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Research Article
Effects of Ferulic Acid Regulation of the PI3k/Akt Signaling Pathway on the Proliferation, Migration or Apoptosis of U87-MG Cells Based on Bioinformatics

Peng Jin, Zhenfei Zhao, Xue Li, Qingbu Mei, Dan Liu, Muqi Liu and Kun Zhao

Qiqihar Medical University, Jianhua, Qiqihar, Heilongjiang 161006, China

Abstract

Background and Objective: Glioma is a common brain tumour. Most deadly brain tumours are malignant. Traditional glioma treatment includes surgical resection, radiation, chemotherapy and biological therapy. Through a series of *in vitro* research studies, this study was intended to assess the effects of ferulic acid (FA) on the proliferation and tumour features of U87-MG cells. **Materials and Methods:** The U87-MG cells were employed in this study. Blank and ferulic acid groups with concentrations of 1.25, 2.50 and 5.00 mmol/L were created. The FA's effect on U87-MG cell apoptosis was examined by AM/PI staining after cell prolifer activity. The ELISA, cell migration, transwell invasion test, intracellular reactive oxygen species, cell apoptosis rate and western blot were assessed. **Results:** The application of FA decreased the proliferation of U87-MG cells, according to the results of the cell counting kit 8 test. Flow cytometry analysis also showed that FA sped up the death of cells and greatly increased the amount of reactive oxygen species inside cells. The phenotype of FA promoting apoptosis in U87-MG cells was consistent with the results of western blot analysis, which demonstrated that ferulic treatment with acid elevated the expression of apoptotic proteins (caspase-3 and caspase-9) and decreased the expression of cell cycle-related proteins (cyclin) in U87-MG cells. The FA suppressed U87-MG cell growth by blocking the PI3K/Akt signalling pathway, according to further detection of this pathway's activity. **Conclusion:** The FA treatment boosted autophagy in U87-MG cells by increasing autophagy protein expression. The clinical treatment of gliomas changed with this discovery.

Key words: Ferulic acid, U87-MG, PI3K/Akt, apoptosis, proliferation

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Corresponding Author: Kun Zhao, Qiqihar Medical University, Jianhua, Qiqihar, Heilongjiang 161006, China

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Neuroectoderm, a frequent malignant nervous system tumour, is where gliomas typically form. They account for about 45% of intracranial tumors[1]. The efficacy of standard treatment, which combines surgical intervention with chemotherapy and radiation, has a negligible impact on the survival rates of glioma patients[2]. However, surgery cannot completely remove the cancerous tissue due to the invasive growth characteristics of gliomas and postoperative recurrence is extremely easy with a high mortality rate[3]. Therefore, new treatment methods for gliomas urgently need to be discovered[4]. Progress in tumour immunology, genetics, cell-signaling pathways and cancer stem cells has significantly improved our understanding of gliomagenesis[5]. Hence, the development of novel anti-glioma medications has significant therapeutic use[6].

Ferulic acid (FA) is a phenolic compound that is often found in plants such as *Chuanxiong*, *Leonurus* and *Angelica sinensis*[5]. It serves as a significant active ingredient in several traditional Chinese remedies. It has both antioxidant and anti-inflammatory properties. It can eliminate excessive reactive oxygen species (ROS) or directly eliminate free radicals and enzymes that generate free radicals. This helps to prevent oxidative damage and decrease inflammatory responses[7,8]. Due to its distinct pharmacological effects, stability, minimal toxicity and abundant natural occurrence, FA and its derivatives have garnered significant interest. The FA and sodium ferulate have recently shown significant anti-cancer properties[9,10] but the effect of FA on brain glioma remains unclear[7].

This investigation concluded that FA also had an anticancer impact on gliomas, which led to the hypothesis. To provide evidence in favour of the concept, this research studied the effects of FA on the invasion, apoptosis and proliferation of U87-MG glioma cells. Additionally, it investigated the potential molecular mechanisms of action for FA in order to contribute to the existing body of information about its anticancer activity and to provide novel ideas for the development of therapeutic medications to treat acute gliomas.

MATERIALS AND METHODS

Study area: The present study was carried out at the Qiqihar-Medical-University, from March-June, 2023.

Cell experiments: In a cell culture incubator set up at 37°C with 5% CO_2, U87-MG cells were regularly cultivated in minimal essential medium (MEM) (Servicebio, Wuhan, China) supplemented with 10% foetal bovine serum (Gibco, Australia). The cells were digested using 0.25% trypsin-EDTA (G4004-100ML; Wuhan, Servicebio) solution, neutralised with MEM and passaged (1:3) once more in a cell culture incubator at 37°C with 5% CO_2 once the cell fusion degree reached 90%. For the experiment, well-maintained logarithmic growth phase cells were isolated and blocked with MEM containing 0.1% foetal bovine serum for a whole day.

Experimental grouping and administration: After being collected from U87-MG cells in the logarithmic growth phase, 2 groups were created: One for FA (0 to 16 mmol/L) and the other for blank control. The MEM was applied to the cells in the blank group without the addition of foetal bovine serum. In 0.1% foetal bovine serum, MEM containing 1.25, 2.50 and 5.00 mmol/L FA was used to treat the cells in the FA group.

Assay of cell proliferation: As 2000 transfected cells per well of 96-well plates were used for culture. To stop the growth, 10 µL of CCK-8 dyes were added to each well and the mixture was incubated for an additional 4 hrs at 37°C. To measure the absorbance at 450 nm, an enzyme-linked analyzer (Thermo Fisher Scientific, USA) was used.

EdU assay: The BeyoClickTM EdU-647 Cell Proliferation Assay Kit (Beyotime, Beijing, China) should be used according to the instructions. The 2 hrs before the cells were collected, an EdU working solution was added. Following labelling, the cells were fixed by the removal of the culture solution, followed by a wash, the addition of permeabilizing solution and a microscope view of the counts.

Application of calcein AM/PI staining to identify the impact of FA on U87-MG cell apoptosis: The cell precipitate obtained by centrifugation was resuspended in 1×assay buffer (Solarbio, Beijing, China) and the cell count of the resuspended cell suspension was set to 1×10^6 cells/mL. Subsequently, 1 µL of calcein AM (stock solution) was added for every 1 mL of cell volume, blown, thoroughly mixed and allowed to incubate for 20-25 min at 37°C in the dark. In addition, 3 µL of the initial PI solution was extracted from the reagent kit and introduced into the previously described labeled cells. For 5 min, the cells were dyed at room temperature in the dark. After 5 min of 450 g incubation, the fluorescent cells were centrifuged to get rid of the staining solution. Under a fluorescent microscope, (Smart-FL2LED, Chongqing Optec Instrument Co., Ltd., Chongqing, China) living cells (green fluorescence) and dead cells (red fluorescence) were simultaneously detected using an excitation filter at 490±10 nm.

Transwell invasion assay: Transwell chambers (8 µm pore size; Millipore) filled with Matrigel (BD Biosciences, USA) were used to detect cell invasion. To put it briefly, the bottom chamber received 600 µL of the whole medium. As 200 µL of the transfected cells' suspension (4×10^4 cells) was added to the upper chamber after the cells were suspended in serum-free media. The cells on the membrane's top surface were scraped off with a cotton swab after 24 hrs and the cells on the membrane's bottom surface were preserved in 95% ethanol and stained with a 4 g/L crystal violet solution. At 200X magnification, the number of cells that adhered to the membrane's bottom surface was counted in three randomly chosen fields.

Detection of cell migration ability: A six-well plate was infected with U87-MG cells that were in the logarithmic growth phase. In the cell monolayer, a "wound" developed. The cells were cultivated in a cell culture incubator at 37°C with 5% CO_2 after being grouped and treated. To compare cell migration rates, wound photos were taken at the start of the experiment along with at regular intervals (0 and 24 hrs). Additionally, each U87-MG cell's scratch healing rate was calculated.

ELISA detection of the changes in cytokine levels: After the cells were sorted the cell supernatant was gathered to perform further cytokine detection. Using commercial kits (Proteintech, Wuhan, China) and following the directions provided by the manufacturer, the ELISA technique was used to measure the quantities of IL-4, IL-6, IL-10 and TNF-α in cell supernatant.

Cell apoptosis rate detection: The dual-labeling flow cytometry apoptosis test for Annexin V–FITC/PI (4A BIOTECH, Beijing, China) was utilised to determine the apoptosis rate of U87-MG cells throughout their logarithmic growth phase. Three wells were included in each batch of cells, which were grouped and given treatment. The Annexin V-FITC/PI apoptosis detection kit's instructions were then followed to assess the cell apoptosis rate using a flow cytometer.

Determination of the amounts of intracellular reactive oxygen species: The DCFH-DA technique was utilised for detection. The intracellular reactive oxygen species (ROS) level was determined by analysing the fluorescence intensity in each set of cells using ImageJ Version 1.8.0 software. The higher the fluorescence intensity was considered to a higher the ROS level in the cell.

Mitochondrial membrane potential detection: Following the directions provided by the JC-1 staining solution (Beyotime, Jiangsu, China), cells were collected and processed. After being resuspended, the cells were stained using the JC-1 staining solution. Following washing, the cells were once again suspended in the appropriate volume of JC-1 staining solution (1x) and then a flow cytometer (FC2040, Perlong Medical Equipment Co., Ltd., Nanjing, China) was used to analyse them.

Western blot analysis: Following a 10% SDS-PAGE separation, equal volumes of protein were transferred to PVDF (Polyvinylidene Fluoride) membranes (Millipore, Bedford, Massachusetts, USA). Incubation with 3% non-fat milk in tris-buffered saline with Tween-20 (TBST) at 4°C for 1 hr prevented non-specific protein interactions. The membranes were then exposed to antibodies against the target proteins for a whole night. Anti-cyclin (ab16663), anti-Bcl-xL (ab32370), anti-caspase-3 (ab4051), anti-caspase-9 (ab32539), anti-Akt (ab8805), anti-p-Akt (ab81283), anti-PI3K (ab182651), anti-p-PI3K (ab302958), Atg (ab109490), Beclin 1 (ab207612), P62 (ab109012) and LC3/(ab192890) were the antibodies utilised in the experiment. After that, secondary antibodies were incubated on the membranes. To develop images, ECL substrate will be applied once the incubation is finished. The endogenous control employed was GAPDH. All of the antibodies were bought from Abcam in the UK.

Statistical analysis: Using SPSS 20.0 software, all data underwent statistical analysis, with measurement data presented as Mean±Standard Deviation. While One-way Analysis of Variance (ANOVA) and least significant variation tests were utilised for comparisons between many groups, t-tests were employed for comparisons between 2 groups. Indicating that the difference was statistically significant was a $p<0.05$.

RESULTS

FA prevents U87-MG cells from proliferating: As U87-MG cells were treated with several dosages of FA (0, 0.98, 1.95, 3.91, 7.81 and 15.625 mM) for 24 hrs (Fig. 1). The FA was shown to have a potent inhibitory impact on U87-MG cells, with an IC_{50} of 4.706 mM (Fig. 1a).

Subsequently, different concentrations of FA (1.25, 2.50 and 5.00 mM) were selected to act on U87-MG cells for 24, 48, 72 and 96 hrs while setting up a blank control group. The OD values were measured and the corresponding cell proliferation

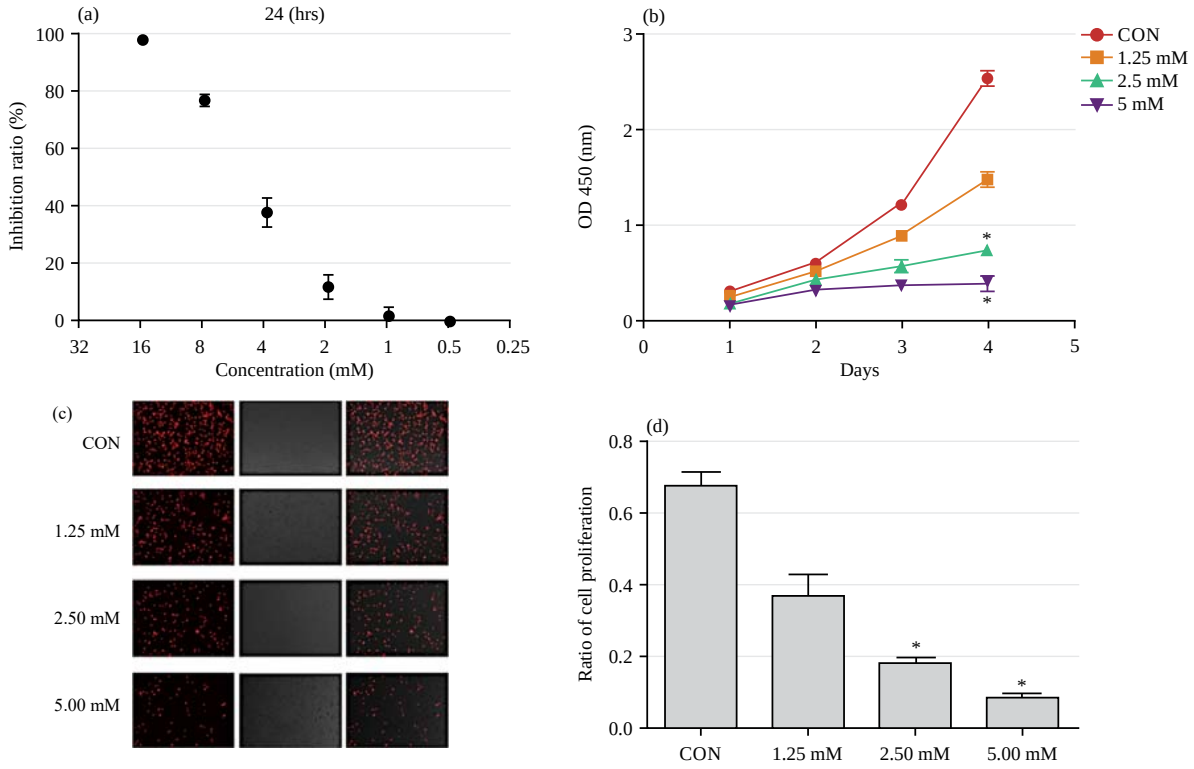

Fig. 1(a-d): Ferulic acid prevents U87-MG cells from proliferating, (a) Cell inhibition experiment to screen drug concentration, (b) Cell proliferation curve, (c) Proliferative cell staining and (d) Bar chart of the proportion of proliferating cells
*p<0.05, correlated with the blank control category

curves were plotted Fig. 1b. The findings demonstrated a dose-dependent, as well as time-dependent inhibitory effect of FA on U87-MG cells, as the cell proliferation rate decreased with an increase in FA concentration and the action time of FA, was prolonged (Fig. 1c-d).

FA induces U87-MG cell apoptosis: The detection of intracellular reactive oxygen species (ROS), apoptosis and alterations in the mitochondrial membrane potential (JC-1) was done using flow cytometry. The FA-treated cohort exhibited a substantial increase in intracellular reactive oxygen species (ROS) ($p<0.05$) when compared to the control cohort (Fig. 2a). Similarly, the group treated with 5 mM FA showed a substantial distinction (Fig. 2b). The apoptosis assay findings demonstrated that, in comparison to the control category, the apoptosis rates of cells in the 1.25, 2.50 and 5.00 mM categories were considerably higher ($p<0.05$), reaching 16.23, 30.02 and 35.06%, correspondingly (Fig. 2c-d). The FA's inhibitory impact on U87-MG cells' apoptosis had a dose-dependent effect and the trend of the data from the mitochondrial membrane potential assay was comparable to that of the ROS and apoptosis assays (Fig. 2e-f). The technique for cell live-dead double labelling also demonstrated a noteworthy rise in dead cells in the 2.50 and 5.00 mM categories relative to the control category. As previously shown, FA has a pro-apoptotic impact. The expression levels of caspase-3, cyclin, caspase-9 and Bcl-xL proteins in the cells were further investigated using the western blot test. It was shown that FA dramatically down-regulated the expression of Bcl-xL and Cyclin proteins, but greatly increased the levels of caspase-3 and caspase-9 protein expression (Fig. 2i-j).

FA inhibits U87-MG cell's epithelial-mesenchymal transition (EMT): The impact of various concentrations of FA on the ability of U87-MG cells to migrate and invade was investigated via the use of the Transwell assay and the Cell Scratch test (Fig. 3a). The metastatic potential of U87-MG cells was significantly reduced by various doses of FA and all 3 groups that received FA treatment had significant changes in cell count (Fig. 3b). The highest concentrations (2.50 mM) of FA were expressed in low cell counts (27). Figure 3c shows that the migrating distance of cells in the FA drug-treated groups was substantially reduced, according to the results of the cell scratch experiment, which showed a considerable reduction

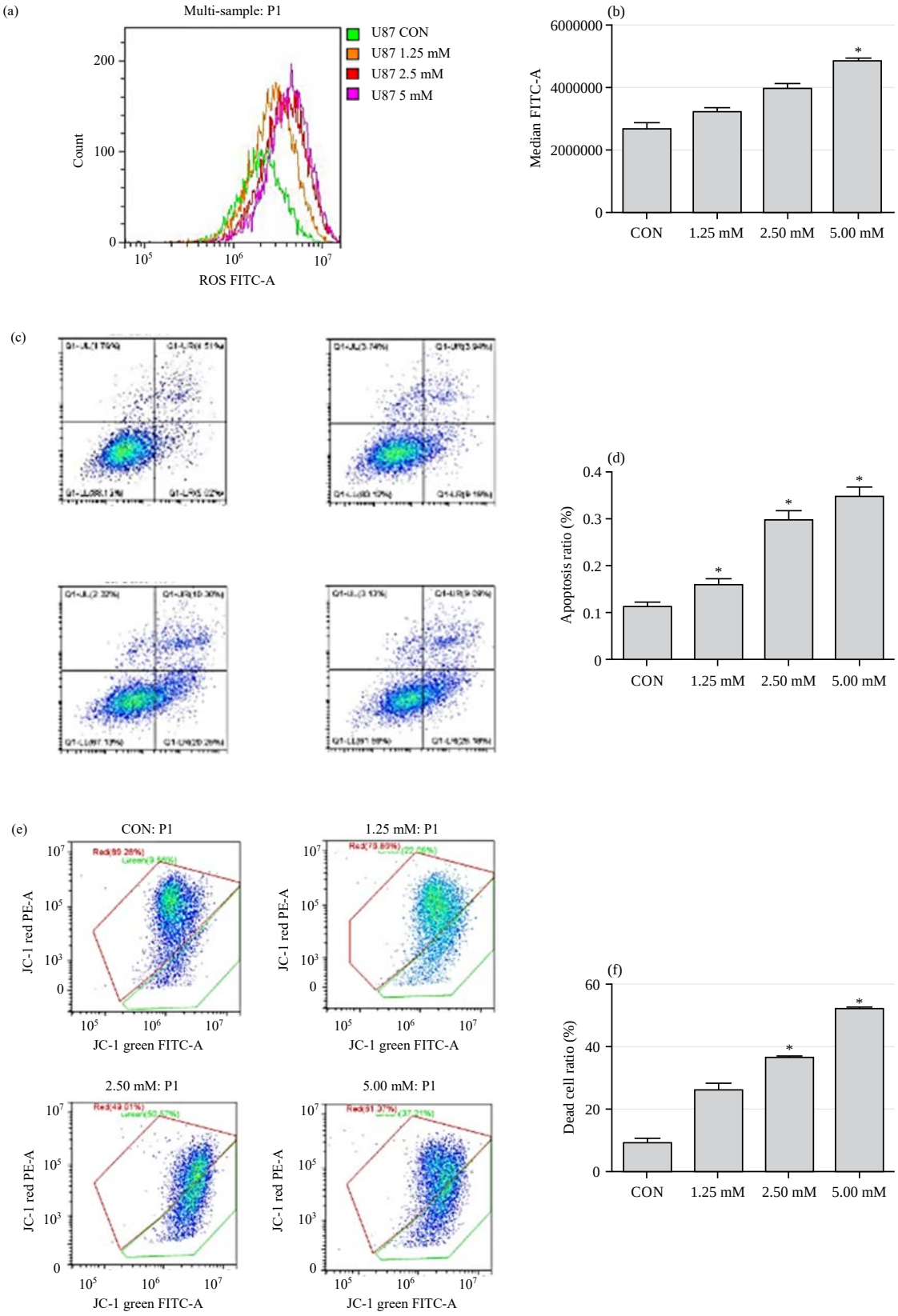

Fig. 2(a-j): Continue

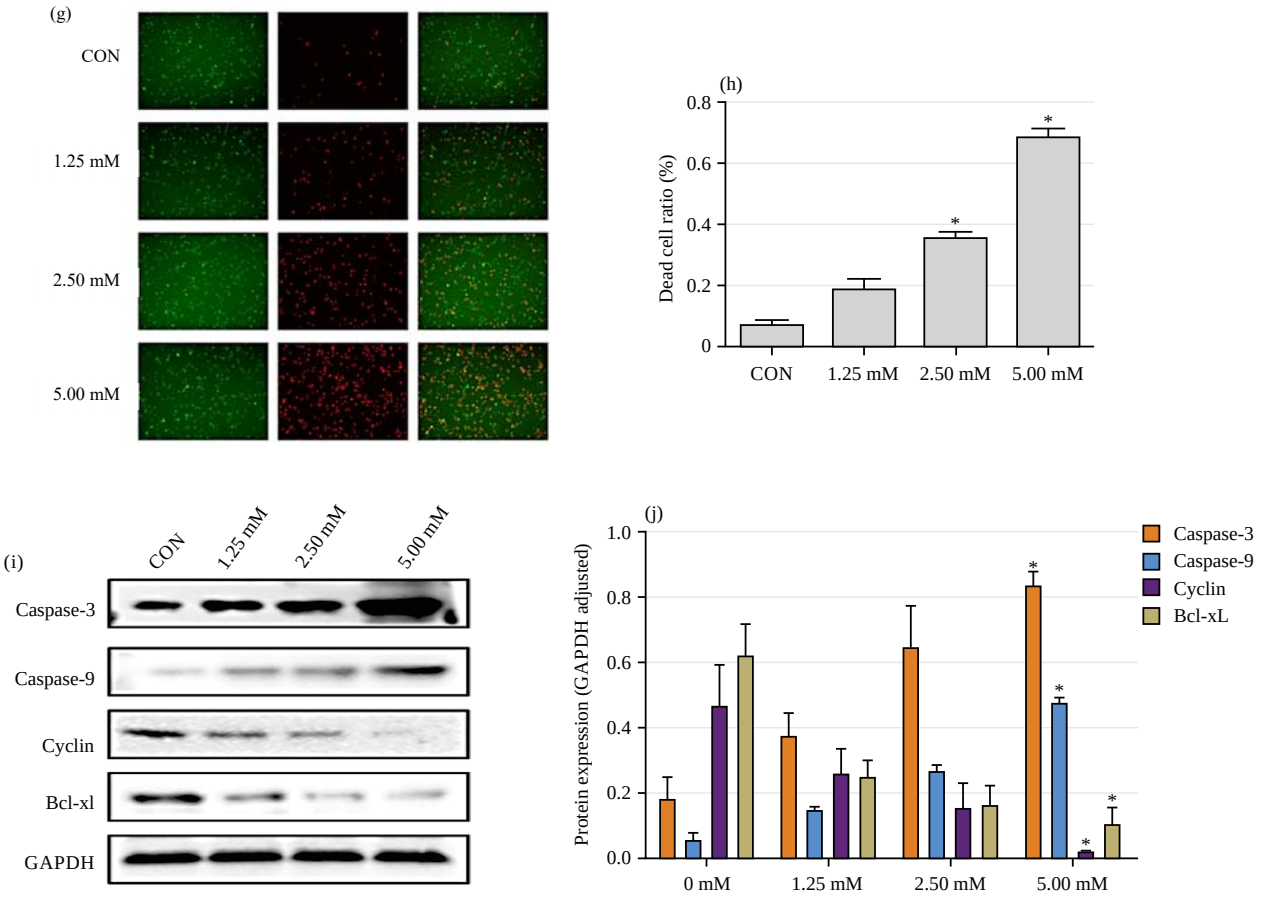

Fig. 2(a-j): Ferulic acid's influence on U87-MG cell proliferation, (a) Reactive oxygen species identification contour map, (b) Bar graph showing the U87-MG cells' measured level of reactive oxygen species upon ferulic acid therapy, (c-d) Flow cytometry detection of ferulic acid's impact on U87-MG cells' programmed cell death, (e-f) Scatter plot of flow, (g) Cell staining, both dead and alive, (h) Bar graph of dead cell percentage, (i) Band formation in Western blot electrophoresis and (j) Western blot detection strip grayscale value quantification using a bar chart
When compared to the blank control group, *p<0.05

in migration capacity. The migration distribution of the quantitative level of U87-MG after FA management is expressed in Fig. 3d. It showed that the highest concentrations (2.5 mM) of FA were expressed in the highest migration distribution of the U87-MG cells.

FA regulates intracellular cytokine levels in U87-MG cells: In this study, an ELISA test was used to find out how much TNF-α, IL-4, IL-6 and IL-10 were being expressed in U87-MG cells so that we could learn more about how FA affected these chemicals. It was discovered that the FA-treated group could greatly raise the expression concentrations of IL-4 and IL-10 while dramatically reducing the expression levels of TNF-α as well as IL-6 when compared to the control category (Fig. 4a-d).

FA regulated proliferation and apoptosis of U87-MG cells via the PI3K/Akt signaling cascade: The PI3K/Akt signalling pathway was examined using a Western blot test to determine whether or not it was activated. The results showed that FA dramatically reduced the expression levels of p-PI3K and p-Akt proteins, but not PI3K or Akt (Fig. 5a-b). According to this, treating U87-MG cells with FA can considerably reduce the activation of the PI3K/Akt signalling pathway, which will prevent the U87-MG cells from proliferating.

Effect of FA on autophagy in U87-MG cells detection: There are two roles for autophagy in the development, growth and treatment of tumours. By causing cell death or reducing the number of cells, autophagy can function as a tumour suppressor mechanism and stop the growth of tumours.

Using Western blot analysis, it was able to determine the expression levels of Atg, Beclin-1, P62 and LC3 to investigate whether autophagy takes place following FA treatment of the tumour cells U87-MG, which was discovered to have an inhibitory proliferation-promoting apoptosis effect. The FA was shown to dramatically up-regulate the expression of Atg, Beclin-1 and LC3 II while down-regulating the expression of P62 and LC3 I. This finding supported the advancement of autophagy in U87-MG (Fig. 6a-b).

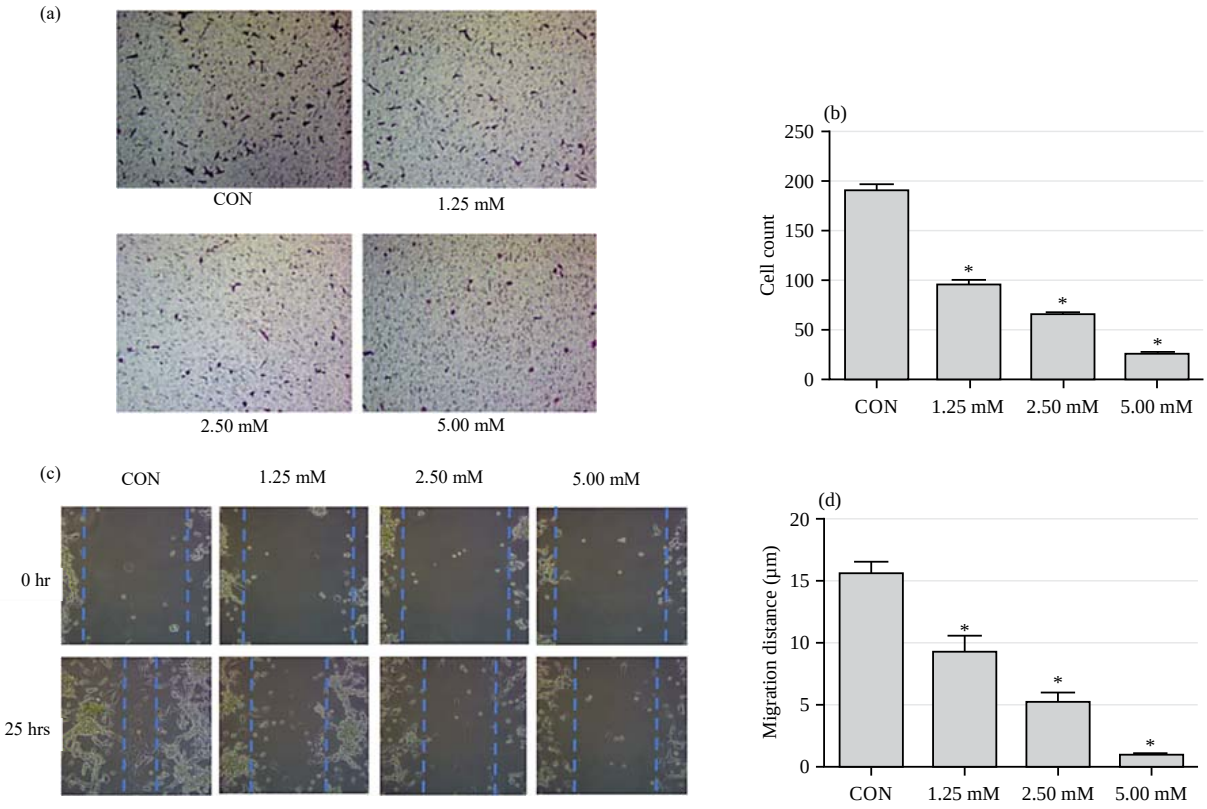

Fig. 3(a-d): Impact of various concentrations of FA on U87-MG cell migration and invasion, (a) Transwell detection of the invasion ability of U87-MG cells, (b) Bar chart of the cell count of the U87-MG cells at tested concentrations, (c) Ferulic acid inhibited U87-MG cell migration at tested concentrations and (d) Bar chart representing the migration distribution of the quantitative level of U87-MG cells after ferulic acid management

*p<0.05 correlated with the blank control category

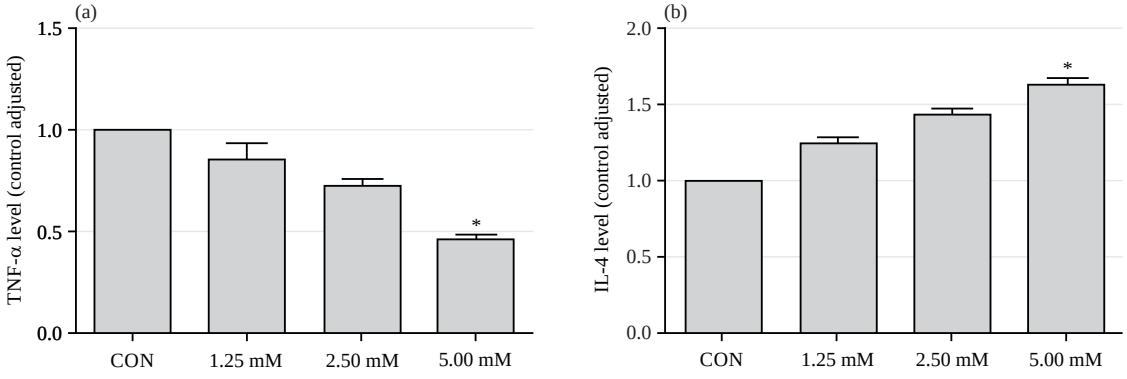

Fig. 4(a-d): Continue

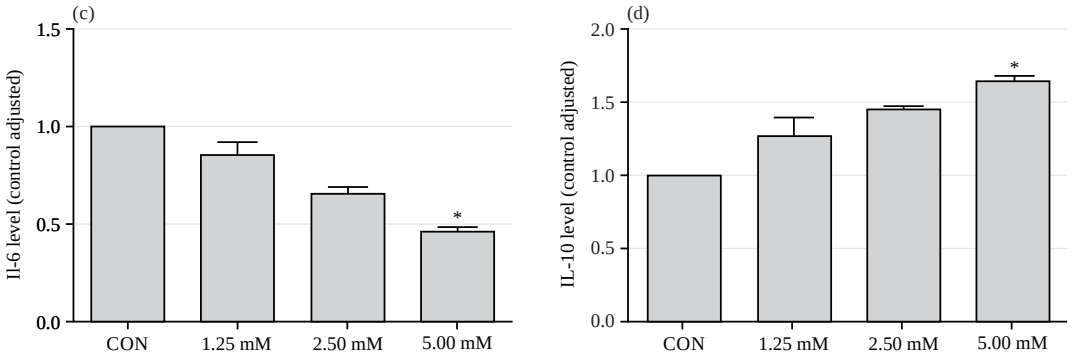

Fig. 4(a-d): ELISA detection of cytokine levels correlated with the blank control category, (a) TNF-α detection, (b) IL-4 detection, (c) IL-6 detection and (d) IL-10 detection

*p<0.05, correlated with the blank control category

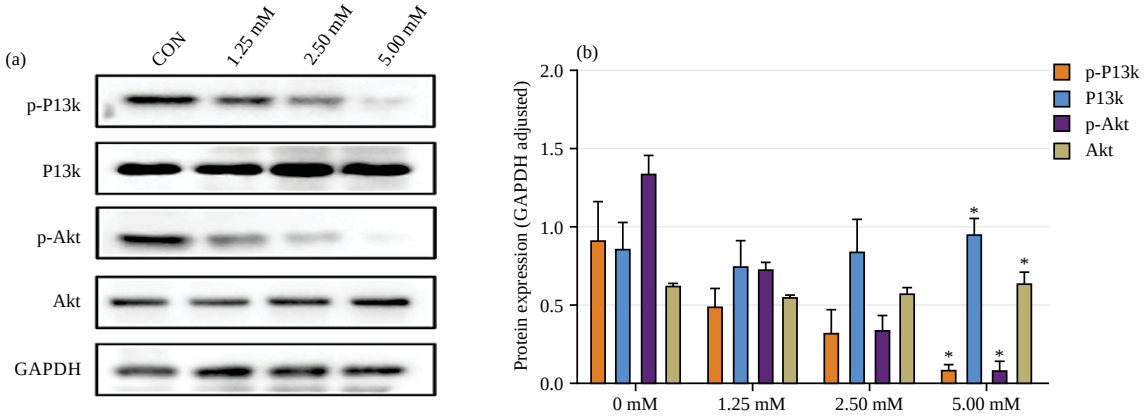

Fig. 5(a-b): Protein expression of Akt, p-Akt, PI3K and p-PI3K on various concentrations, (a) Western blot was used to measure Akt, p-Akt, PI3K and p-PI3K protein levels and (b) Bar chart of protein expressions of the levels of Akt, p-Akt, PI3K and p-PI3K

*p<0.05, relative to the blank control population

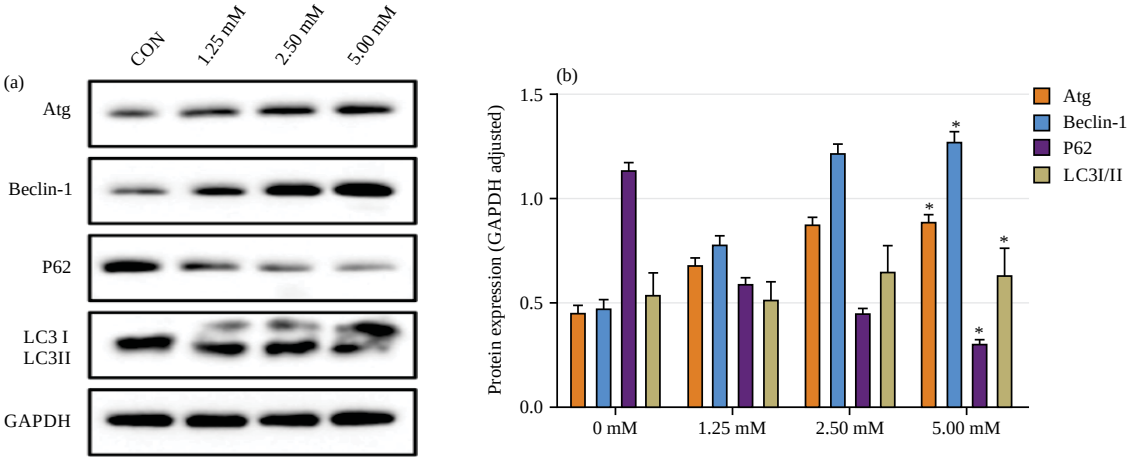

Fig. 6(a-b): Protein expression levels of Atg, Beclin-1, P62 and LC3. Effect of FA on autophagy in U87-MG cells, (a) Western blot assessment to measure the protein expression levels of Atg, Beclin-1, P62 and LC3 I/LC3 II and (b) Bar chart of protein expressions of the levels of Atg, Beclin-1, P62 and LC3 I/LC3 II

*p<0.05, relative to the blank control population

DISCUSSION

The effects of FA on glioma cells were examined by treating U87-MG cells for 24, 36, 48 and 72 hrs, respectively, with 0, 1.25, 2.50 and 5.00 mM FA. It was investigated if U87-MG cells could survive. The findings demonstrated that FA considerably reduced glioma cell survival in a concentration- and time-dependent manner, with 5.00 mM FA having the greatest inhibitory effect on U87-MG cell survival.

Gliomas form in a very complicated way. It is intimately associated with both the rise in cell activity and the breakdown of cell cycle control. Cyclins bind to associated kinases to control the cell cycle. Usually, tumour medications fix damaged DNA by stopping the tumour cell cycle in the G1/S or G2/M stages. In this work, U87-MG cells were exposed to 0, 1.25, 2.50 and 5.00 mM FA for a whole day. Using flow cytometry, the cell cycle distribution was identified. The findings demonstrated that following FA administration, the percentage of cells undergoing apoptosis rose dramatically, the amount of intracellular ROS increased considerably the mitochondrial membrane potential dropped. The Bcl-xL is a member of the Bcl-2 family and is essential for cell death. The cell apoptosis can be inhibited by Bcl-2. A vital part of cell apoptosis is played by the caspase family. Caspase-3 is the junction point of caspase apoptosis-dependent pathways, situated downstream of the caspase cascade process. Cell death is aided by caspase-3 activation. The therapy of U87-MG cells with 5 mM FA for 24 hrs greatly accelerated apoptosis, according to the results of flow cytometry analysis. Western blot examination revealed that following FA treatment, the expression of caspase-3 and caspase-9 proteins dramatically increased whereas that of cyclin and Bcl-xL proteins significantly decreased. This demonstrated that FA-induced U87-MG cell death via controlling the expression of proteins linked to apoptosis. The impact of FA therapy on the expression of PI3K, p-PI3K, Akt and p-Akt proteins was examined in this work using Western blot. The findings demonstrated that FA administration dramatically decreased the expression levels of p-PI3K and p-Akt, but did not significantly alter the expression levels of PI3K or Akt. This suggests that FA inhibits the PI3K/Akt signalling pathway. Treatment with FA markedly reduced U87-MG cell growth, halted cell cycle progression and increased cell apoptosis.

A primary tumour that frequently appears in the human brain is called glioma. It is the brain tumour that is most likely to be malignant and to be fatal[11]. The traditional treatment for gliomas is mainly surgical resection, supplemented by various treatment methods such as radiotherapy, chemotherapy and biological therapy[12]. Surgery to completely remove cancerous tissue is difficult due to the extremely high malignancy of gliomas, which exhibit invasive growth in the intracranial area and have unclear boundaries with normal brain tissue[13]. Therefore, the recurrence rate of postoperative gliomas is extremely high.

Modern pharmacological research has shown that natural drugs have enormous potential value in the development of anticancer drugs[14]. One phenolic acid that is frequently found in nature is FA, which has several biological properties including anti-inflammatory, anti-cancer and antioxidant properties[15]. Recent studies have shown that FA is a multi-target natural antitumor drug with stable properties and low toxicity. The FA was shown to be beneficial in promoting autophagy in esophageal cancer cells, inhibiting cell proliferation and inducing cell cycle arrest in the G0/G1 phase, as demonstrated by Guzmán-López *et al.*[16]. According to Yue *et al.*[9], FA derivatives controlled several signalling pathways, prevented human lung cancer A549 cells from proliferating and migrating and offered a significant body of knowledge for the development of FA-derived anticancer medications.

Widely distributed throughout cells, the PI3K/Akt signalling system regulates the growth, differentiation, apoptosis and advancement of the cell cycle[17]. Studies verify that the PI3K/Akt pathway is activated in a way that stimulates the growth of cancer cells while preventing them from dying[18]. It investigated how the PI3K/Akt pathway controls cell apoptosis. demonstrating that the Bcl family member Bad was phosphorylated by activated Akt, which prevented the formation of the Bad/Bcl-2 complex and therefore directly controlled cell death[19]. Moreover, it phosphorylated NF-κB to encourage NF-κB transcription, which in turn enhanced Bcl-xL expression and cell viability[20]. Moreover, Akt contributed to the advancement of the cell cycle by phosphorylating mTOR[21]. The mechanism of action may be associated with the suppression of the PI3K/Akt signalling pathway, offering novel research opportunities for the application of FA in the treatment of gliomas[7].

Maintaining cellular homeostasis and adapting to environmental changes depend heavily on autophagy, a self-degradation and recycling function of cells[22]. It has several functions in the genesis, growth and management of tumours[23]. In some circumstances, it may even act as a mechanism to encourage the survival and growth of tumour cells[24]. Inhibiting tumour growth in its early stages may be facilitated by autophagy. Eliminating possible carcinogens and damaged organelles aids in limiting the development and multiplication of tumour cells[25]. In addition, autophagy can also induce apoptosis or necrosis of tumor cells, thereby directly inhibiting the development of tumors[26]. Using protein immunoblotting to assess the expression levels of autophagy-

related proteins, this study discovered that FA therapy increased autophagy in U87-MG cells. This finding offered a fresh approach to the clinical management of gliomas.

CONCLUSION

This investigation used protein immunoblotting to assess the expression levels of autophagy-related proteins. The findings revealed that FA treatment induced upregulation of autophagy in U87-MG cells by enhancing the expression of autophagy proteins. This finding brought about a significant shift in the clinical management of gliomas. This discovery presented a novel strategy for the medical treatment of gliomas.

SIGNIFICANCE STATEMENT

Most of the lethal brain tumours are malignant. Surgical removal, radiation, chemotherapy and biological therapy are traditional glioma treatments. *In vitro* investigations will determine how ferulic acid affects U87-MG cell proliferation and tumour growth. Ferulic acid inhibited U87-MG cell growth, according to the cell counting kit 8 test. Flow cytometry demonstrated that ferulic acid accelerated cell death and substantially elevated reactive oxygen species. Western blot analysis showed that ferulic acid increased the expression of apoptotic proteins (caspase-3 and caspase-9) and decreased the expression of cell cycle-related proteins (cyclin) in U87-MG cells. Further analysis showed that ferulic acid blocked the PI3K/Akt signalling pathway to inhibit U87-MG cell proliferation.

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