

MYRICETIN INHIBITION ON MURINE GLIOMA GL261 CELL LINE

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Abstract

This study aimed to investigate and compare myricetin activity on mouse glioma GL261 cell line such as inhibition of the cytoactivity, block of cell proliferation and induction of GL261 apoptosis. Two samples, namely myricetin and myricetin plus myricetrin, were used to compare the effect on cell proliferation after 24 h, 48 h and 72 h respectively. Cytoactivity of GL261 cells was inhibited by myricetin in concentration of 25 μ M. The inhibition of cytoactivity was more obvious when concentration increased and the action time prolonged. When myricetin was applied in G1 stage, the cell cycle was blocked. We noticed that the number of surviving GL261 cells was reduced by myricetin and apoptosis occurred. The association between myricetin and myricetrin had a synergistic inhibitory effect on cell proliferation that was superior compared with myricetin alone. Regarding the cells survival we observed that the apoptosis of GL261 cells was related to the dose of myricetin and myricetrin and also to the ratio of their combination.

Rezumat

Acest studiu a urmărit investigarea activității miricetinei asupra liniei celulare murine GL261 prin analizarea inhibării citoactivității, blocarea proliferării celulare și inducerea apoptozei. S-au utilizat două probe, miricetina și miricetina + miricetrină, pentru a compara proliferarea celulară după 24 h, 48 h și respectiv 72 h. Miricetina în concentrație de 25 μ M a inhibat activitatea celulelor GL261. Efectul a fost mai pronunțat cu creșterea concentrației și a timpului de acțiune. Administrarea miricetinei în stadiul G1 al ciclului celular a produs creșterea în volum și blocarea acestuia. S-a constatat de asemenea că miricetina reduce numărul celulelor GL261 prin inducerea apoptozei. Asocierea miricetinei cu miricetrină a avut un efect inhibitor sinergic asupra proliferării celulare comparativ cu miricetina. În ceea ce privește supraviețuirea celulară, apoptoza celulelor GL261 a fost corelată cu doza de miricetina și miricetrină, precum și cu raportul dintre acestea.

Keywords: myricetin, myricetrin, GL261 cell line, cell proliferation, apoptosis, synergistic effect

Introduction

Glioma is a common tumour, highly malignant, hardly to be cured and easy to relapse [4]. To confirm the diagnosis of glioma, it is recommended to perform the histological and immunohistological examination [5, 24].

Clinical signs of gliomas can be headache, syncope, nausea, vomiting, and cognitive dysfunction including memory problems and personality changes. Therefore, neuroimaging should be considered to exclude a cerebral tumour in patients without a history of depression that develop depressive or debilitating symptoms without an obvious cause [15]. In some cases, severe vitamin K deficiency coagulopathies may occur [23].

The regular treatment is excision assisted by chemotherapy. But all chemotherapeutic regimens can cause immunosuppression, leading to a decrease of white blood cells, red blood cells and platelets. Therefore, chemotherapy in gliomas may be complicated by opportunistic fungal infections such as *Aspergillus* or *Fusarium* [22] or severe bacterial infections with resistant germs [2, 3].

A lot of biomolecules are produced by tumour stroma cells when tumour is growing including growth factors, angiogenine, and matrix metalloproteinases (MMPs) [1] that contribute to tumour angiogenesis, immune reactions inhibition and promotion of tumour proliferation.

Blood vessels supply tumour cells the need to survival and assists them to proliferate, grow, invade normal cells and move locations. Compared to normal blood vessels, tumour blood vessels [21] are different in structure and function. Tumour vascular structure has distorted morphology, thin vascular wall and gaps on the walls.

One of the anti-cancer strategies is represented by the use of anti-angiogenic therapy. These drugs stop the tumour from developing their own blood vessels; most of them by inhibiting MMPs. MMPs are vital for tumour angiogenesis and cell migration because they support blood vessel development.

MMPs can degrade the basilar membrane and adjust the relations among cells endothelium and the cytomembrane of the endothelial cells (ECs). With adhesion on matrix, tumour cells invade endothelial cells (ECs) through MMPs. There are

two kinds of MMPs inhibitors, natural inhibitors and synthetic inhibitors. Natural inhibitors, mainly tissue inhibitors of metalloproteinases (TIMPs) [6, 7] are able to inhibit MMPs activity while induces generation of pro-MMP-2 [9]. In the category of synthetic inhibitors there are included the bisphosphonates [8] that have a lot of side effects and the peptidomimetics inhibitor batimastat [15], its use being restricted because intraperitoneal injection is needed.

Myricetin [10], an extractive of *Myrica rubra* leaf, is a pure natural compound and is widely spread in tea, fruits and vegetables [20]. Myricetin has anti-tumoural [13], antioxidant and antithrombotic activity. Myricetin can induce the apoptosis of tumour cells, disrupt signal transmission and prevent cells migration. Researches showed that myricetin can induce the death of HepG2 cells [12] through mitochondria and blood vessel basement and inhibit tumour growth by blocking the cell cycle in G2/M period [14]. Researches also proved that myricetin is able to inhibit the generation of tumoural blood vessels by inhibiting VEGF (vascular endothelial growth factor) [11].

Materials and Methods

Materials

Myricetin, extracted from *Myrica rubra* leaf with purity over than 90% (Sigma Aldrich, USA), in absolute ethyl alcohol at -20°C. 150 g - 200 g glioma GL261 cells from mouse brain was obtained from State Key Laboratory of Biology School in the Army Medical University (Chongqing City, China). Cells were cultured in RPMI-1640 medium (Gibco, USA) with 10% foetal calf serum (Gibco, USA) in 3% CO₂ incubator at 37°C. Cells in a certain growing period were extracted during the experiments.

Methods

Cell Counting Kit-8 (CCK-8) experiments

A part of GL261 cells were extracted and digested with 0.3% pancreatin (Beyotime, China). Then, the cells number was calculated and adjusted to 1×10^5 cell/mL. A cell suspension of 45 μ L and fresh nutrient solution of 55 μ L were infused to 96-well plates. Myricetin nutrient solutions of concentrations of 10 μ M, 15 μ M, 25 μ M, 35 μ M, 45 μ M and 55 μ M were added. Nutrient solution with no myricetin was used as control group.

The well plates were taken out after 24 h and 48 h respectively and 15 mL CCK-8 solution (Beyotime, China) were added. After cultured for 1 - 2 h, at dark, the colour of the nutrient solution was assayed at 450 nm.

Flow cytometry of cell cycle

On 96-well plate, we added 400 μ L nutrient solution and GL261 cells. When the number of cells increased 10 times and the degree of cell fusion reached 85%,

we added new nutrient solutions that contained 10 μ M or 40 μ M myricetin. The plates were incubated in 3% CO₂ atmosphere at 37°C.

After 24 h, pancreatin was used to digest cells. Nutrient solution was centrifuged at 1000 rpm for 5 min. Cold phosphate-buffered saline (PBS) (Beyotime, China) was used to wash twice the cells and then 550 μ L 75% cooled ethylic alcohol (Beyotime, China) was added. After fully blending, the cells were kept in refrigerator at 3.8°C. 7.5 μ L of RNA enzyme (RNA-ase) (Beyotime, China) was added and cultured for 40 min at 37°C. In the final step propidium iodide (PI) (Beyotime, China) staining solution was added to detect the evolution of the cell cycle.

Western-blotting

We added a fresh nutrient solution to the test group with 260 μ M myricetin when the degree of cell fusion reached 85%. Nutrient solution for the control group didn't contained myricetin. We washed the cells and filtered the nutrient solution, we performed the fragmentation reaction with 120 μ L decomposition fluid, we centrifuged protein solution and stored it at -85°C in deep freezer. The protein solution was mixed with $5 \times$ Loading Buffer solution, boiled, washed and mixed up with 6% of skim milk powder and incubated for the second time. Finally, ECL luminescence kit (Beyotime, China) was used for the chromogenic reaction.

Cell survival

400 μ L cells suspension was infused on 96-well plate and solutions of 5 μ M, 30 μ M, 35 μ M and 50 μ M myricetin respectively, were added.

After 24 h, the cells were washed twice for 5 min with PBS, fixed with paraformaldehyde and 250 μ L of diluted Hoechst 33342 staining fluid (Beyotime, China) was added. Finally, cells survival was observed under microscope.

Cells proliferation and apoptosis after the association of myricetin with myricetrin

A suspension of 400 μ L cells was infused in a 96 well-plate. We used four cell groups: myricetin, myricetrin, myricetin + myricetrin and the control group. The concentration of myricetin and myricetrin were 40 μ M, 75 μ M, 160 μ M and 320 μ M respectively. The concentrations of the combination myricetin + myricetrin were 20 μ M, 37.5 μ M, 80 μ M and 160 μ M respectively, mixed in a 1:1 ratio.

50 μ L of thiazolyl blue-tetrazolium bromide, 5.6 mg/mL was added and the cell proliferation was detected after 24 h, 48 h and 72 h respectively. Cells' survival was observed after 5 μ L Annexin V-FITC and 10 μ L propidium iodide were added.

Statistical analysis

SPSS17.0 statistical software was used for data analysis. All numerical data are shown as mean \pm standard deviation and Student t test was conducted

to compare means. The statistical significance was set to $p < 0.05$.

Results and Discussion

CCK-8 kit was used to test the influence of different concentration of myricetin on GL261 cells [18]. Results showed different cell resistance to different myricetin concentrations, as depicted in Figure 1.

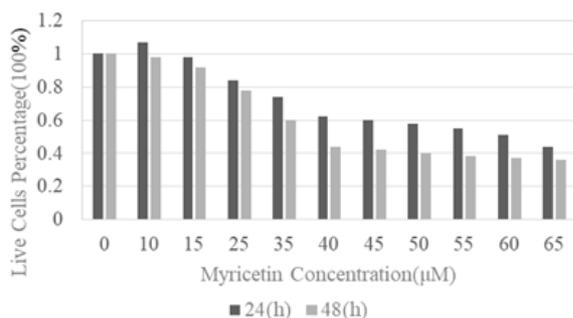


Figure 1.

The effect of myricetin concentrations on cell survival

It can be observed that the effect of myricetin in concentration below 25 µM on GL261 cells was very low, even an increased activity of the cells could be observed when myricetin concentration was lower than 10 µM. However, at 25 µM, the cell survival rate showed an obvious downwards trend, especially when the contact time was prolonged to 48 h. With the increase of myricetin concentration, more cells were inhibited, the cell survival rate dropped to less than 40% with a GL261 cells concentration of 65 µM ($p < 0.01$).

CCK-8 experiment proved that GL261 cells activity can be inhibited by myricetin. Besides, the number of survived cells was controlled by further inhibiting cell proliferation through blocking the cell cycle in different stages.

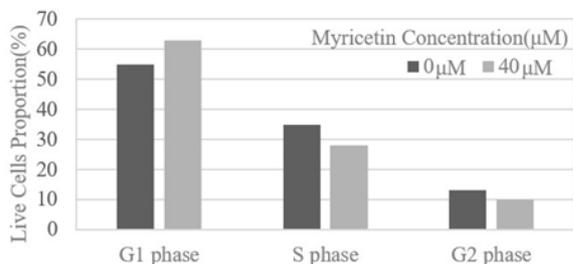


Figure 2.

The effect of myricetin on cycle stages of GL261 cells

As was shown in Figure 2, a concentration of 40 µM myricetin determined an increase of GL261 cells in G1 cell stage, accompanied by a decrease of GL261

cells in S and G2 stages of the cell cycle. This showed that GL261 cells can be blocked in G1 stage. In order to further explain how GL261 cells proliferation was blocked in G1 period, Western-Blot was used to detect the protein content. Lower proteic metabolism meant cell growth was inhibited. Cycline D1 can promote cell proliferation and therefore it was selected to detect how GL261 cells were inhibited in G1 stage by myricetin. The results are presented in Figure 3.

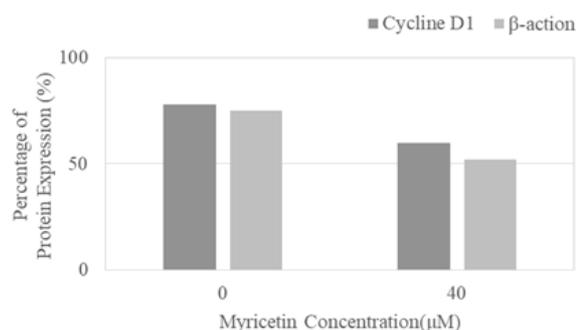


Figure 3.

Myricetin effect on Cycline D1 and Myricetin effect on β-action

The results showed that the expression of Cycline D1 of GL261 cells with myricetin decreased. Grey value imaging analysis showed that there was statistical significance in terms of colour difference between the experimental group with myricetin and the control group ($p < 0.05$).

β-action in eukaryocyte promotes cell movement and cell division growth. Thus, the expression of β-action factor was detected. When myricetin concentration increased, the expression ratio of β-action factor was decreased, thus the proliferation of GL261 cells was inhibited.

Hoechst33342 staining was used to detect cells' apoptosis. Myricetin concentrations of 10 µM, 15 µM, 25 µM, 35 µM, 45 µM and 55 µM were mixed with GL261 cells suspension. After 24 h, the change of cell colour was observed, thus the death of cells was identified.

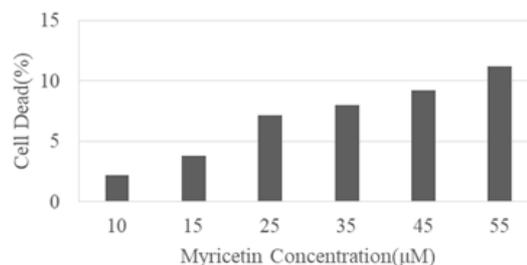


Figure 4.

The apoptotic effect of myricetin on GL261 cells

In Figure 4 it can be seen that the apoptosis rate of GL261 cells increased with myricetin concentration.

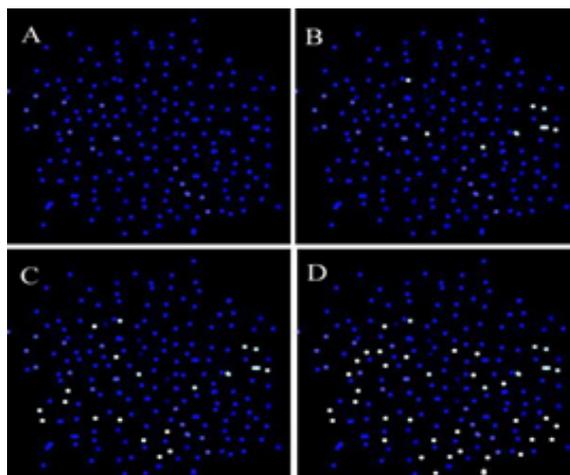


Figure 5.

GL261 Cells Colour Change using Hoechst33342 dye (A: 0; B: 40 μ M; C: 50 μ M; D: 70 μ M; $\times 300$; $p < 0.05$)

When the concentration reached 55 μ M, the ratio of apoptotic bodies of GL261 cells was over above 10%. Microscope and Hoechst33342 detection were combined to observe cells colour.

From Figure 5 we can observe under different myricetin concentration the colour of GL161 cells changed into a brighter colour, showing the apoptosis of a part of cells. These results showed that myricetin can induce the death of GL261 tumour cell to a certain degree.

In the next step, myricetrin was associated with myricetin. The activity of four groups, including myricetin, myricetrin, myricetin + myricetrin and the control group was determined after 24 h, 48 h and 72 h. The concentrations of myricetin and myricetrin were 40 μ M, 75 μ M, 160 μ M and 320 μ M respectively. Concentrations of the combination of myricetin + myricetrin were 20 μ M, 37.5 μ M, 80 μ M and 160 μ M respectively. They were mixed in a ratio of 1:1.

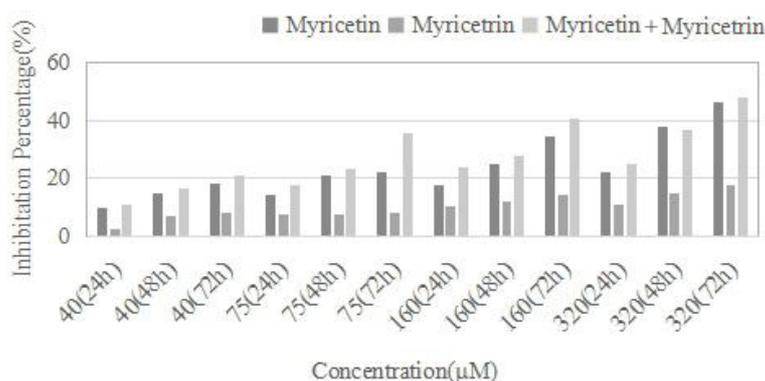


Figure 6.

The inhibitory effect of myricetin, myricetrin and myricetin + myricetrin on GL261 cells growth after 24 h, 48 h and 72 h

It was shown in Figure 6 that the percentages of inhibition of GL261 cells growth corresponding to myricetin concentrations of 40 μ M, 75 μ M, 160 μ M and 32 μ M were 9.8%, 13.2%, 18.7% and 21.2% respectively. When myricetrin concentration was 320 μ M, the inhibitory effect was poor and the inhibition ratio was only 10.3%. When myricetin and myricetrin were combined, the inhibition was strengthened and the inhibitory percentages corresponding to the concentrations of 20 μ M, 37.5 μ M, 80 μ M and 160 μ M were 10.7%, 17.2%, 22.4% and 24.8% respectively. After 48 h, it was found that the inhibition of the three reagents on GL261 cells proliferation was higher. When single myricetin concentration was 40 μ M, 75 μ M and 160 μ M respectively, inhibition of the mixture was increased compared with the compounds alone and its inhibition percentage was 17.5%, 23.2% and

27.8% respectively. After 72 h, the inhibition performances of the dual reagents were the best with a percentage of 56.4% at 320 μ M. As the working time was longer and myricetin concentration was higher, the inhibition on GL261 cells improved.

We made some changes to the above three groups of reagents: the corresponding myricetin concentration and myricetrin concentration were set as 80 μ M, 150 μ M, 320 μ M and 640 μ M and the concentration of the mixture of myricetin and myricetrin was set as 40 μ M, 75 μ M, 160 μ M and 320 μ M respectively.

It was concluded from previous analysis that as myricetin concentration was higher, its corresponding inhibitory effect on GL261 cells proliferation was higher. Annexin V-FITC/PI staining was used to test the effect of the three groups under the maximum concentration (640 μ M myricetin and myricetrin).

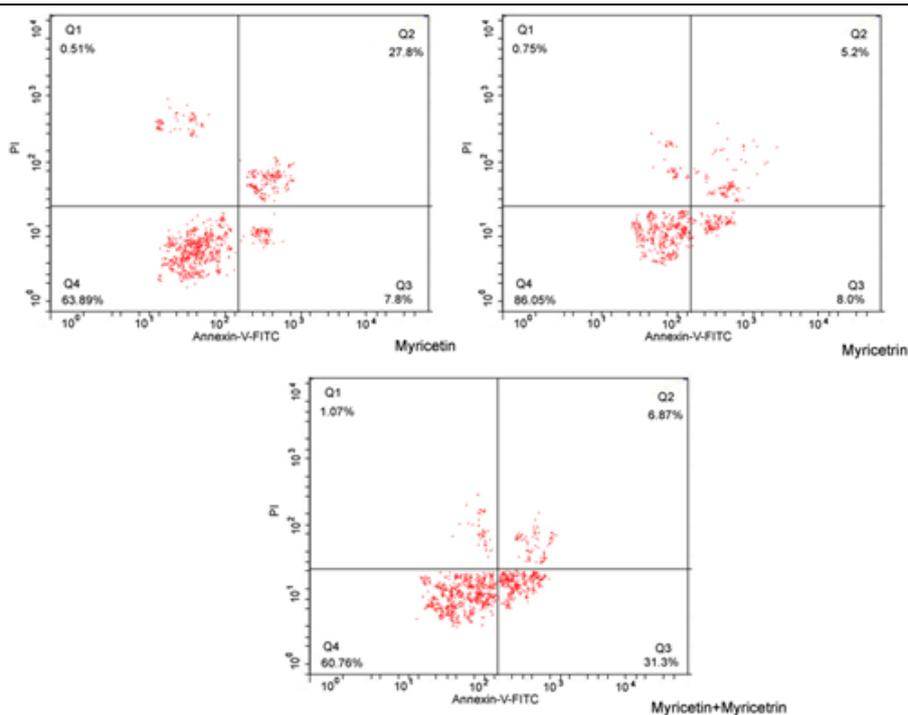


Figure 7.

The pro-apoptotic effect of myricetin, myricetrin and myricetin + myricetrin on GL261 cells growth after 24 h of exposure

In Figure 7 the plots are divided into four areas. Q1 denotes the percentage of cell death, Q2 denotes the percentage of late apoptosis, Q3 denotes the percentage of early apoptosis and Q4 denotes the percentage of the remaining normal cells. Results showed that among them, myricetrin had the lowest effect on GL261 cells apoptosis and less than 640 μ M myricetrin the percentage of apoptosis was 13.95%. On other hand the concentration of 640 μ M myricetrin induced 36.11% apoptosis. The association of the two compounds was synergistic, with an apoptosis rate of 39.24%.

Brain glioma is a common neural tumour with severe malignant forms, hard to be cured and easy to metastasize. Excision is a common treatment but it is hard to be done because tumour features disordered growing and thin wall, easy to stick to normal cells. Not few studies were focused on chemotherapy and immunological therapy with drugs, such as carmustine and temozolomide. Even though they can inhibit tumour cells to a certain degree, there are many side-effects.

Conclusions

Myricetin can inhibit blood vessel MMPs of tumour effectively, but there are no researches on adopting it to brain glioma.

Glioma GL261 cells [17] from mouse brain was used to test the inhibitory effect of myricetin, myricetrin and myricetin + myricetrin. Tumour growth is based both on cell proliferation and apoptosis inhibition. I

our study, CCK-8 [18] was used as an apoptosis marker for GL261 cells in a flow cytometry assay [19].

The various experimental approaches used in this study suggested that myricetin inhibits GL261 cells proliferation and induces apoptosis in a dose dependent manner. Association of myricetin with myricetrin showed an increased effect on both GL261 cells proliferation inhibition and apoptosis.

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