ORIGINAL RESEARCH

Effects and Mechanism of Luteolin on Proliferation and Apoptosis of Glioma

Xiaobing Yuan, PhD; Jian Ouyang, PhD; Chunping Long, PhD

ABSTRACT

Objective • To explore and analyze the efficacy of luteolin on the proliferation and apoptosis of cerebral glioma and its possible mechanism, providing a theoretical basis for luteolin in glioma treatment.

Methods • The cell line of Human cerebral glioma U87 was utilized for our current research. The study group were added with 0, 20, 40, and 80 μmol/L luteolin, respectively. Luteolin's Inhibitory function in the proliferation of U87 cells was detected by CCK-8, the Transwell chamber test was used to measure cell migration and invasion, while flow cytometry was used to assess apoptosis and Western Blot to gauge the expression of JNK/STAT3 pathway proteins.

Results • In comparison with the control group, the intervention of various doses of luteolin could effectively inhibit glioma cell proliferation, the inhibitory rate uplifted remarkably with an increase of dose as well as intervention time (95% CI. 92.160-107.494, $P < .05$), which presented a significant time and dose dependence. The apoptosis rate of the low-dose, medium-dose, and high-dose categories was higher than that of the comparison group after 2 days of luteolin intervention ($P < .05$), and the apoptosis rate appeared to increase with the increase in intervention dose ($P < .05$). After 2 days of luteolin intervention, there were significantly more migratory and invasive cells in 3 categories than that in the control group ($P < .05$), and the number increased as the luteolin intervention dose was raised ($P < .05$). Bax and Cyt-c expressions dropped after 2 days of luteolin treatment when compared with the control set ($P < .05$), and the expression of Bax and Cyt-c fell considerably with increasing luteolin dose. Bcl-2, Caspase-3 and PARP expressions were significantly elevated in comparison to the control group ($P < .05$), and the increase was more obvious with the rise of the luteolin dose.

Conclusion • Luteolin has a good inhibiting function on the proliferation of glioma cells, inhibiting cellular invasion and migration and promoting the apoptosis of glioma cells and the expression of apoptosis-related proteins, which has laid a good foundation for the application of luteolin to treat glioma cells. (*Altern Ther Health Med.* 2024;30(7):284-288).

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INTRODUCTION

The most prevalent neurocutaneous intracranial tumor is glioma. Epidemiological investigations have shown that glioma accounts for about 40% of central nervous system (CNS) carcinoma and 81% of malignant carcinoma in the central nervous system.[1] Since glioma cells are prone to develop resistance to radiotherapy and chemotherapy, current treatments such as surgery, radiotherapy, or chemotherapy cannot cure it completely. At the same time, glioma cells have strong invasion and migration capabilities, can often transfer to the surrounding tumor tissues, and promote tumor recurrence, which largely contributes to the patient's poor prognosis for glioma.[2-3] To increase the effectiveness of chemotherapy, researchers are concentrating on finding medications that efficiently suppress the proliferation, invasion, and migration of glioma cells.

As a representative component of natural flavonoids, luteolin belongs to weak acidic tetrahydroxy flavonoids and is a natural pigment component widely distributed in plants [4]. Studies have proved that luteolin has antiviral, anti-infection, antioxidant, and other effects. It acts as an anticancer agent against various types of human malignancies such as lung, breast, glioblastoma, prostate, colon, and pancreatic cancers.[5] Several studies found that it inhibits the growth of malignant tumor cells such as lung cancer and cervical cancer by anti-proliferation and induction of apoptosis.[6-7] Lee et al.[8] have found that luteolin induces apoptosis and autophagy in malignant glioblastoma cells,

and that luteolin-induced autophagy promotes cell survival, implying that appropriate combination treatment with luteolin and autophagy. In addition, several studies have suggested that luteolin has anti-glioma effects.[9-11]

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway was discovered more than a quarter-century ago. As a fulcrum of many vital cellular processes, the JAK/STAT pathway constitutes a rapid membrane-to-nucleus signaling module and induces the expression of various critical mediators of cancer and inflammation. Growing evidence suggests that the JAK/STAT pathway dysregulation is associated with various cancers and autoimmune diseases.[12]

Aiming at further confirming luteolin's inhibitory effect on glioma cells, this study explored and investigated luteolin's effects on the apoptosis and proliferation of glioma cells as well as its potential mechanism.

MATERIALS AND METHODS

Cells and Main Reagents

Glioma cell U87 was purchased from the Cell Bank of the Typical Cultures Preservation Committee of the Chinese Academy of Sciences. Luteolin (L812409, Shanghai MacLean Biochemical Technology Co., LTD., CAS No. : 491-70-3); Dulbecco's Modified Eagle Medium (DMEM) High glucose Medium (Gibco, 11995040, USA); Penicillin-streptomycin (15140148, Gibco, USA); Fetal bovine serum (Gibco, 1010047, USA); CCK-8 kit (C0038, Shanghai Biyuntian Biotechnology Co., LTD.); Annexin V-FITC/PI Apoptosis Detection Kit (CA1020, Beijing Solibo Technology Co., LTD.); Cell lysate (P0013, Shanghai Biyuntian Biotechnology Co., LTD.); BCA protein concentration assay kit (P0012S, Shanghai Biyuntian Biotechnology Co., LTD.); SDS-PAGE gel preparation kit (P0012A, Shanghai Biyuntian Biotechnology Co., LTD.); Polyvinylidene Fluoride (PVDF) membrane (HVLP04700, Millipore); Anti-Bax antibody (AB53154, Abcam); Recombinant Anti-Bcl-2 antibody (AB32124, Abcam); Anti-Cyt-c antibody (ab13575, Abcam, USA); Recombinant Anti-Caspase-3 antibody (ab32351, Abcam, USA); Recombinant Anti-PARP antibody (ab191217, Abcam, USA).

Methods

Detection of Cellular Proliferation by CCK-8 assay. The 10% Fetal Bovine Serum (FBS) DMEM high glucose medium containing penicillin-streptomycin was set in a cell culture incubator at 37°C and 5% CO_2 concentration. Human glioma U87 cells, which were good and in the logarithmic-growth phase, were prepared in a single cell suspension and planted into a 96-well plate with 100 μl per void, by each well of 1×10^4. The different concentrations of luteolin were added after 24h of adherent growth with concentration gradients of 0, 10, 20, 40, 80, 160, and 320 μmol/L, respectively. Dimethyl sulfoxide (DMSO) was put into the control group, and 5 wells were set in each group. After 24h and 48h of drug treatment, the well-received 10 μl of CCK-8 reagent, was then incubated for an additional 2 hours at 37°C. Using a microplate reader, the absorbance value at 450 nm for each group was determined and the cell survival curve and IC50 value were calculated.

Detection of Apoptosis. The Annexin V-FITC/PI apoptosis detection kit was used to identify cells that had died. Cells were injected into 6-well plates following the kit's instructions with the number of cells 4×10^5 per well and cultured for 48h. The three groups were set up and then added with 0, 20, 40, and 80 μmol/L luteolin, respectively. After 48h of treatment, the drug was rinsed 3 times with Phosphate Buffered Saline (PBS), digested with trypsin, and centrifuged (1000 rpm, 5 min, 4°C). After the cells were harvested and rinsed twice in PBS, the supernatant was removed, and 1×10^5 cells were then resuspended in 100 μL of the buffer. Following a 15-minute incubation with PI (5 μL) and Annexin V-FITC (5 μL) at room temperature in the dark, the cells were then given 400 μL of 1 binding buffer and each group's apoptosis was detected by flow cytometry.

Detection of Cell Migration and Invasion Ability by Transwell Chamber Experiment

U87 cells in the logarithmic growth phase were designed at a cell density of 3×10^4 cells per well and seeded in a chamber containing membrane filter inserts with a membrane pore diameter of 8 μmol/L. Grouped and treated the cells according to the abovementioned method, the duration of drug treatment was 48h. For intrusion detection, the gel matrix was pre-coated onto the insert of the membrane filter and 30% FBS was present in the medium in the lower chamber. Cells were incubated for 36h in serum-free medium at 37°C, then fixed 4% paraformaldehyde, stained 2% crystal violet, and counted from three random fields with a microscope for statistical analysis.

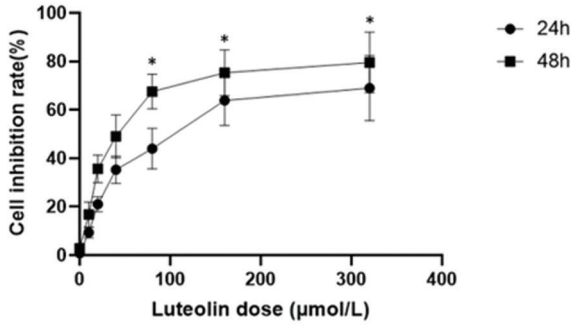
Western blot detection of apoptotic pathway protein expression

With 4105 cells in each well of a 6-well plate, the cells were planted and cultured for 24h. Grouped and treated the cells according to the abovementioned method, and the duration of drug treatment was 48h. Cell lysate was subsequently added and centrifuged for 20min (16000g, 4°C). The supernatant was taken as the extracted cell protein, then the BCA protein quantification kit was used to determine the sample's total protein concentration. A Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) gel preparation kit was used to prepare to separate gel and stacking gel. SDS-PAGE was performed by adding 30-70 μg protein samples and 5 μL prestained Marker into each well. After being transferred to a PVDF membrane, the material was blocked for 2 hours with 5% skim milk powder, and washed five times with TBST, and then Bax antibody (1:1000) and Bcl-2 antibody were added (1:1000).

Statistical Analysis

SPSS version 25.0 was adopted for data processing and analysis. Comparison of measured data among groups was performed using ANOVA, and pairwise comparisons using

Figure 1. Inhibitory effect of luteolin on glioma cells



LSD *t* test. $P < .05$ was used to indicate that the data had statistical significance.

RESULTS

Luteolin's Inhibitory Effect

The intervention of various doses of luteolin, compared to the control group, can inhibit cell proliferation, and its inhibition rate increased remarkably with the rise of dose and intervention time ($P < .05$, Figure. 1), which presented a significant time and dose dependence. The IC50 was 99.770 μmol/L (95% CI. 92.160-107.494) at 24 h of intervention, and 44.835 (95% CI. 36.226-57.286) μmol/L at 48 h of intervention.

Promotion of Luteolin on Apoptosis of Glioma Cells

The three groups' apoptosis rates were significantly greater than the control group's after 2 days of luteolin intervention ($P < .05$, Figure 2), and the apoptosis rate appeared to rise with increasing intervention dose ($P < .05$, Figure 2).

Luteolin Inhibits Cell Migration and Invasion

Upon 48 hours of luteolin intervention, the low-dose, medium-dose, and high-dose groups all showed significantly higher numbers of migrating cells and invasive cells compared to the control team ($P < .05$), and the number of migrating cells and invasive cells increased significantly as the luteolin intervention dose raising ($P < .05$). (Figure 3, 4).

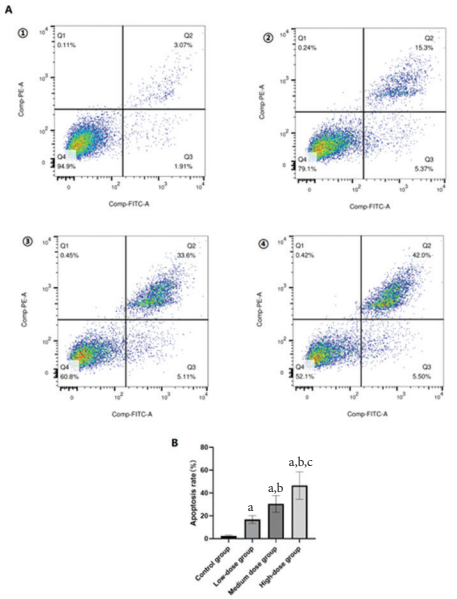
Luteolin activates the apoptosis pathway of glioma cells

Bax and Cyt-c protein expressions reduced considerably with an increase in luteolin dose after 2 days of luteolin intervention compared to the control group ($P < .05$, Figure 5). Bcl-2, Caspase-3, and PARP protein expressions were all considerably higher in the luteolin group than in the control class ($P < .05$, Figure 5), and the rise became more pronounced as the luteolin dose was increased.

DISCUSSION

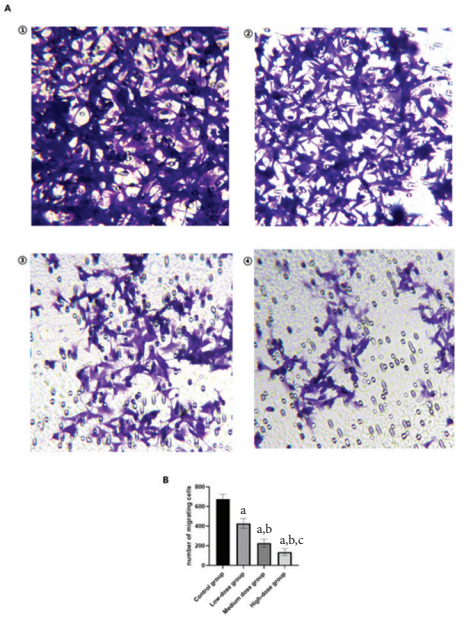
As one of the most common and fatal brain tumors, glioma has high malignancy and patient mortality.[13] Related epidemiological studies show that 3-8 cases are suffering from the disease among the 100 000 population. Brain tumors can squeeze into the surrounding tissues and secrete harmful

Figure 2. The apoptosis rate of luteolin in human glioma cells **(%, ± s).** A: Apoptosis rate of luteolin in human glioma cells. 1. Control category. 2. Low-dose category. 3. Medium-dose category. 4. High-dose category. B: Histogram of the effect of luteolin on apoptosis rate of human glioma cells.



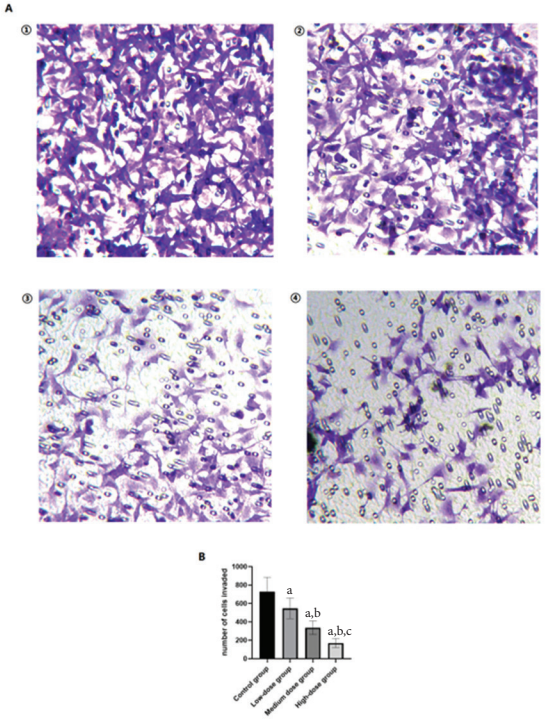
[a]Compare with the control category, $P < .05$
[b]Compare with the low-dose category, $P < .05$
[c]Compared with the medium-dose category, $P < .05$

Figure 3. luteolin Impact over quantity of glioma cells that migrate. A: Effects of Luteolins on the quantity of migrating human glioma cells. 1. Control category. 2. Low-dose category. 3. Medium-dose category. 4. High-dose category. B: Histogram of the effect of luteolin on the migration cell number of human glioma cells.



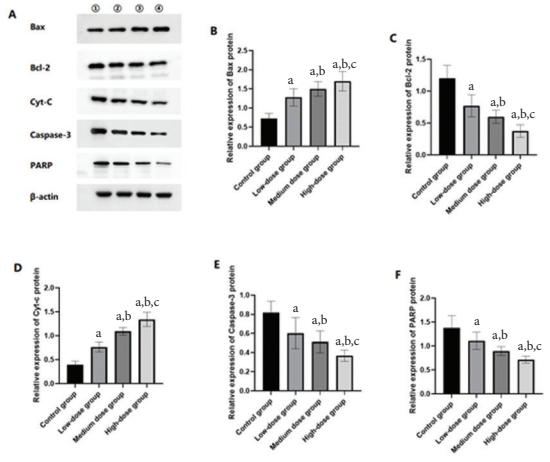
[a]Compared with the Control category, $P < .05$.
[b]Compared with the Low-dose category, $P < .05$
[c]Compared with the Medium-dose category, $P < .05$

Figure 4. Luteolin impacts the number of glioma cells that are being invaded. A: Effects of Luteolins on the number of invasive cells in human glioma cells. 1. Control category. 2. Low-dose category. 3. Medium-dose category. 4. High-dose category. B: Histogram of the effect of luteolin on the migration cell number of human glioma cells.



[a]Compared with the Control category, $P < .05$.
[b]Compared with the Low-dose category, $P < .05$.
[c]Compared with the Medium-dose category, $P < .05$.

Figure 5. Luteolin activates the apoptosis pathway of glioma cells. A: Apoptosis pathway protein expression band. 1. Control category. 2. Low-dose category 3. Medium-dose category. 4. High-dose category. B: Relative expression of Bax protein. C: Relative expression of Bcl-2 protein. D: Relative expression of Cyt-c protein. E: Relative expression of Caspase-3 protein. F: Relative expression of PARP protein.



[a]Compared with the control category, $P < .05$.
[b]Compared with the low-dose category, $P < .05$.
[c]Compared with the medium-dose category, $P < .05$.

substances, which results in severe brain damage and complications such as brain edema and epilepsy.[14-15] Patients often have a high recurrence rate after surgery and poor clinical prognosis due to the strong invasiveness of glioma cells.

As a representative component of natural flavonoids, luteolin exhibits significant antitumor effects through a variety of mechanisms by inhibiting the growth of different tumor cell lines.[17-18] The impact of luteolin on cell proliferation and apoptosis, as well as potential mechanisms, were reviewed and evaluated in this study and provided a basis for seeking therapeutic drugs for human glioma. The results demonstrated that the intervention with different doses of luteolin, compared to the control group, inhibited cell proliferation, and the inhibition rate increased significantly with a triose of dose and intervention time. This is consistent with the previous reports,[19-20] which suggested that luteolin can notably inhibit the proliferation of glioma cells and further supplement the anti-tumor effect of luteolin. The observation under the microscope showed that the growth state of U87 cells after adding luteolin was very poor, with obvious apoptotic morphology and cell shrinkage.

Apoptosis is a complex biological process with a series of cascade-activated active cell death processes that occur after various death signal stimulation.[21] Since the occurrence of a variety of diseases (such as a tumor) is directly or indirectly related to the disorder of the apoptosis process, induction of tumor cells apoptosis is of great significance in anti-tumor effects.[22] In this study, the apoptosis rate of experiment groups was found to be considerably higher than that of the control group after 2 days of luteolin intervention, and it appeared that the apoptosis rate increased with increasing intervention dose. This referred that luteolin notably promoted glioma cell apoptosis, which may be one of the anti-tumor mechanisms of luteolin. In addition, cell proliferation, migration, and invasion are the main characteristics in the process of tumor occurrence and development. Human glioma is characterized by high invasiveness. The study found that the number of migratory cells and invasive cells in the low-dose, medium-dose, and high-dose groups was considerably higher than in the control group after a 48-hour luteolin intervention with the uplifting of luteolin intervention dose. There is evidence that luteolin can successfully prevent glioma cells from migrating and invading other tissues, which has positive implications for clinical treatment and improving the prognosis of patients.

Mitochondria exert a crucial role in regulating cell apoptosis by increasing permeability and decreasing membrane potential. Pro-apoptotic factors such as Cyt-c and Bax were released from mitochondria into the cytosol, which then triggers the Caspase cascade and ultimately leads to apoptosis. Therefore, the reduction of mitochondrial membrane potential is generally regarded as an early hallmark event of apoptosis.[23-25] The Caspase family of proteins, the Bcl-2 family of proteins, and cytochrome C are only a few of the proteins that control mitochondria-dependent apoptosis. Caspase-3 is at the core of the Caspase family cascade

reaction. As an executive protein of apoptosis, the activation of Caspase-3 can expand the Caspase cascade reaction, activate PAPR protein, and cause cell DNA fragmentation, cytoplasm concentration, and cell apoptosis. Bcl-2 and Bax are the two most vital members in the Bcl-2 family, which is primarily distributed on mitochondria in the cytoplasm, regulating apoptosis by forming homodimers.[26-27] Cyt-c is an indispensable factor in cell apoptosis and plays a pivotal signal amplification role in cell apoptosis.[28-29] In this study, the results showed that the protein expressions of Bax and Cyt-c were dramatically reduced after 48 hours of luteolin intervention compared to the control group and that the expression of Bax and Cyt-c reduced significantly with increasing doses of luteolin. In comparison with the control group, there was a considerable increase in the protein expression of Bcl-2, Caspase-3, and PARP; this increase became more pronounced with an increase in luteolin dose. As a result, it was assumed that luteolin may trigger the mitochondrial pathway leading to apoptosis in T24 cells.[30–31]

CONCLUSION

The result of this study suggested that luteolin has an apparent inhibiting function on the proliferation of glioma cells by inhibiting cellular invasion and migration and promoting the apoptosis of glioma cells and the expression of apoptosis-related proteins, which laid the foundation for its becoming one of the therapeutic agents in glioma.

DATA AVAILABILITY

The data used to support this study is available from the corresponding author upon request.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING

No funding was received for this study.

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