension was treated with 100 μL RNase (0.01 mol/L, Sigma Chemical Co., St. Louis, MO, USA) at 37°C for 30 minutes, washed with phosphate buffered saline (PBS), stained with 0.5 mg/L propidium iodide (PI) staining solution (Boster Biology Co., Wuhan, China) for 30 minutes at 4°C in the dark.

The stained cell suspension was analyzed using FACSCalibur cell analyzer and software (Becton Dickinson, Franklin Lakes, NJ, USA). Each experiment was done at least quintic.

Proliferation Assay

After caffeine or DMSO treatment for 24 h, 5 × 10^3 glioblastoma C6 and U87MG cells were propagated in a 5% CO_2 atmosphere at 37 °C, respectively. Then, Cell Counting Kit-8 (CCK-8, Zhongshan Co., Beijing, China) was used to detect the cell proliferation. After the cells were inoculated for 0 d, 1d, 2 d, 3 d and 4 d, CCK-8 was applied to the culture medium for 1 h, and later the absorbance was measured at 450 nm through the multiwell spectrophotometer. Each experiment was done at least quintic.

Apoptosis Assay

Flow cytometry detection was carried out to study the apoptosis of glioblastomas by calculating the value of apoptosis ratio (AR). After treated with caffeine or DMSO for 24 h, rat C6 and human U87MG glioblastoma cells were harvested and counted. Cells were resuspended in the PBS (Boster Biology Co., Wuhan, China). Then 5 ml of PI staining solution was added, and the mixture was cultured at room temperature for 30 minutes, away from light. After that, flow cytometric analysis was used to measure the AR values of the C6 and U87MG cells, respectively, through Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Protein Preparation

The extraction of Caspase-3, Cytochrome C (Cyt-C), Bax and Bcl-2 proteins was conducted as previously demonstrated[22]. In short, cells were treated on ice with the phosphate-buffered saline plus 1% Nonidet P-40 (Boster Biology Co., Wuhan, China), 0.1% Sodium Dodecyl Sulfonate (SDS, Sigma Chemical Co., St. Louis, MO, USA), 20 μl/mL protease inhibitor cocktail (Zhongshan Co., Beijing, China) and 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO, USA). After that, the concentrations of the proteins were measured using the Bradford method[23], and the spare proteins were saved up at −40 °C for further use. Each experiment was done at least quintic.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

Equal amounts of C6 and U87MG cell lysates were fractionated by SDS-PAGE and, then, transferred onto acetic nitrocellulose sheets electrophoretically. The sheets were treated in TBST buffer (20 mM Tri-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) supplemented with 5% nonfat dried milk. Then, the cells were incubated at 4 ° C for 12 h with the primary antibodies (Zhongshan Co., Beijing, China) against Caspase-3 (1:200), Cyt-C (1:200), Bax (1:200), Bcl-2 (1:200) and GAPDH (1:500), respectively. At the next morning, the sheets was washed with the TBST buffer and cultured with the specific secondary antibodies (Zhongshan Co., Beijing, Chi-

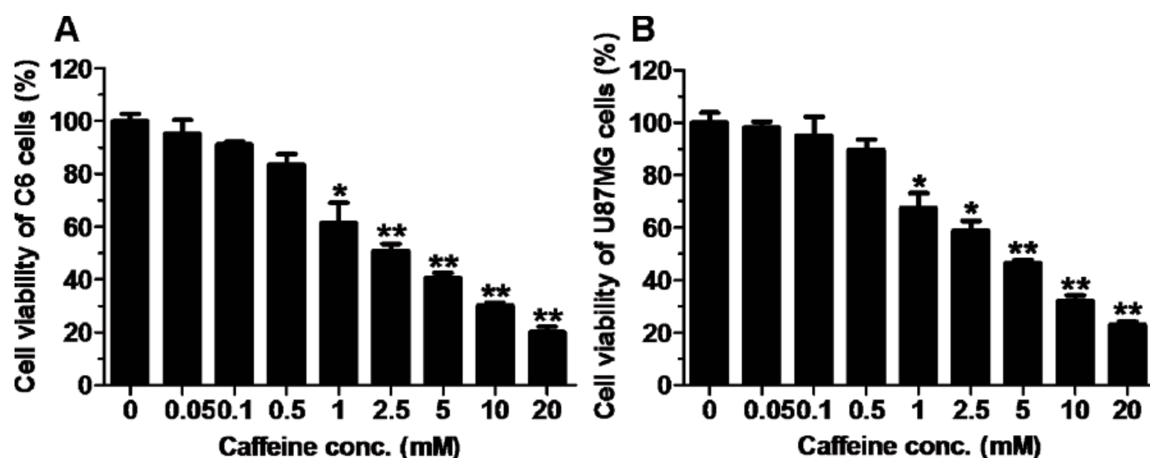

Figure 1. The effects of caffeine on the cell viability in rat C6 and human U87MG glioblastoma cells. ***A,*** The cell viability of C6 cells after the application of different doses of caffeine for 24 h detected by MTT colorimetric assay. The data expresses the means ± SD. $^{*}p<0.05$, $^{**}p<0.01$ vs. the control. ***A,*** The cell viability of U87MG cells after the application of different doses of caffeine for 24 h detected by MTT colorimetric assay. The data expresses the means ± SD. $^{*}p<0.05$, $^{**}p<0.01$ vs. the control.

na). The enhanced Pierce chemiluminescence kit (Sigma Chemical Co., St. Louis, MO, USA) was used to visualize the specific protein bands. The optical density of the bands (normalized with those of GAPDH) was determined by Image Pro Plus image analysis system. Each experiment was done at least quintic.

Statistical Analysis

Data were shown as the mean ± standard deviation (SD). Statistical analysis of variance was used to evaluate the results. SPSS IBM software for Mac was used for statistical analysis of the results (SPSS Inc., Chicago, IL, USA). Statistical significance was calculated by using the oneway ANOVA with the Bonferronni correction. $p<0.05$ was regarded as a statistically significant difference.

Results

Caffeine Reduced the Cell Viability of Glioblastoma Cells in the Dose-Dependent Way

After treated with caffeine for 24 h, MTT colorimetric assay was conducted to measure the percentage of viable cells in glioblastoma C6 and U87MG cells. Our results demonstrated that caffeine decreased the cell viability of glioblastoma C6 and U87MG cells in the dose-dependent way (Figure 1 A and B). Caffeine at 1 mM reduced the cell viability of the both cell lines to less than 70%. Therefore, to avoid any effects on cell viability, the maximal non-cytotoxic concentration of caffeine on both the two-glioblastoma cell lines was 0.5 mM and, thereby, this kind of concentration was used in our following experiments.

Caffeine Changed the Cell Cycle Dynamics oof Glioblastoma Cells

To study the effects of caffeine on the cell cycle dynamics in glioblastoma C6 and U87MG cells, flow cytometric analysis was conducted. The results showed that treatment for 24 h with caffeine significantly blocked the cell cycle in the G0/G1 phase, compared with the normal control (C6, U87MG: $p<0.01$, $p<0.05$, Figure 2 E). Meanwhile, the percentage of cells in S phase obviously lower, when comparing to that of the normal control (C6, U87MG: $p<0.01$, $p<0.01$, Figure 2 F). These dates revealed that caffeine might change the cell cycle dynamics and inhibit the proliferation of rat C6 and human U87MG glioblastoma cells.

Caffeine Inhibited the Proliferation of Glioblastoma Cells

The changes in the proliferation of both rat C6 and human U87MG glioblastoma cells by caffeine or DMSO treatment were further examined in a CCK-8 assay. As shown in Figure 3 A, after the cells were inoculated for 0 d, 1 d, 2 d, 3 d and 4 d, the number of rat C6 cells significantly decreased in caffeine treatment group, when comparing to that of the normal control (day2, day3, day4: $p<0.05$, $p<0.01$, $p<0.01$, Figure 3 A). Similar with rat C6 cells, we also found that the proliferation of U87MG cells was significantly restrained by caffeine treatment (day2, day3, day4: $p<0.05$, $p<0.05$, $p<0.01$, Figure 3 B). Our findings further confirmed that the proliferation of the glioblastoma cells was suppressed by the treatment of caffeine.

Caffeine Promoted the Apoptosis of Glioblastoma Cells

To observe the influence of caffeine on apoptosis of both C6 and U87MG glioblastoma cells, the value of AR was calculated by flow cytometry detection. Our results demonstrated that after caffeine treatment the percentage of apoptotic cells remarkably increased in C6 cells, when comparing to that of the normal control ($p<0.01$, Figure 4). Moreover, our results also demonstrated that the application of caffeine induced much higher apoptosis of glioblastoma U87MG cells, when comparing to the normal control ($p<0.05$, Figure 4). In short, our above results found that the apoptosis of glioblastoma cells was accelerated by the introduction of caffeine.

Caffeine Induced The Apoptosis of Glioblastoma Cells Through Activating the Caspase-3 Signaling Pathway

To investigate the mechanisms of the apoptosis induced by caffeine further, Western blot analysis was used to detect the protein expression of Caspase-3, Cyt-C, Bax and Bcl-2. As shown in Figure 5, the expression of protein Bcl-2 in caffeine treated group markedly reduced, when compared with those in the normal control (C6, U87MG: $p<0.01$, $p<0.01$, Figure 5 A and B). Nevertheless, as for the expression of protein Bax, no statistically significant differences were existed between the two groups (C6, U87MG: $p>0.05$, $p>0.05$ Figure 5 A and B). Furthermore, increas-

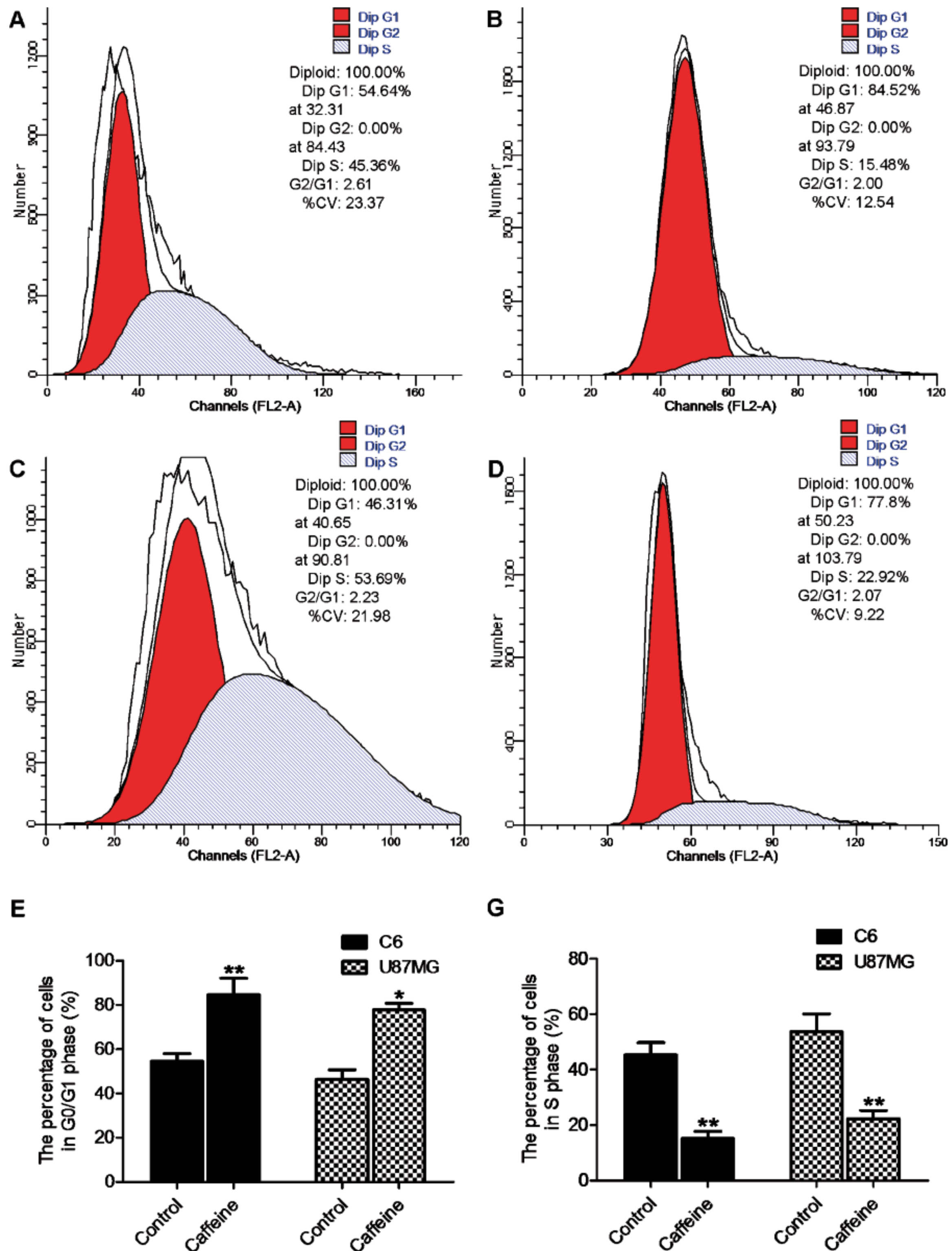

Figure 2. The influence of caffeine on the cell cycle dynamics in rat C6 and human U87MG glioblastoma cells. (A), (B) Flow cytometry cell cycle profiles of C6 cell suspension in the normal control group and the caffeine treated group, respectively. (C), (D) Flow cytometry cell cycle profiles of U87MG cell suspension after the application of caffeine or DMSO for 24 h. (E) The percentage of C6 and U87MG cells in G0/G1 phase is depicted in the bar graphs, respectively. The data expresses the means ± SD. *$p<0.05$, **$p<0.01$ vs. the control. (F) The percentage of C6 and U87MG cells in S phase is depicted in the bar graphs, respectively. The data expresses the means ± SD. *$p<0.05$, **$p<0.01$ vs. the control.

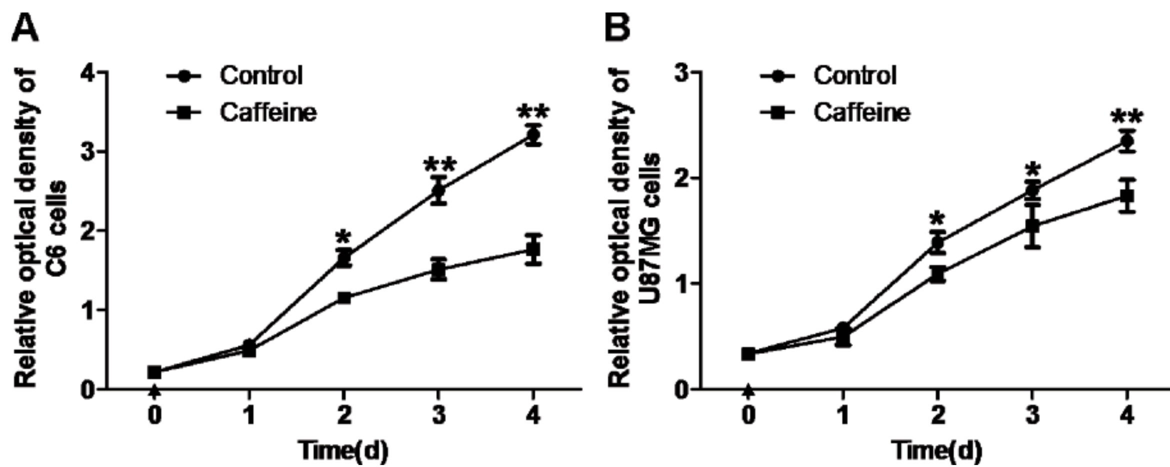

Figure 3. The influence of caffeine on cell proliferation of glioblastomas. ***A,*** The relative optical density of C6 cells is depicted in the broken line graph. The data expresses the means ± SD. $^{*}p<0.05$, $^{**}p<0.01$ vs. the control. ***B,*** The relative optical density of U87MG cells is depicted in the broken line graph. The data expresses the means ± SD. $^{*}p<0.05$, $^{**}p<0.01$ vs. the control.

es in the expression of protein Cyt-C were observed in the caffeine treated group when comparing to those in the normal control (C6, U87MG: $p<0.05$, $p<0.01$, Figure 5 A and B). Similar to the expression of protein Cyt-C, our results also showed that the expression of protein Caspase-3 in the caffeine treated group remarkably enhanced, compared with those in the normal control (C6, U87MG: $p<0.01$, $p<0.01$, Figure 5 A and B). These results suggested that caffeine decreased the expression of apoptosis related protein Bcl-2, but did not elevate the expression level of Bax protein, with the ratio of Bax/Bcl-2 increased. After that, the elevated rate of Bax/Bcl-2 activated increasing release of Cyt-C through mitochondrial pathway. Ultimately, the release of Cyt-C activated the caspase-3 pathway, accelerating the apoptosis of glioblastoma cells.

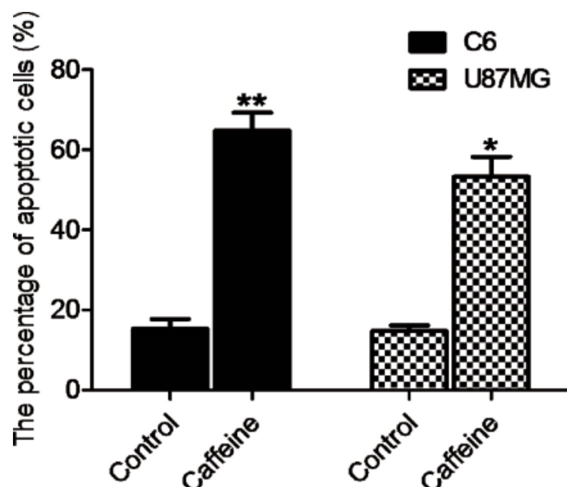

Figure 4. The effects of caffeine on the apoptosis in glioblastoma C6 and U87MG cells. The percentage of apoptosis cells in the glioblastoma C6 and U87MG cells is depicted in the bar graphs, respectively. The data expresses the means ± SD. $^{*}p<0.05$, $^{**}p<0.01$ vs. the control.

Discussion

Glioma, one the most ordinary primary malignant neoplasms in human CNS, is increasingly studied by the researchers all over the world. As is know to every researcher, the treatment for gliomas, especially the glioblastomas, is one of the most difficult challenges because of its infiltrative and aggressive nature, so that it cannot be fully defeated by surgical interference followed by chemotherapy, making it the main death of brain tumors[24]. Because of the BBB, several therapeutic agents are remarkably effective against glioblastomas *in vitro* while ineffective *in vivo*[25]. In addition, as a result of the high metabolic function of brain endothelial cells and shortage of pinocytic vesicles, the access of the medications for the treatment of cancer is inhibited from the plasma to the CNS[26]. Previous studies have demonstrated that caffeine can penetrate the BBB due to its low molecular weight and good lipid solubility. Recently, some research has also found that caffeine shows a wide range of phar-

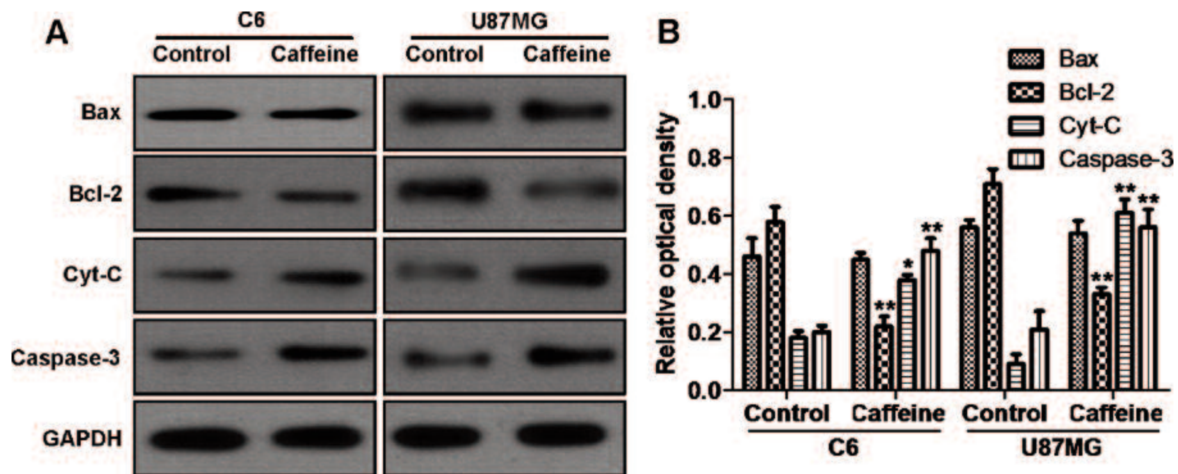

Figure 5. The influence of caffeine on expression of apoptosis-related proteins. ***A,*** The protein expression of Caspase-3, Cyt-***C,*** Bax and Bcl-2 in the normal control and the caffeine treated groups detected by Western blot analysis. ***B,*** Relative quantification of western blot analysis is depicted in the bar graphs. The data expresses the means ± SD. $^{*}p<0.05$, $^{**}p<0.01$ vs. the control.

macological effects on the nervous system, especially the therapeutic influence on Parkinson's and Alzheimer's diseases[10]. Nevertheless, the effects and associated mechanisms of caffeine on cell viability, cycle dynamics, proliferation and apoptosis of glioblastomas have not been thoroughly studied. Hence, in this present research, we studied the effects and associated mechanisms of caffeine on glioblastomas through investigating the cell viability, cycle dynamics, proliferation and apoptosis both in glioblastoma C6 and U87MG cell lines. Our results found that caffeine inhibits the proliferation through arresting the cell cycle in G0/G1 phase and accelerates the cell apoptosis both in C6 and U87MG cell lines. Similar with our findings, it has also been reported the anti-cancer effects of caffeine on breast cancer, liver cancer and so on via inducing the apoptosis and inhibiting the proliferation of tumor cells[13,27].

The major aim of glioblastoma treatment is causing the cell death of tumors through accelerating the apoptosis, which makes the detection of apoptosis become a vital diagnostic parameter in tumor tissues[28]. The proteins in Bcl-2 family act as the key regulators modulating the common apoptosis pathway in the chondriosome[29]. The activation of the mitochondrial apoptosis pathway through pro-apoptotic Bcl-2 proteins is able to activate different cell death pathways including apoptosis[30]. The key upstream event that leads to the activation of these different pathways is mitochondrial outer membrane permeabilization. This process is triggered by the membrane insertion and oligomerization of the apoptosis-related members Bak and Bax, with subsequent release of apoptosis-activating factors, such as cytochrome c (Cyt-C), disrupting the integrity of the external chondriosome membrane and increasing its permeability[31]. Bcl-2, one of the most important proteins that against the tumor-related apoptosis, can protect cells through suppressing the release of Cyt-C[32]. Thereby, the ratio of Bax/Bcl-2 is an essential index suggesting the apoptosis progression of tumor cells[33]. Consistent with former findings, we demonstrated that caffeine reduced expression level of Bcl-2, while did not elevate the expression of Bax, leading to the enhancement of the ratio of Bax/Bcl-2. After that, the elevated rate of Bax/Bcl-2 induced Cyt-C releasing.

Activating the apoptosis in cells is regarded as a key protective way against development and progression of glioblastomas. Apoptosis, commonly known as the way of programmed cell death, is regulated by the caspase families. Caspases, with one of the most vital role in transducing the signals of apoptosis, are the family members of cysteinyl aspartate-requiring proteases[34]. These kinds of proteins are generally classified into two groups: the transducing caspases consisting of caspase-2, caspase-8 and caspase-10 as well as the effect caspases consisting of caspases-3, caspase-6 and caspase-7[35]. In addition, recent evidence showed that caspase-3, the "executor" protease that is the key machinery of cellular

death and functions at the terminal stages of apoptosis, hold the key for mobilizing tissue stem and progenitor cells and promoting tumor regeneration[36]. When activating the caspase-3 pathway, some specific substrates for caspase-3 such as PARP are cleaved, causing the apoptosis. Similar with previous researches, we also found that the release of Cyt-C induced by caffeine could activate the caspase-3 pathway, accelerating the apoptosis and restraining the proliferation of glioblastomas.

Conclusions

Caffeine has effects on inhibiting the growth of glioblastomas by promoting their apoptosis. This kind of apoptosis may be accelerated by the motivation of the caspase-3 signaling pathway, which is induced by the increasing release of Cyt-C as well as the elevated rate of Bax/Bcl-2. Caffeine could improve the efficacy of the treatment for glioblastomas. Caffeine is, therefore, a promising option for the long-term interstitial therapy for glioblastomas. Our results provide the experimental bases for further clinical investigations of caffeine, which could be one hopeful strategy for the therapy of glioblastomas in future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Zhu T, Shen Y, Tang Q, Chen L, Gao H, Zhu J. BCNU/PLGA microspheres: a promising strategy for the treatment of gliomas in mice. Chin J Cancer Res 2014; 26: 81-88.
2) Gritsenko PG, Ilina O, Friedl P. Interstitial guidance of cancer invasion. J Pathol 2012; 226: 185-199.
3) Kalinina J, Peng J, Ritchie JC, Van Meir EG. Proteomics of gliomas: initial biomarker discovery and evolution of technology. Neurooncology 2011; 13: 926-942.
4) Rousseau A, Mokhtari K, Duyckaerts C. The 2007 WHO classification of tumors of the central nervous system--what has changed? Curr Opin Neurol 2008; 21: 720-727.
5) Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 2007; 114: 97-109.
6) Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S, Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG, Mirimanoff RO; European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 2009; 10: 459-466.
7) Linz U. Commentary on Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial (Lancet Oncol 2009;10:459-466). Cancer 2010; 116: 1844-1846.
8) Morelli M, Frau L, Simola N. Alteration in the progression of dopamine neuron degeneration: may caffeine offer new perspective? Exp Neurol 2012; 237: 218-222.
9) Tanaka H, Mizojiri K. Drug-protein binding and blood-brain barrier permeability. J Pharmacol Exp Ther 1999; 288: 912-918.
10) Daly JW. Caffeine analogs: biomedical impact. Cell Mol Life Sci 2007; 64: 2153-2169.
11) Bode AM, Dong Z. The enigmatic effects of caffeine in cell cycle and cancer. Cancer Lett 2007; 247: 26-39.
12) Jang MH, Shin MC, Kang IS, Baik HH, Cho YH, Chu JP, Kim EH, Kim CJ. Caffeine induces apoptosis in human neuroblastoma cell line SK-N-MC. J Korean Med Sci 2002; 17: 674-678.
13) Okano J, Nagahara T, Matsumoto K, Murawaki Y. Caffeine inhibits the proliferation of liver cancer cells and activates the MEK/ERK/EGFR signalling pathway. Basic Clin Pharmacol Toxicol 2008; 102: 543-551.
14) Conney AH, Zhou S, Lee MJ, Xie JG, Yang CS, Lou YR, Lu Y. Stimulatory effect of oral administration of tea, coffee or caffeine on UVB-induced apoptosis in the epidermis of SKH-1 mice. Toxicol Appl Pharmacol 2007; 224: 209-213.
15) Qi W, Qiao D, Martinez JD. Caffeine induces TP53-independent G(1)-phase arrest and apoptosis in human lung tumor cells in a dose-dependent manner. Radiation Res 2002; 157: 166-174.
16) Nomura M, Ichimatsu D, Moritani S, Koyama I, Dong Z, Yokogawa K, Miyamoto K. Inhibition of epidermal growth factor-induced cell transformation and Akt activation by caffeine. Mol Carcinogenesis 2005; 44: 67-76.
17) Lu G, Liao J, Yang G, Reuhl KR, Hao X, Yang CS. Inhibition of adenoma progression to adenocarcinoma in a 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis model in A/J mice by tea polyphenols and caffeine. Cancer Res 2006; 66: 11494-11501.

18) Yang H, Rouse J, Lukes L, Lancaster M, Veenstra T, Zhou M, Shi Y, Park YG, Hunter K. Caffeine suppresses metastasis in a transgenic mouse model: a prototype molecule for prophylaxis of metastasis. Clin Exp Metastasis 2004; 21: 719-735.

19) Malerba S, Galeone C, Pelucchi C, Turati F, Hashibe M, La Vecchia C, Tavani A. A meta-analysis of coffee and tea consumption and the risk of glioma in adults. Cancer Causes Control 2013; 24: 267-276.

20) Holick CN, Smith SG, Giovannucci E, Michaud DS. Coffee, tea, caffeine intake, and risk of adult glioma in three prospective cohort studies. Cancer Epidemiol Biomarkers Prev 2010; 19: 39-47.

21) Ku BM, Lee YK, Jeong JY, Ryu J, Choi J, Kim JS, Cho YW, Roh GS, Kim HJ, Cho GJ, Choi WS, Kang SS. Caffeine inhibits cell proliferation and regulates PKA/GSK3beta pathways in U87MG human glioma cells. Mol Cells 2011; 31: 275-279.

22) Kamsteeg M, Rutherford T, Sapi E, Hanczaruk B, Shahabi S, Flick M, Brown D, Mor G. Phenoxodiol – an isoflavone analog – induces apoptosis in chemoresistant ovarian cancer cells. Oncogene 2003; 22: 2611-2620.

23) Kruger NJ. The Bradford method for protein quantitation. Methods Mol Biol 1994; 32: 9-15.

24) Ostrom QT, Gittleman H, Farah P, Ondracek A, Chen Y, Wolinsky Y, Stroup NE, Kruchko C, Barnholtz-Sloan JS. CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006-2010. Neuro Oncol 2013; 15 Suppl 2: ii1-56.

25) Harn HJ, Lin SZ, Lin PC, Liu CY, Liu PY, Chang LF, Yen SY, Hsieh DK, Liu FC, Tai DF, Chiou TW. Local interstitial delivery of z-butylidenephthalide by polymer wafers against malignant human gliomas. Neuro Oncol 2011; 13: 635-648.

26) Reese TS, Karnovsky MJ. Fine structural localization of a blood-brain barrier to exogenous peroxidase. J Cell Biol 1967; 34: 207-217.

27) Al-Ansari MM, Aboussekhra A. Caffeine mediates sustained inactivation of breast cancer-associated myofibroblasts via up-regulation of tumor suppressor genes. PLoS One 2014; 9: e90907.

28) Liu H, Schmitz JC, Wei J, Cao S, Beumer JH, Strychor S, Cheng L, Liu M, Wang C, Wu N, Zhao X, Zhang Y, Liao J, Chu E, Lin X. Clove extract inhibits tumor growth and promotes cell cycle arrest and apoptosis. Oncology Res 2014; 21: 247-259.

29) Li GY, Xie P, Li HY, Hao L, Xiong Q, Qiu T. Involment of p53, Bax, and Bcl-2 pathway in microcystins-induced apoptosis in rat testis. Environ Toxicol 2011; 26: 111-117.

30) Lindsay J, Esposti MD, Gilmore AP. Bcl-2 proteins and mitochondria--specificity in membrane targeting for death. Biochim Biophys Acta 2011; 1813: 532-539.

31) Kilbride SM, Prehn JH. Central roles of apoptotic proteins in mitochondrial function. Oncogene 2013; 32: 2703-2711.

32) Chipuk JE, Fisher JC, Dillon CP, Kriwacki RW, Kuwana T, Green DR. Mechanism of apoptosis induction by inhibition of the anti-apoptotic BCL-2 proteins. Proc Natl Acad Sci U S A 2008; 105: 20327-20332.

33) Ghate NB, Hazra B, Sarkar R, Mandal N. Heartwood extract of Acacia catechu induces apoptosis in human breast carcinoma by altering bax/bcl-2 ratio. Pharmacogn Mag 2014; 10: 27-33.

34) Troy CM, Jean YY. Caspases: therapeutic targets in neurologic disease. Neurotherapeutics 2015; 12: 42-48.

35) Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 2004; 5: 897-907.

36) Mao P, Smith L, Xie W, Wang M. Dying endothelial cells stimulate proliferation of malignant glioma cells via a caspase 3-mediated pathway. Oncology Lett 2013; 5: 1615-1620.