Int J Clin Exp Med 2015;8(10):18434-18440
www.ijcem.com /ISSN:1940-5901/IJCEM0013635

Original Article

Combined treatment of fasudil and glutamate decreased the viability of human glioblastoma cells by excitotoxicity through NMDAR in vitro

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Received July 29, 2015; Accepted September 28, 2015; Epub October 15, 2015; Published October 30, 2015

Abstract: Glioblastoma (GBM) is the most common brain tumor with high abilities of proliferation, migration and invasion. As is well-known, the peritumoral excitotoxic neuronal cell loss caused by glutamate, secreted by GBM cells, through activated N-methyl-D aspartate receptor (NMDAR) of neuronal cell. What's more, glutamate benefits the migration of GBM cells. However, the glutamate will not kill the GBM cells itself, which may be due to the deficiency of NMDAR. Fasudil, a ROCK inhibitor, was applied for subarachnoid hemorrhage (SAH) in clinic for many years. And it was found to be of potential to inhibit the proliferation, migration and invasion of GBM cells. In present study, we applied fasudil on the primary human GBM cells to further investigate the reduction of cell viability combined with glutamate. Combination treatment of glutamate and fasudil could significantly decrease the cell viability and elevate the level of LDH compared with fasudil treatment alone. What's more, MK-801, a NMDAR antagonist, could partially abolish this death caused by combination treatment. Further study found that the expression level of NMDAR-2B was elevated after treatment with fasudil in GBM cells. These results demonstrated fasudil could increase the expression level of NMDAR, which is necessary for glutamate to work. In a word, our research has provided a new sight of medicine combination in the treatment of GBM.

Keywords: Glioblastoma, fasudil, NMDAR, glutamate

Introduction

Glioblastoma (GBM) (Word Health Organization grade IV) is the most common tumor in central nervous system (CNS), and it has a median survival time of only 12 to 14 months [1]. Although there is a great progress in the treatment of GBM including surgical excision, chemotherapy and radiation, the prognosis is not satisfactory [2].

Rho/ROCK pathway plays an important role in cell biology behavior such as the reconstruction of the cytoskeleton, cell differentiation, migration, and apoptosis [3, 4]. ROCK1 and ROCK2 are the two isoforms of ROCK. The excessive activation of ROCK has been involved in many diseases. And inhibition of ROCK could improve the symptom of some diseases such as PD [5], AD [6] and SAH [7]. As a clinical medicine, fasudil is applied for SAH by the potent function of dilating artery through inhibiting ROCK. There are researches showing that fasudil could inhibit the migration, invasion and proliferation of GBM cell lines [8, 9]. The present studies just focus on the effect of fasudil alone on the GBM. There is little researches has reported fasudil was involved in the combination treatment to stop GBM growth.

NMDAR, a glutamate receptor, is the predominant molecular device for controlling synaptic plasticity and memory function [10]. NMDAR is composed of three subunits, NMDAR-1, NMDAR-2 and NMDAR-3 [11]. NMDAR-1 binds to the co-agonist glycine and NMDAR-2 binds to the neurotransmitter glutamate [12]. NMDAR is a non-specific cation channel that can allow the passage of Ca^{2+} and Na^{+} into the cell and K^{+} out of the cell. Ca^{2+} is tightly associated with neural

excitotoxicity, which could result in the death of neural cells [13]. GBM cells could release glutamate to cause the loss of peritumoral excitotoxic neuronal cell [14], and GBM cells has a lower expression level of NMDAR than that in normal neural cells to escape the excitotoxicity of glutamate [15, 16].

In the present study, the synergistic effects of the combination treatment with fasudil and glutamate on primary human glioblastoma cells as well as its potential mechanisms were investigated.

Materials and methods

Primary human GBM cell culture and drug treatment

Human glioma tissues were obtained immediately after surgical removal with informed consent from each patient. Tissues were washed with PBS solution and minced into pieces of 2 mm in diameter. The tissue fragments were suspended in 0.25% trypsin (Invitrogen) and digested for 10 min at 37°C with a magnetic stirring bar. Supernatant cells were harvested, washed, filtered with a 200 mesh filter, and then resuspended in RPMI medium 1640 (Invitrogen) containing 10% FBS, 100 units/ml penicillin, and 100 μg/mL streptomycin. The remaining tissues were redigested for 10 min until cells were well dispersed. The cultures were kept at 37°C in a humidified atmosphere of 5% CO_2, and the medium was replaced every third day.

Assessment of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell proliferation and viability was measured using an MTT (MO, USA) assay. Briefly, cells (5×10^4 cells/ml, 100 μL per well) were seeded in 96-well plates incubated with medium 24 h before being treated with fasudil (0, 50 and 100 μM) for 48 h. And then glutamate (MO, USA) with the final concentration of 0 or 500 μM was added into each concentration of fasudil treatment group. Meantime, MK-801 0 or 10 μM was used to antagonize NMDAR. MTT, 5 g/L, 10 μL was added into each well and then cells were cultured in the incubator for 2 h, followed by removal of the culture medium and the addition of 100 μL of DMSO. The absorbance was measured at 570 nm. All experiments were performed in triplicate.

LDH release assay

GBM cells were plated in 96-well plates at a density of 5×10^4 cells/ml. On the following day, cells were exposed to various concentrations of fasudil for 48 h. And then glutamate with the final concentration of 0 or 500 μM was added into each concentration of fasudil treatment group. Meantime, MK-801 0 or 10 μM was used to antagonize glutamate. The medium was collected and assayed for LDH activity using a cytotoxicity detection kit (Nanjing, China). Briefly, the release of LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt into red-colored formazan. The amount of formazan synthesized positively correlated with LDH activity. The formazan product was measured with a microplate reader at 450 nm. Results were expressed as the percentage of LDH release and the absorbance of control cells was set as 100%. This experiment was performed in triplicate.

Apoptosis analysis by flow cytometry

Four groups of cells were harvested and centrifuged at 1,000 rpm for 5 min and then fixed in 5 mL 70% pre-refrigerated ethanol for 24 h at -20°C. After washing the cells with PBS twice, 1 mg/mL RNase (Sigma Chemical Co., St. Louis, MO) was added and incubated for 30 min at 37°C. Then, cells were stained with 300 μL 50 μg/mL propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO) in the dark for 30 min at 4°C. Cell apoptosis was analyzed by flow cytometry (FCM, FACScan, Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Western blot

The expression level of NMDAR-2B (Cell signaling technology, 1:1000) was examined by Western blot in GBM cells after fasudil treatment. Cells were harvested at the indicated time-points and then incubated in the lysis buffer with protease inhibitor tablet for 30 min at 4°C. Cell lysates were centrifuged at 12,000 g at 4°C for 15 min, and the supernatant collected and stored at -80°C for further analysis by western blot. Protein extracts (20 μg, quantitation performed by BCA method, Thermo Scien-

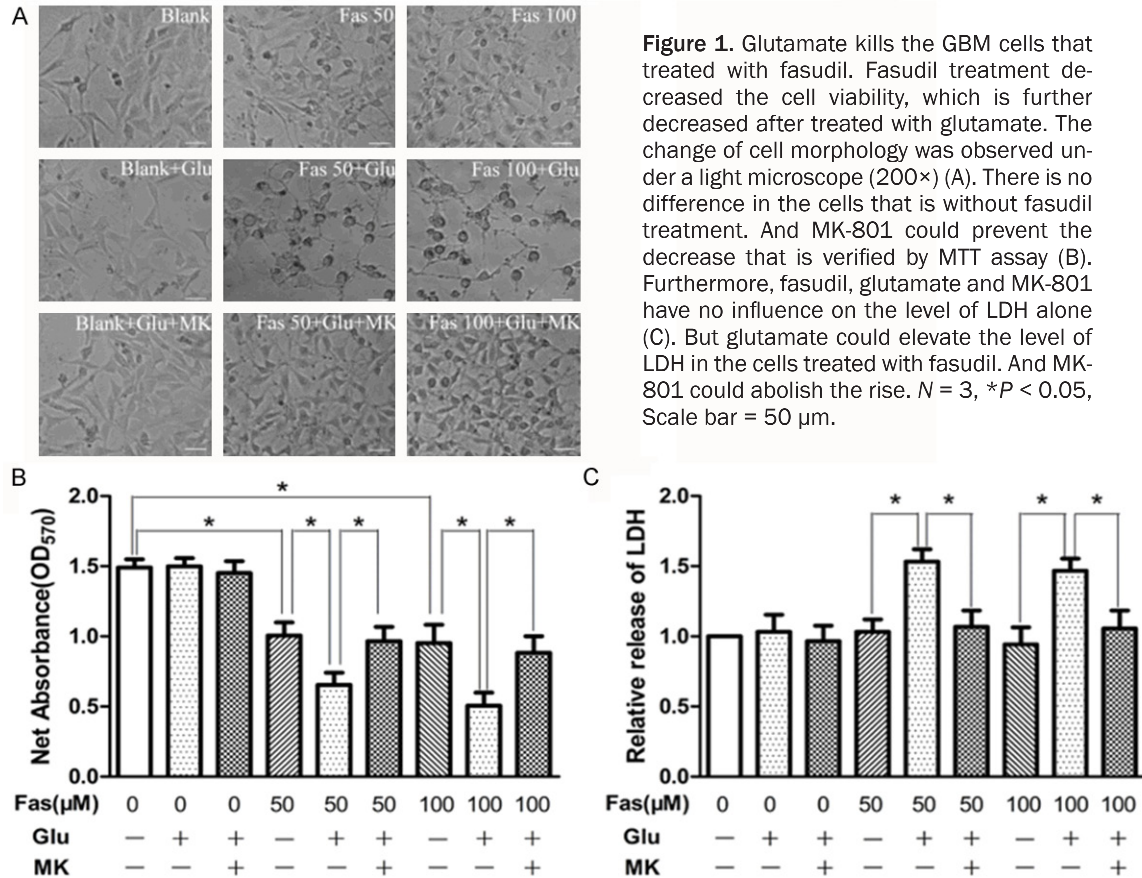

Figure 1. Glutamate kills the GBM cells that treated with fasudil. Fasudil treatment decreased the cell viability, which is further decreased after treated with glutamate. The change of cell morphology was observed under a light microscope (200×) (A). There is no difference in the cells that is without fasudil treatment. And MK-801 could prevent the decrease that is verified by MTT assay (B). Furthermore, fasudil, glutamate and MK-801 have no influence on the level of LDH alone (C). But glutamate could elevate the level of LDH in the cells treated with fasudil. And MK-801 could abolish the rise. $N = 3$, $^{*}P < 0.05$, Scale bar = 50 μm.

tific) were fractionated by electrophoresis on 10% and 15% polyacrylamide gels and transferred to PVDF membranes. After blocked by 5% skim milk at room temperature for 1 hour, membranes were incubated with primary antibodies overnight at 4°C. Then the membranes were followed by incubation with secondary antibodies. The membranes were also incubated with anti-α-tubulin or anti-β-actin for loading control. Proteins were detected with an ECL kit, and signals were quantified using scanning densitometry.

Immunofluorescence

GBM cells were fixed by 4% paraformaldehyde for 30 minutes at room temperature after 48 hours treated with fasudil. Then 0.3% TritonX-100 was used for 15 minutes to drill hole on the cell membranes before blocked by normal goat serum for 1 hour at room temperature. Then cells were incubated with NMDAR-2B antibody (Cell signaling technology, 1:300) at 4°C overnight. Subsequently, a Alexa 555-conjugated secondary antibody (Thermo Scientific, 1:500) was added to the cells for 1 hour at room temperature. Cells were counterstained with Hochest 33258 (MO, USA) to visualize nuclei. Images were observed under a Confocal Laser Scanning Microscopy (Zeiss LSM710, Germany).

Statistical analysis

The data were presented as the means ± standard error. Statistical analyses between two groups were performed by unpaired Student's t-test. Differences among groups were tested by one-way analysis of variance (ANOVA). Following ANOVA analyses, the Tukey's test was used and $P < 0.05$ was accepted to be statistically significant.

Results

Cell viability was decreased after fasudil treatment and further decreased by glutamate treatment

The GBM cells were incubated with fasudil in various concentrations for 48 hours, and the cell viability was measured by MTT assay (**Figure 1B**). Viability of the cells was decreased

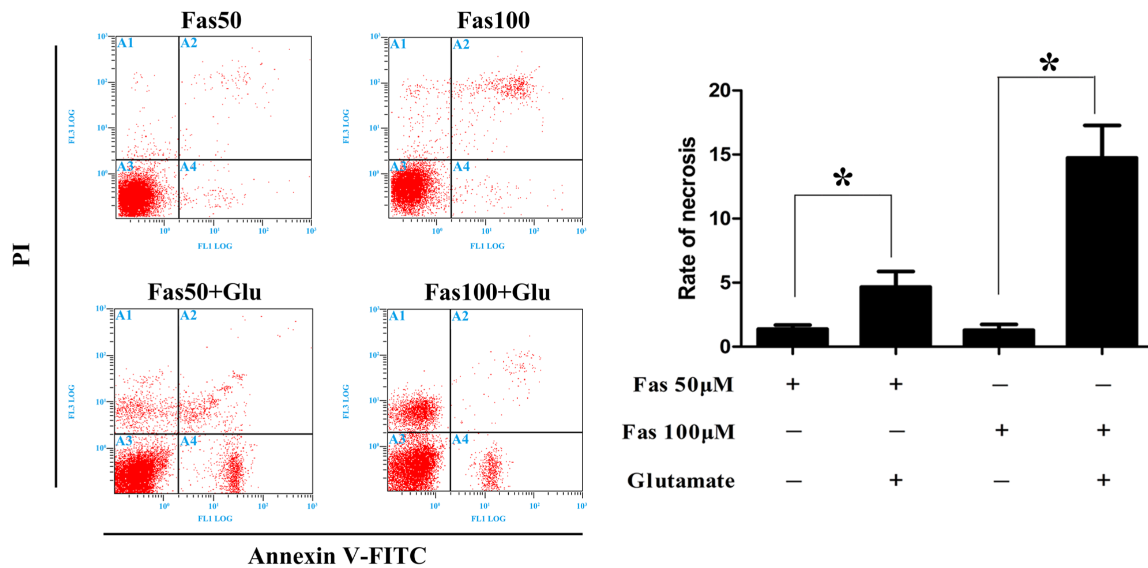

Figure 2. Detection of necrosis using flow cytometry after annexin V-FITC/propidium iodide (PI) staining for fasudil and glutamate treatment. Viable cells are in the lower left quadrant (A3); early apoptotic cells are in the lower right quadrant (A4); late apoptotic cells are in the upper right quadrant; and non-viable necrotic cells are in the upper left quadrant (A1). $N = 3$, $^*P < 0.05$.

after fasudil treatment ($P < 0.05$) compared with untreated cells. Furthermore, glutamate treatment could further decreased the viability of the cells in the present of fasudil ($P < 0.05$), which was abrogated by MK-801 pre-treatment. However, there is no difference in the cells without fasudil treatment.

Glutamate treatment elevated the level of LDH

To investigation whether the decrease of cell viability in MTT assay is through the inhibition of cell proliferation or the death of cells, LDH assay was performed (**Figure 1C**). There is no difference between the cells with or without fasudil treatment. And glutamate treatment could elevate the level of LDH in the present of fasudil ($P < 0.05$) but not in the cells treated with glutamate alone, and the synergistic effect could be abolished by MK-801 treatment. This indicates that fasudil treatment decreased the cell viability by inhibiting cell proliferation, and combination treatment of fasudil and glutamate decreased the cell viability by cytotoxicity.

Glutamate could induce GBM cells necrosis but not promote apoptosis

For further distinguish the reason of the death of cells. PI staining followed by flow cytometry was applied to determine the rate of apoptosis. There is no difference in the rate of both early and late apoptosis between cells whose viabilities were decreased by cytotoxicity (**Figure 2**). But the rate of necrotic cells were significant raised ($P < 0.05$), which in the cells treated with both fasudil and glutamate compared with cells treated with fasudil alone. The necrotic rate was 2.14±0.11% and 1.91±0.19% in the cells treated with fasudil 50 μM and 100 μM respectively. However, the rate was raised to 4.89±0.23% and 15.04±4.77% after adding with glutamate.

The expression level of NMDAR-2B was elevated after treated with fasudil

The expression level of NMDAR-2B in GBM cells was examined after treated with fasudil by immunofluorescence and western blot (**Figure 3**). In immunofluorescence assay, the percentage of NMDAR-2B positive cells (**Figure 3A**, **3B**) were significantly elevated to 59.5% and 63.4% in the cells treated with fasudil 50 μM and fasudil 100 μM respectively compared with 17.9% in control group ($P < 0.05$). Similarly, the protein level of NMDAR-2B determined by western blot was significantly increased in GBM cells after treated with fasudil ($P < 0.05$).

Discussion

In the present study, the viability of GBM cells was decreased by excitotoxicity caused by glutamate which could activate NMDAR, and the

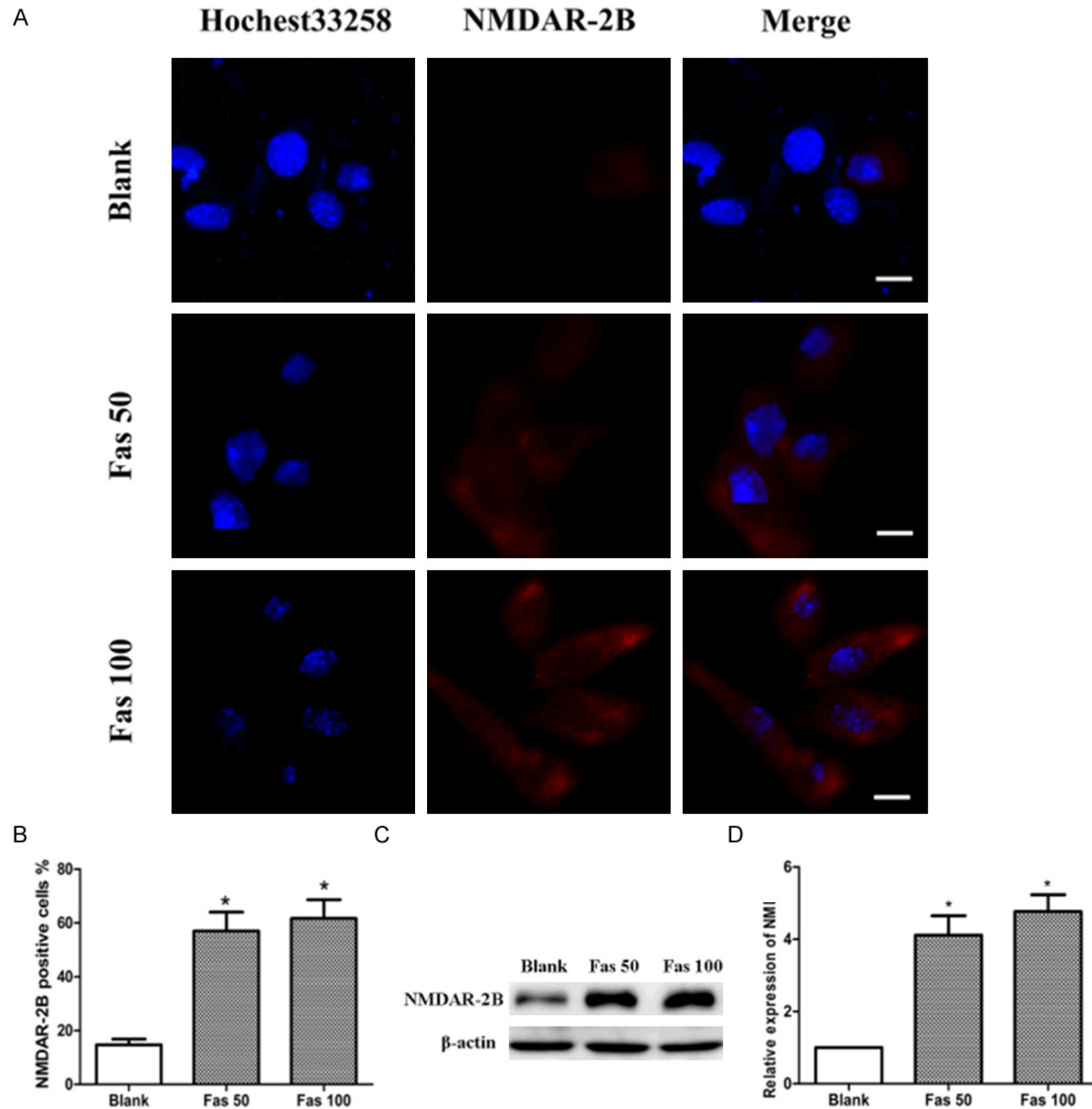

Figure 3. Expression level of NMDAR-2B was elevated after treated with fasudil in GBM cells. The percentage of NMDAR-2B positive cells was significantly increased in the cells treated with fasudil, which was counted by immunofluorescence (A/B). And the expression level of NMDAR-2B was increased in the cells treated with fasudil verified by western blot (C/D). $^*P < 0.05$ compared with blank group. Scale bar = 20 μm.

expression level of NMDAR was elevated after treatment with fasudil. Additionally, MK-801, a NMDAR antagonist, could prevent the death caused by combination treatment of fasudil and glutamate. The MTT assay and LDH assay (**Figure 1**) indicated fasudil treatment decreased the cell viability by inhibiting cell proliferation, and glutamate treatment decreased the cell viability by cytotoxicity. Moreover, the PI staining followed by flow cytometry (**Figure 2**) demonstrated glutamate treatment could induce GBM cells necrosis but not apoptosis.

NMDAR is the predominant molecular device for controlling synaptic plasticity and memory function [10]. And now it is regarded as a possible target for glioma treatment [17]. However, the expression level of NMDAR is lower in gliomas than that in normal tissues [15, 16]. In our research, the expression level of NMDAR in GBM cells was elevated after fasudil treatment (**Figure 3**). That may be resulted from the effect of differentiation involving in the inhibiting of Rho kinase [18-20], which induced the activation of myosin phosphatase. Rho kinase and

myosin phosphatase are the main streams in the signal pathway of differentiation [21]. Two ROCK isoforms ROCK1 and ROCK2 have been identified, ROCK1 is mainly expressed in the lung, liver, spleen, kidney and testis. However, ROCK2 is distributed mostly in the brain and heart [22]. As in GBM, the two isoforms play different roles in cell migration. Inhibition of ROCK1 could singly inhibit cell migration. But inhibition of ROCK2 has no influence in cell migration. And ROCK1 knockdown reduced cell proliferation, whereas ROCK2 knockdown enhanced it [23]. Fasudil could inhibit GBM cells proliferation and migration effectively by inhibiting both ROCK1 and ROCK2.

Furthermore, fasudil could decrease the viability of GBM cells verified by MTT assay and has no cytotoxicity in LDH assay (**Figure 1**), which indicates that fasudil decreased viability just by inhibiting the proliferation of GBM cells but not kill it directly. Glutamate treatment could further decreased the viability of GBM cells in the present of fasudil in MTT assay and elevated the LDH level, but MK-801 could abolish the synergistic effect. Combination treatment killed the cells by excitotoxicity, and this excitotoxicity may be involved in the activating NMDAR. The constantly activation of NMDAR resulted in the opening of ion channel that is nonselective to cations including Na^+ and K^+ and Ca^{2+}. Among them, Ca^{2+} serves as a second messenger and it is necessary for the physiology and biochemistry of organisms and cells [24]. However, excessive entry of calcium into a cell may damage it or even cause it to undergo apoptosis or death. The break of the balance of osmotic stress between inside and outside cells was thought to be the main reason, and it is more pronounced when the Ca^{2+} flux rapidly pour into cells [25]. As for neural cells, excessive stimulation by neurotransmitters such as glutamate and similar substances could cause NMDAR over-activated to allow pathological high level of Ca^{2+} to enter the cells, which leads intracellular Ca^{2+} overloaded and the death of cells [26]. By elevating the expression level of NMDAR-2B (**Figure 3**), combination treatment of fasudil and glutamate could kill the GBM cells through excitotoxicity (**Figure 1**).

However, there are some limitations in our research. First, we did not verify the elevation of NMDAR induced by fasudil and the death of GBM cells induced by combination treatment in animal models. Second, fasudil is applied for SAH patients in clinic but not patients with glioma. Even so, the present study has provided a new sight of medicine combination in the treatment of GBM.

In summary, combination treatment of fasudil and glutamate could kill GBM cells through NMDAR whose expression level was elevated by fasudil. And MK-801 could prevent the synergistic. Although there are limitations of our research, it has provided a new vision in the treatment of GBM.

Acknowledgements

This research was supported by grants from Fundamental Research Funds for Guangdong Provincial Project of Science & Technology (No. 2014A020212096) and Guangdong Natural Science Foundation (No. S2011010002638) to Anmin Liu.

Disclosure of conflict of interest

None.

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