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The *in vitro* effects of caffeine on viability, cycle cycle profiles, proliferation, and apoptosis of glioblastomas

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Abstract. – OBJECTIVE: We studied the effects of caffeine on cell viability, cell cycle profiles, proliferation, and apoptosis in rat C6 and human U87MG glioblastoma cell lines.

MATERIALS AND METHODS: Cell viability was quantified by the methyl thiazolyl tetrazolium (MTT) assay. Flow cytometry was used to quantify the relative number of cells in different phases of the cell cycle, while cell proliferation was quantified using the Cell Counting Kit-8. The proportion of apoptotic cells was determined by flow cytometry, and expression of apoptosis-related proteins Caspase-3, Cyt-C, Bax and Bcl-2 by Western blot.

RESULTS: Caffeine at doses of up to 0.5 mM did not affect cell viability in both rat C6 and human U87MG glioblastoma cells. Further studies were done using the dose of 0.5 mM. Percentage of cells in the G0/G1 phase was markedly increased, while percentage of cells in the S phase decreased, after cell treatment with caffeine. Cell proliferation was significantly inhibited by caffeine. Furthermore, caffeine induced cell apoptosis, decreased expression of Bcl-2, and increased expression of Cyt-C and Caspase-3.

CONCLUSIONS: Caffeine inhibits proliferation and induces apoptosis in glioblastoma cells. Our results provide the experimental basis for further studies of potential role of caffeine in the treatment of glioblastomas.

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

Introduction

Gliomas account for more than 40% of brain tumours and are the most common primary tumors of the central nervous system[1]. Glioma invasion into other brain tissues impede disease treatment. Despite surgery and chemotherapy, the mean survival rate for glioma patients is usually less than one year[2].

Gliomas are classified on the basis of histological tumour type (astrocytic, oligodendroglial, mixed oligoastrocytic, or ependymal gliomas) and based on malignancy grades (I, II, III, or IV)[3]. The grade I and II gliomas are considered as low-grade, or benign, tumours, while grade III and IV gliomas are regarded as high-grade and malignant tumours[3,4]. The grade IV astrocytomas, also known as "glioblastomas multiforme" or "glioblastomas", are the most frequent primary malignancy of the adult central nervous system and the most malignant form of gliomas[5]. Despite aggressive multimodal treatment, which includes maximal surgery resection, tribuzone and radiation therapy, the prognosis for glioblastomas remains dire, with a 3-year survival rate of only 10%[6,7]. Therefore, there is an urgent need for novel anti-glioma therapies.

Caffeine, a methylxanthine, is a common component of many drinks, such as tea, coffee, or soft drinks, and is the most commonly ingested neuroactive substance in the world[8]. Caffeine can penetrate the blood-brain barrier (BBB) which normally restricts the movement of chemotherapeutic agents or drugs[9]. Caffeine has a diverse range of pharmacological effects on the nervous system. It was shown that caffeine and its analogues exert analgetic effects, and are effective in Alzheimer's and Parkinson's diseases[10]. Recently, anti-cancer effects of caffeine were reported. Specifically, caffeine induces cell apoptosis and inhibits proliferation of several cancer types[11], including neuroblastoma[12], lung adenocarcinoma[13], and skin cancer[14]. Caffeine also induces the p53-independent G1 phase arrest in lung adenocarcinoma cells[15]. Furthermore, it was reported that

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caffeine block metastasization in a mouse model of mammary tumour, prevent the transformation of lung adenoma into adenocarcinoma, and inhibits cell transformation induced by epidermal growth factor in JB6 cells[16-18]. Studies demonstrated negative relationship between caffeine consumption and the risk of gliomas[19,20]. Still, the effects of caffeine on glioblastomas have not been fully elucidated. To address this knowledge gap, we examined the effects of caffeine on cell viability, cell cycle profiles, proliferation and apoptosis in rat C6 and human U87MG glioblastoma cell lines. These findings may provide the experimental basis for future pharmacological treatments of glioblastomas.

Materials and Methods

Cell Lines

Rat C6 and human U87MG glioblastoma cell lines were obtained from the First Affiliated Hospital of Chongqing Medical University and maintained in Dulbecco's Modified Eagle Medium (DMEM; Boxter Biologics, Wuhan, China) supplemented with 10% fetal bovine serum (FBS; Boxter Biologics). The cells were seeded onto 24-well plates at a concentration of 6×10^4 cells per well. The stock solution of caffeine (Sigma, Saint Louis, MO, USA) was prepared in DMSO (Sigma) and further diluted in complete culture medium. Medium containing DMSO, but no caffeine, was used as a control.

Cell Viability Assay

Similar to previous studies[21], the effects of caffeine on glioblastoma cells growth were determined using the methyl thiazolyl tetrazolium (MTT) assay (Boxter Biologics, Pleasanton, CA, USA). Cells were seeded at a density of 5000/well onto 96-well plates and cultured overnight at 37°C. Then, cells were treated with caffeine at the doses of 0, 0.05, 0.1, 0.5, 1, 2.5, 5, 10, or 20 mM for 24 hours. Cell growth was measured by adding 20 µl of 5 mg/ml MTT (Boxter Biologics) to each well. The plates were incubated at 37°C for 4 hours. The supernatant was removed, and absorbance was measured at 570 nm using a multiwell spectrophotometer (Bio-Rad, Hercules, CA, USA). Cell viability was calculated using this following formula: (experimental well absorbance value / control well absorbance value) × 100%. Each experiment was repeated at least five times.

Cell Cycle Analysis

Flow cytometry was used to quantify the relative number of cells in different phases of the cell cycle. After cells were treated with caffeine or DMSO for 24 hours, they were collected and lifted with 0.25% trypsin (Sigma) for 15 minutes at 37°C. Cells were passed through a 100 µm mesh sieve and fixed with 75% ethanol at 4°C. Cells were then treated with 100 µl RNase (0.01 mol/L; Sigma) for 30 min at 37°C, washed with phosphate-buffered saline (PBS, Boxter Biologics), and stained for 30 min (4°C in the dark) with 0.5 mg/L propidium iodide (PI) staining solution (Boxter Biologics). Then, cells were analyzed using FACSCalibur cell analyzer (Becton Dickinson, Franklin Lakes, NJ, USA). Each experiment was repeated at least five times.

Proliferation Assay

After treatments, the cells were propagated at 37°C. Then, cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) kit (Zhongshan, Beijing, China). After the cells were grown for 0, 1, 2, 3, or 4 days, CCK-8 was added to the wells and incubated for 1 hour. Then, absorbance was measured at 450 nm. Cell proliferation was compared by cell counting under microscope to calculate the Inhibitory Concentration (IC) 50. Each experiment was repeated at least five times.

Apoptosis Assay

Flow cytometry analysis was used to study the apoptosis of glioblastomas. Specifically, we calculated the apoptosis ratio. After treatments, the cells were harvested and counted. One million cells were resuspended in 100 µl of PBS. Then, 5 µl of PI staining solution were added, and the mixture was incubated for 30 min at room temperature in the dark. The cells were subjected to flow cytometry to determine the apoptosis ratio using Cell Quest software (Becton Dickinson).

Protein Extraction

Protein extraction was done as previously described[22]. Briefly, cells were lysed on ice in 1× PBS supplemented with 1% Nonidet P-40 (Boxter Biologics), 0.1% SDS (Sigma Chemical), 20 µl/mL of freshly added protease inhibitor cocktail (Zhongshan), and 2 mM phenylmethylsulfonyl fluoride (Sigma). Protein concentration was measured using the Bradford method[23], and proteins were stored at -40°C pending analysis. Each experiment was repeated at least five times.

SDS PAGE and Western Blot

Equal amounts of sample lysates were separated on 30% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dried milk in TBS-T buffer (20 mM Tri-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) and incubated overnight at 4°C with primary antibodies (all from Zhongshan) against Caspase-3 (1 : 200 dilution), Cyt-C (1 : 200), Bax (1 : 200), Bcl-2 (1 : 200), or GAPDH (1 : 500). The membrane was then washed with TBS-T buffer and incubated with secondary antibody (HRP-conjuaged goat anti-rabbit IgG (H+L); Zhongshan). Protein bands were visualized using enhanced Pierce chemiluminescence kit (Sigma). Band optical densities were normalized to those of GAPDH using Image Pro Plus image analysis system. Each experiment was repeated at least five times.

Statistical Analysis

The SPSS 17.0 statistical software was used for statistical analyses (SPSS Inc., Chicago, USA). Data are presented as mean ± SD for continuous variables and as frequency / percentage for categorical variables. The differences were tested evaluated by one-way ANOVE. The $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Caffeine Dose-Dependently Reduces Viability of Glioblastoma Cells

Two glioblastoma cell lines (C6 and U87MG) were treated for 24 hours with caffeine. The viability was detected by MTT assay. Our results demonstrate that caffeine dose-dependently reduced cell viability in both glioblastoma cell lines (Figures 1A and 1B). Specifically, at 1 mM, caffeine reduced cell viability to less than 70% in both cell lines. To minimize confounding effects due to reduced cell viability, caffeine was used at the maximal non-cytotoxic concentration (i.e., 0.5 mM) in subsequent experiments.

Caffeine Alters Cell Cycle Profiles by Causing Cell Cycle Arrest in the G0/G1 Phase

As shown in Figure 2, treatment for 24 hours with caffeine significantly blocked the cell cycle in the G0/G1 phase, compared with control cells (C6, U87MG: $p < 0.01$, $p < 0.05$, respectively; Figures 2A, 2B and 2E). The percentage of cells in the S phase was markedly lower than in control cells (both C6 and U87MG cells: $p < 0.01$; Figures 2C, 2D, and 2F). Thus, our results demonstrate that caffeine appears to alter the cell cycle profiles in both C6 and U87MG glioblastoma cells.

Caffeine Inhibits Proliferation of Glioblastoma Cells

The effects of caffeine on proliferation of glioblastoma cells were examined using the CCK-8 assay. As shown in Figure 3A, the number of C6 cells significantly decreased in the caffeine-treated group, compared with control cells, and the differences were statistically significant on days 2, 3, and 4 (Figure 3A). Similarly, proliferation of U87MG cells was significantly inhibited by caffeine treatment on all tested days (Figure 3B). These results confirmed that proliferation of glioblastoma cells is suppressed by caffeine.

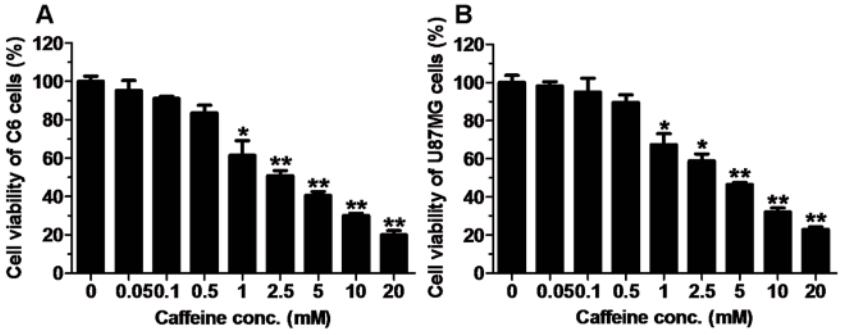

Figure 1. Effects of caffeine on viability of rat C6 and human U87MG glioblastoma cells. ***A,*** Viability of C6 cells 24 hours after the treatment with different doses of caffeine. The viability was assessed by MTT assay. Values are mean ± SEM of five experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control cells, treated only with DMSO. ***B,*** Cell viability of U87MG cells 24 hours after the treatment with different doses of caffeine. The viability was assessed as above. Values are mean ± SEM of five experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control cells.

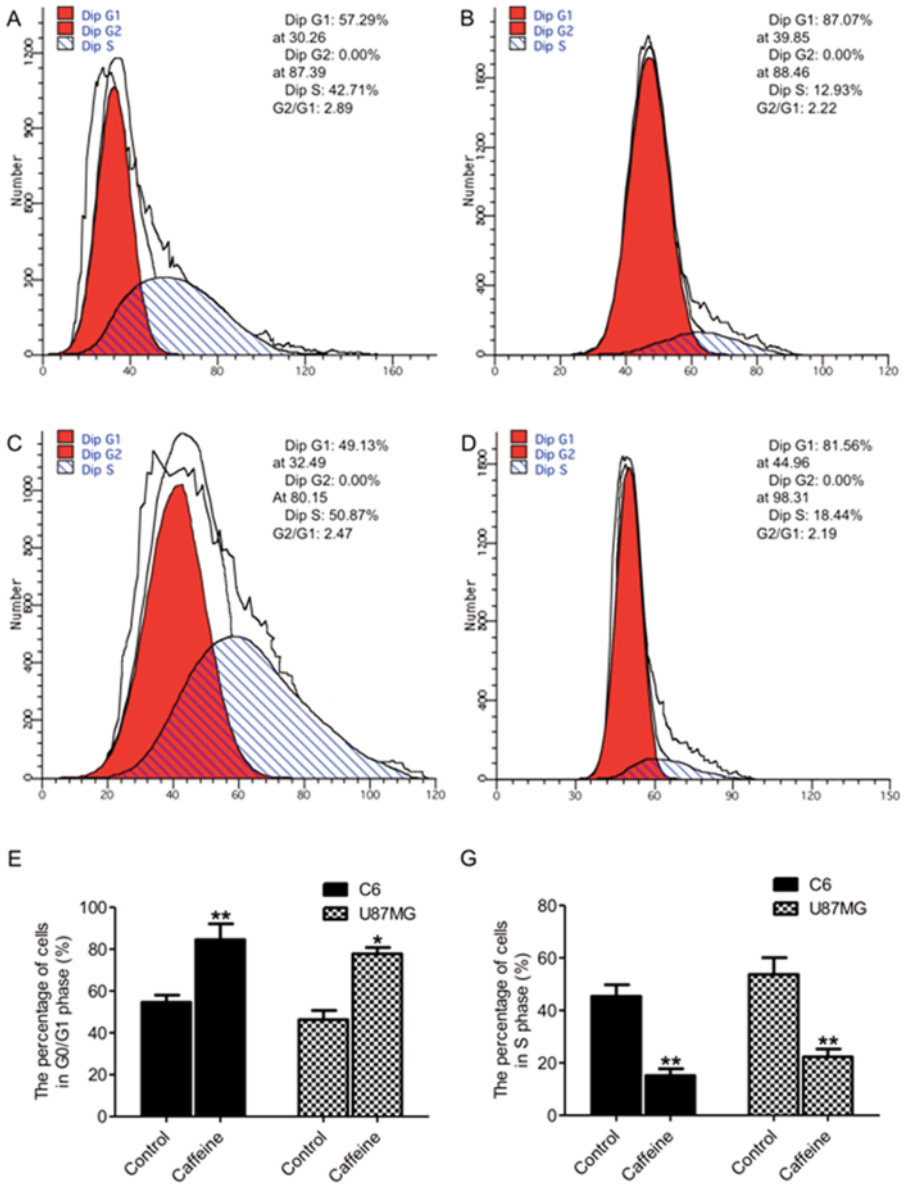

Figure 2. Effects of caffeine on cell cycle profiles in rat C6 and human U87MG glioblastoma cells. ***A*** and ***B*** Respectively, flow cytometry analyses of cell cycle profiles of control C6 cells (DMSO treatment) or cells treated with 0.5 mM caffeine. ***C,*** and ***D,*** Flow cytometry analyses of respective cell cycle profiles of control and caffeine–treated U87MG cells. (E) Percentage of C6 and U87MG cells in the G0/G1 phase. Values are mean ± SEM of five experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control cells. ***F,*** Percentage of C6 and U87MG cells in the S phase. Values are mean ± SEM of five experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control cells.

Caffeine Promotes Apoptosis of Glioblastoma Cells

We next tested the effects of caffeine on apoptosis of C6 and U87MG glioblastoma cells. Caffeine treatment markedly increased the percentage of apoptotic cells in both tested cell lines ($p < 0.05$, both comparisons; Figure 4). Thus, apoptosis of glioblastoma cells is accelerated by caffeine.

Caffeine Modulates Expression of Caspase-3, Cyt-C, Bax and Bcl-2

To further elucidate the mechanisms of cell apoptosis induced by caffeine, we carried out Western blot analyses of expression of Caspase-3, Cyt-C, Bax, and Bcl-2. As shown in Figure 5, expression of Bcl-2 in the caffeine-treated cells was significantly reduced compared with control cells ($p < 0.01$ for both C6 and U87MG cells; re-

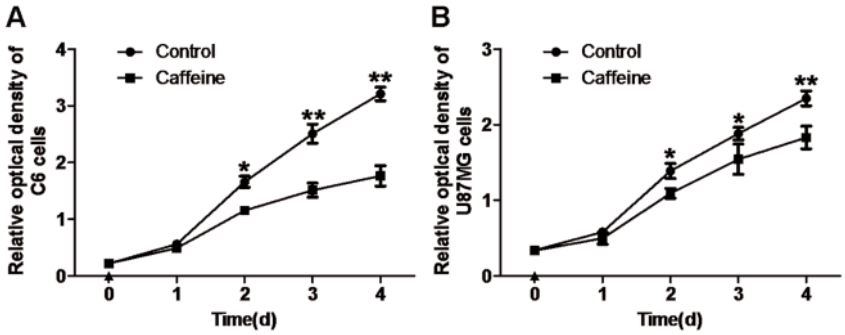

Figure 3. Effects of caffeine on proliferation of glioblastoma cells. ***A,*** Relative optical density of C6 cells treated with either DMSO (control) or 0.5 mM caffeine. Values are mean ± SEM of five experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control cells. ***B,*** Relative optical density of U87MG cells treated with either DMSO (control) or 0.5 mM caffeine. Values are mean ± SEM of five experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control cells.

spectively, Figures 5A and 5B). In contrast, Bax protein expression was not significantly altered by caffeine in either cell line (Figures 5A and 5B). Similar to Bcl-2, the expression of Cyt-C and Caspase-3 was modulated by caffeine ($p < 0.05$ for all comparisons; Figures 5A and 5B). Therefore, our observations indicate that caffeine treatment decreases expression of apoptosis-related protein Bcl-2, thereby increasing the ratio of Bax/Bcl-2.

Discussion

Gliomas are the most common primary malignant brain tumours. The treatment of gliomas, especially glioblastomas, is very challenging because of their infiltrative and aggressive nature. A full cure cannot be achieved by surgical intervention, even if combined with chemotherapy. It is not surpising that gliomas are the main cause of death among brain tumours[24]. Due to the existence of BBB, many therapeutic agents effective against glioblastomas *in vitro* are not effective *in vivo*[25]. In addition, deficiency in pinocytic vesicles and high metabolic capacity of cerebral endothelial cells limit the transition of anticancer agents from the plasma into the central nervous system[26].

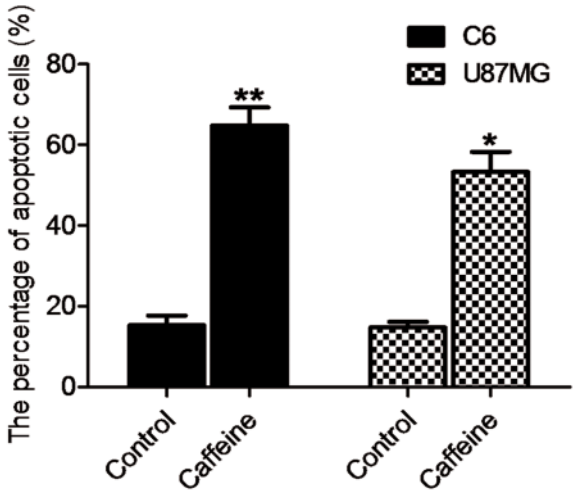

Figure 4. Effects of caffeine on apoptosis of rat C6 and human U87MG glioblastoma cells. Values are mean ± SEM of five experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control cells.

Previous studies demonstrated that caffeine penetrates BBB because of its low molecular weight and good lipid solubility. Caffeine shows a wide range of pharmacological effects toward diseases of the central nervous system, especially Alzheimer's and Parkinson's diseases[10]. Until now, the effects of caffeine on cell viability, cell cycle prolifes, proliferation and apoptosis of glioblastomas have not been thoroughly studied. To address this, we devised the present study using rat C6 and human U87MG glioblastoma cell lines. Our findings indicate that caffeine inhibits proliferation through a cell cycle arrest in the G0/G1 phase and promotes cells apoptosis in the tested cell lines. Others reported anti-cancer effects of caffeine in breast cancer and liver cancer, with the main effector mechanism being enhancement of apoptosis and inhibition of tumour cell proliferation[13 27]. Therefore, quantification of apoptotic processes is a reasonable measure to evaluate anticancer therapies[28].

Proteins in the Bcl-2 family act as key regulators of intrinsic or "mitochondrial" apoptosis pathway[29]. Activation of this pathway through

pro-apoptotic Bcl-2 proteins leads to cell apoptosis[30]. Initial step is membrane insertion and oligomerization of pro-apoptotic proteins Bax and Bak, with a subsequent release of apoptosis-activating factors, such as cytochrome c (Cyt-C), from the mitochondrial intermembrane space to the cytosol as a result of disruption of the integrity of the outer mitochondria membrane and increased permeability[31]. Bcl-2 is an anti-apoptotic protein that protects cells by suppressing the release of Cyt-C[32]. The ratio of Bax/Bcl-2 is, therefore, a valid measure to gauge the progression of apoptosis in tumour cells[33 34 35]. Consistent with previous findings[32 33], our results demonstrate that caffeine reduces the expression level of Bcl-2 and does not affect expression of Bax, causing an elevated ratio of Bax/Bcl-2. This, in turn, activates the release of Cyt-C.

Caspases play a central role in the transduction of apoptotic signals, and caspase-3 is the key enzyme at the terminal stages of apoptosis[36 37]. When caspase-3 is activated, specific substrates (e.g., PARP) are cleaved, causing cell apoptosis[38]. In line with previous studies[35-37], we found that release of Cyt-C induced by caffeine activates the caspase-3 pathway, accelerating cell apoptosis and inhibiting proliferation of glioblastomas.

Conclusions

Caffeine inhibits the growth of glioblastomas by promoting cell apoptosis. Thereby, caffeine may improve the efficacy of anti-cancer treatment for glioblastomas. Our data provide the experimental basis for further studies of potential role of caffeine in the treatment of glioblastomas.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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