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Ferulic Acid Increases Temozolomide Sensitivity in Glioblastoma Cells, Causing DNA Damage and Inhibiting Cell Proliferation

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ABSTRACT

AIM: To investigate the antitumor effects of ferulic acid (FA) on glioblastoma multiforme (GBM) cells both alone and in combination with temozolomide (TMZ), and also to determine the potential of this synergy for treatment processes.

MATERIAL and METHODS: Human glioblastoma U87-MG cells were used in this study. To evaluate the potential constructive interaction between temozolomide (TMZ) and ferulic acid (FA), a sequential treatment protocol was applied in which cells were first treated with TMZ (20, 40, and 80 μM) for 48 hours, followed by FA (1000 μM and 1500 μM) for an additional 24 hours. Cell viability was assessed using the MTS assay, clonogenic capacity was evaluated by the clonogenic assay, and nuclear morphological changes were examined by Hoechst 33258 staining. The expression levels of Cyclin D1 and PARP were also analyzed to explore the molecular mechanisms underlying the treatment effects.

RESULTS: FA treatment reduced cell viability and increased DNA damage in U87-MG cells. It suppressed the expression of Cyclin D1 and PARP. Furthermore, the combination of FA and TMZ almost completely inhibited cell proliferation and colony formation and significantly increased DNA damage.

CONCLUSION: Although FA has demonstrated antitumor activity at high concentrations, this may limit its clinical applicability. However, its ability to enhance the effects of TMZ suggests that FA could be used as a supportive treatment strategy in GBM therapy.

KEYWORDS: Glioblastoma Multiforme, Temozolomide, Ferulic Acid, DNA Damage, U87-MG

ABBREVIATIONS: GBM: Glioblastoma Multiforme, **TMZ:** Temozolomide, **FA:** Ferulic Acid, **PARP:** Poly (ADP-ribose) polymerase, **BBB:** Blood-Brain Barrier, **MGMT:** O6-Methylguanine-DNA Methyltransferase, **BER:** Base Excision Repair, **PI3K/AKT:** Phosphoinositide 3-kinase/Protein kinase B, **TG2:** Transglutaminase 2, **EMT:** Epithelial-Mesenchymal Transition, **(DMEM)/F12:** Dulbecco's Modified Eagle's Medium, **DMSO:** Dimethyl Sulfoxide, **MTS:** 3-(4,5 dimethylthiazol-2-yl) 5-(3-carboxymethoxyphenyl)-2-(4 sulphophenyl)-2H-tetrazolium), **PBS:** Phosphate Buffered Saline, **SDS:** Sodium Dodecyl Sulphate, **TBS-T:** Tris-Buffered Saline-Tween 20, **DNA:** Deoxyribonucleic Acid

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INTRODUCTION

Glioblastoma Multiforme (GBM) is the second leading cause of cancer-related deaths worldwide (8). GBM originating from glial cells is the most common and aggressive type of human brain tumor and accounts for 81% of malignant brain tumors (9,23). Surgical resection followed by chemotherapy and radiotherapy is currently the standard treatment used in the clinic for GBM (3,16). However, the therapeutic efficacy of standard treatment is low due to the invasive nature of glioblastoma, and 90% of patients experience tumor recurrence within 6-9 months after initial treatment, with a median survival time of approximately 15 months (3).

Temozolomide (TMZ), approved by the Food and Drug Administration (FDA) and widely used in the treatment of GBM, is a DNA alkylating agent that can cross the blood-brain barrier (BBB) (7,20). TMZ shows its cytotoxicity through the formation of DNA helix breaks by transferring methyl groups to the N3 region on adenines and N7, O6 regions on guanines (15,21,29), and then induces cell cycle arrest in G2/M leading to cell apoptosis (15). However, O6-methylguanine methyltransferase (MGMT) and base excision repair (BER)-based repair systems eliminate TMZ-mediated double helix breaks and methylation that reverses cell cycle arrest (10,24,28). This mechanism renders GBM resistant to TMZ and leads to decreased treatment efficacy. Therefore, finding effective therapeutic strategies and alternative compounds for the treatment of GBM is of foremost importance.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) is a hydroxycinnamic acid and an abundant phenolic phytochemical with antioxidant and antitumor activities in vegetables and fruits. It has been detected in plants such as Angelica sinensis, Cimicifuga heracleifolia, and Ligusticum chuangxiong (7). It has been reported that FA can inhibit the expression and activity of many cytotoxic enzymes, including nitric oxide synthase, caspases, and cyclooxygenase (31).

FA has been reported to have a wide range of effects, including anti-inflammatory, antidiabetic, anticarcinogenic, antiapoptotic, hepatoprotective, neuroprotective, radioprotective, pulmonary protective, antiatherogenic, hypotensive, and vasodilatation effects (6). Recently, the high therapeutic potential of FA has attracted considerable interest in terms of research. The therapeutic effects of FA on various cancer types have been demonstrated in numerous studies. In osteosarcoma cells, FA stopped the cell cycle and induced apoptosis by suppressing the PI3K/Akt (Phosphoinositide 3-kinase/ Protein kinase B) pathway (27). In breast cancer cell lines, it showed cytotoxic effect by activating caspase-8 and caspase-9 (5). It was reported that nanoparticle forms of FA increased apoptosis by decreasing tissue transglutaminase 2 (TG2) expression in glioblastoma cells and inhibited proliferation by suppressing DNA synthesis in glioblastoma cells (4). In metastatic breast cancer cells, it has been shown to prevent metastasis by inhibiting epithelial-mesenchymal transition (EMT) (30).

These findings show that the anticarcinogenic effects of FA are multifaceted, and when used together with chemotherapeutic agents, they may create a potential synergistic effect. Considering the limitations in glioblastoma treatment and the prevalence of TMZ resistance, the present study focused on whether ferulic acid can improve the efficacy of TMZ and how it can stop the proliferation of glioblastoma cells. Our results showed that FA alone and in combination with TMZ suppressed cell proliferation and induced DNA damage by suppressing Cyclin D1 and PARP expression. Our data suggest that the combination of these two agents may offer a new and effective way to combat glioblastoma, making a significant contribution to current treatment strategies.

MATERIAL and METHODS

Cell Line, Culture Conditions, and Reagents

U87-MG (cat# HTB-14) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). U87-MG cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO). Cells were cultured at 37°C in an incubator humidified with 5% CO2 (12). Ferulic acid and temozolomide were purchased from Sigma-Aldrich (St. Louis, MO), and stock solution was prepared by dissolving them in 100% Dimethyl Sulfoxide (DMSO). They were then diluted with FBS-free medium before application to cells.

Cell Viability and Replication Experiments

Cell viability and proliferation were measured using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) assay (Promega, Madison, WI) (1,11). To observe the effects of the agents accurately, we spread the experiment protocol over a total period of 96 hours.

To start the experiment, we seeded U87-MG cells at a density of 1.20 x 10^3 cells per well in 96-well plates and designated the first 24 hours as the incubation phase to ensure that the cells adhered completely to the surface. On day 1 of the experiment, we treated the cells with increasing doses of temozolomide (10, 20, 40, and 80 μM) during a 48-hour incubation period. On the third day of the experiment, without removing the existing medium or TMZ from the medium, we proceeded to the final 24-hour incubation phase by directly adding ferulic acid (different doses ranging from 100-1500 μM) or the specified combination groups (FA 1000+TMZ 40 μM, FA 1500+TMZ 40 μM, FA 1000+TMZ 80 μM, FA 1500+TMZ 80 μM) to the final 24-hour incubation phase. After completing the 96-hour protocol, we added a marker solution containing MTS and Phenazine Methosulfate (20:1 v/v) to the cells. We incubated the cells at 37 °C for 1 hour for formazan formation. In the ultimate step, we measured absorbance at 490 nm using an ELISA reader to determine the density of viable and proliferating cells (2,12).

Colony Formation Assays

To determine the long-term effects of temozolomide and ferulic acid on the proliferation capacity and colony-forming ability of GBM cells, we selected the clonogenic assay method (2,12). For this purpose, we seeded U87-MG cells at a density of 1.5 x 10^3 cells per well in 6-well plates and left them to incubate for 48 hours to allow the cells to adhere completely to the surface.

Following this preparation step, the cells were treated with increasing doses of temozolomide (20, 40, and 80 μM), ferulic acid (1000 and 1500 μM), and their combinations (FA 1000+TMZ 40 μM, FA 1500+TMZ 40 μM). Plates were incubated at 37°C for approximately 2 weeks until colonies in the control group reached sufficient density (confluency). After the process was complete, we carefully removed the medium and washed the cells with Phosphate Buffered Saline (PBS); then, we made the colonies visible through fixation and crystal violet staining steps. During the analysis phase, we counted colonies consisting of more than 50 clearly distinguishable cells and reported our data as a percentage relative to the control group.

Western Blot Analysis

To examine changes at the molecular level, we performed Western blot analyses. We seeded the cells in T-25 flasks and incubated them for 24 hours to allow the cells to adhere. We performed our experiment using a 96-hour sequential protocol: In the first step, we treated the cells with 80 μM temozolomide for 48 hours from day 1 to day 3. On day 3 of the experiment, we added ferulic acid (1000-1500 μM) and the corresponding combination doses (FA 1000+TMZ 40 μM, FA 1500+TMZ 40 μM) to the cells and allowed the interaction to complete with a final 24-hour incubation.

At 96 hours, we harvested the cells, washed them twice with cold PBS, and lysed them in lysis buffer at 4°C. We performed precise measurements to determine protein concentrations using a commercial kit (DC kit; Bio-Rad, Hercules, CA). A 40-μg protein load from each sample was run on a 4–20% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes.

To block nonspecific binding on the membranes, we incubated them for 60 minutes in a blocking buffer containing 5% nonfat milk powder (0.1% Triton X-100, TBS-T). After washing, we targeted the membranes with the following primary antibodies: PARP (Cell Signaling, cat# 9532), Cyclin D1 (Cell Signaling, cat# 9542S), and Beta Actin (Proteintech, cat# 60008-1-Ig) as a loading control. Following the washing steps, we proceeded with appropriate secondary antibodies (anti-rabbit or anti-mouse; Bio-Rad). We performed chemiluminescence detection using Clarity Western ECL Substrate (Bio-Rad) to visualize the bands; the resulting signals were visualized and analyzed using the ChemiDoc MP Imaging System (Bio-Rad) (2,12,13,14).

Apoptosis Analysis (Host Staining)

We performed Hoechst 33258 staining to observe changes in the nuclear morphology of U87-MG GBM cells and to provide morphological evidence of apoptotic cell death. Cells were seeded in 6-well plates at a density of 100,000 cells/well and allowed to adhere for 24 hours. Treatments were administered according to the 96-hour sequential protocol: cells were first treated with temozolomide (40 and 80 μM) for 48 hours, followed by the addition of ferulic acid (1000 and 1500 μM) and their combinations (FA 1000+TMZ 40 μM, FA 1500+TMZ 40 μM) for the final 24 hours. Untreated cells served as the control group. Following the 96-hour treatment, cells were fixed with 4% paraformaldehyde, washed with PBS, and stained with 200 μl Hoechst 33258 (Sigma, 0.5 mg/mL) for 15 minutes in the dark.

After staining, the solution was removed, and cells were washed three times with ice-cold PBS. Changes in nuclear morphology were assessed using a fluorescence microscope with 320-350 nm filters (Eclipse Ti, Nikon). Apoptotic cells were identified by hallmark morphological changes, such as highly condensed chromatin and fragmented nuclei (12,26).

Statistical Analysis

All experiments were performed at least in triplicate, and results were summarized as means with standard deviation. Statistical significance was determined using Student's t-test. P-values less than 0.05 were considered statistically significant. GraphPad Prism (ver. 8.0.2) software was used for data evaluation and graphing.

RESULTS

Ferulic Acid Inhibits GBM Cell Proliferation and Enhances TMZ Efficacy

To evaluate the effects of ferulic acid (FA) and temozolomide (TMZ) treatment on glioblastoma (GBM) cell proliferation and viability, MTS analysis was performed 96 hours after treatment. The results showed that U87-MG cell viability was significantly reduced in a dose-dependent manner compared to the control group (Figure 1).

TMZ (10-80 μM) treatments administered from 24 to 72 hours showed a marked decrease, particularly at the 80 μM concentration (Figure 1B). For FA application, cells were exposed to different doses (100-1500 μM) during the final 24-hour period following a 48-hour pre-incubation. FA application alone showed a potent inhibitory effect at concentrations of 700, 1000, and 1500 μM (Figure 1A).

In combination groups treated with the same sequential protocol (48 hours of TMZ followed by 24 hours of FA co-incubation), a synergistic decrease in cell viability was observed. The lower viability rate observed in the combination treatment groups compared to the control group and single FA or TMZ treatments (Figure 1C) confirms that ferulic acid enhances the cytotoxic effect of TMZ in GBM cells.

Ferulic Acid and Temozolomide Synergistically Inhibit Colony Formation

We performed clonogenic assays to observe the effects of FA and TMZ, as well as their combinations, on the clonogenic capacity of GBM cells. The results showed that when we increased the TMZ dose (20, 40, and 80 μM), the colony-forming ability of U87-MG cells decreased significantly in a dose-dependent manner compared to the control group (Figure 2A). However, the most striking results were observed with FA. We found that colony formation was completely inhibited in cells treated with FA alone (1000–1500 μM) (Figure 2B). However, the most noteworthy aspect of the study was the combination groups; when FA (1000 μM or 1500 μM) was combined with TMZ (40 μM), colony formation was eliminated (Figure 2C).

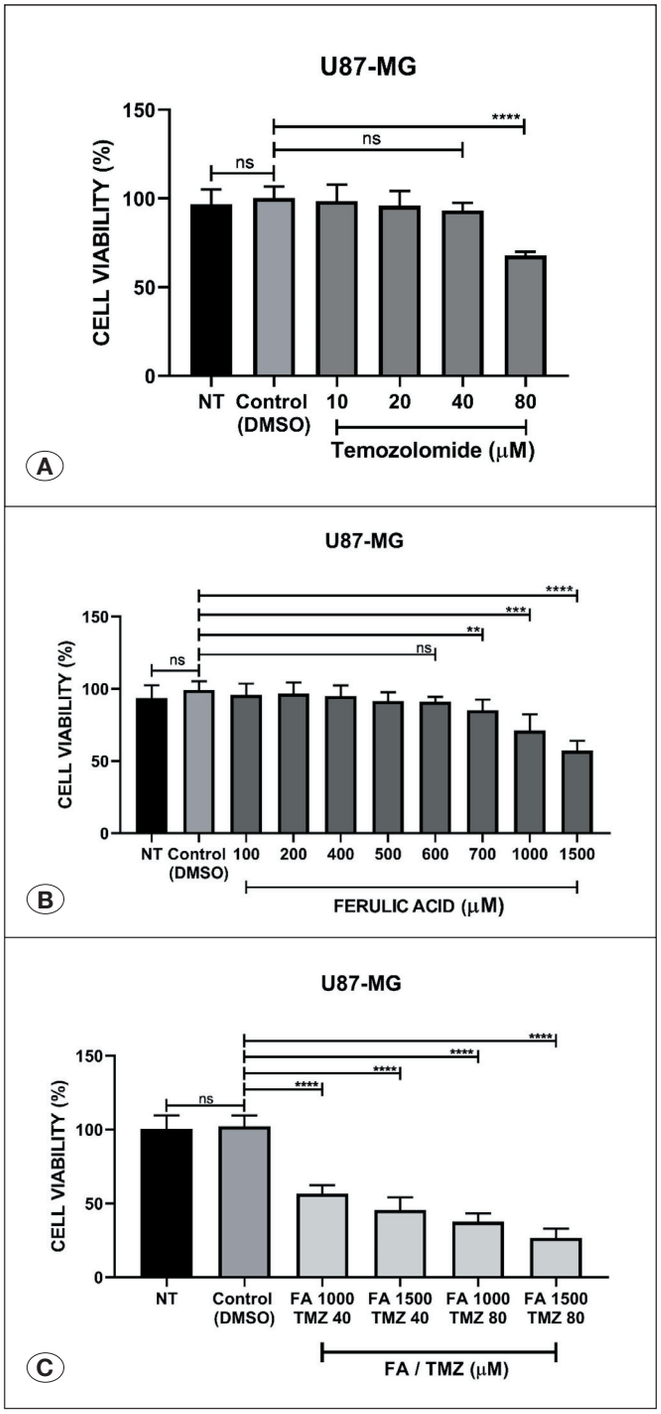

Figure 1: Effects of Ferulic Acid (FA) and Temozolomide (TMZ) on the viability of U87-MG glioblastoma cells, evaluated using the MTS assay following a 96-hour sequential treatment protocol. **A)** Cells treated with 100-1500 μM FA for the final 24 hours of the protocol showed a dose-dependent decrease in viability, with significance at 1000-1500 μM. **B)** Cells treated with 10-80 μM TMZ for a total of 72 hours exhibited a significant viability decrease at 80 μM. **C)** Sequential combination of FA (1000-1500 μM) and TMZ (40-80 μM) demonstrated a synergistic reduction in cell viability compared to the control group (ns: p>0.05; *p<0.05; **p<0.0001).

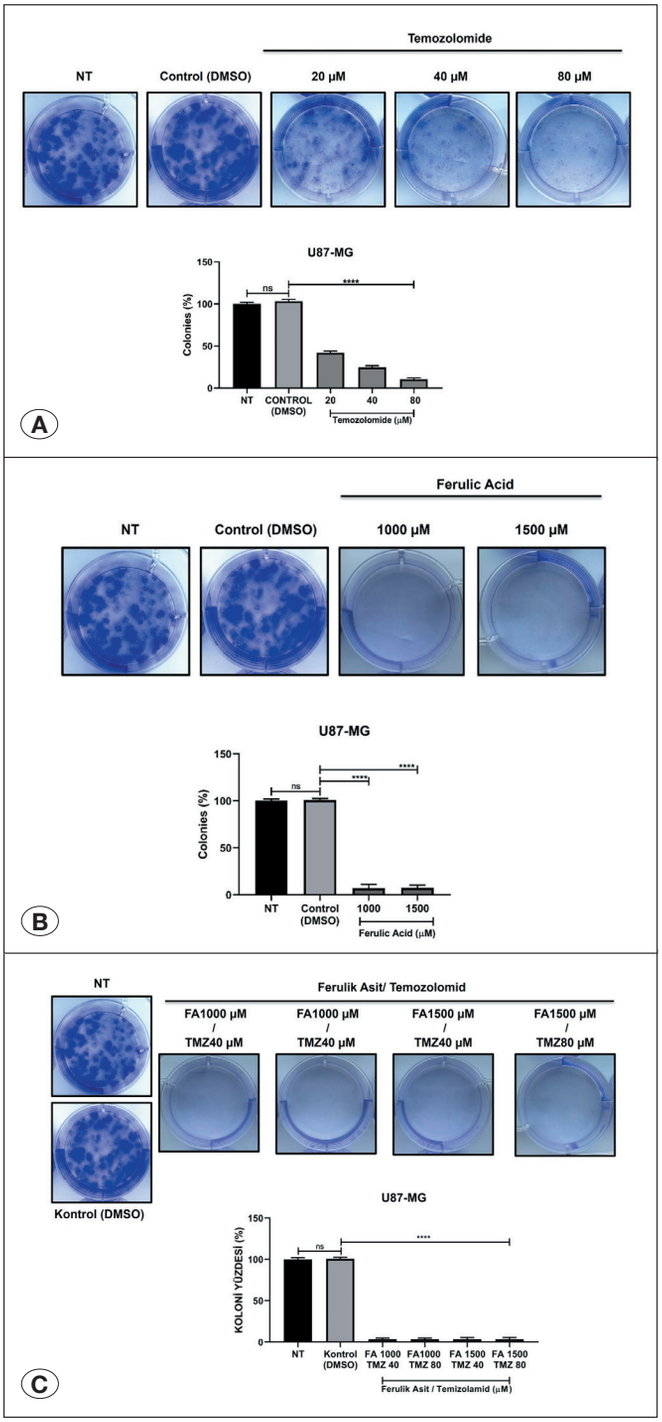

Figure 2: Impact of FA and TMZ on the clonogenic capacity of U87-MG cells. **A)** Treatment with 20, 40, and 80 μM TMZ resulted in a dose-dependent suppression of colony formation. **B)** Colony formation was completely inhibited in cells treated with 1000 and 1500 μM FA alone. **C)** The combination of FA (1000-1500 μM) and TMZ (40 μM) led to the total abolishment of colony formation, indicating a complete loss of proliferative integrity (ns: not significant; p>0.05; **p<0.01; ***p<0.001; ****p<0.0001).

These data indicate that the strategic addition of FA to TMZ treatment directly targets both the self-renewal mechanism and proliferation of U87-MG cells. This intervention results in the complete loss of the cells' clonogenic potential.

Ferulic Acid and Temozolomide Combination Suppresses Cyclin D1 and PARP Expression

To demonstrate the underlying molecular mechanisms of the anti-proliferative effect we observed, we performed Western blot analysis following a 96-hour sequential treatment protocol. At this stage, we focused on the expression levels of the PARP enzyme, which plays a key role in both DNA repair mechanisms (BER pathway) and cell death processes, and the Cyclin D1 protein, which controls the G1/S transition of the cell cycle (Figure 3A).

Our quantitative analyses revealed that the combination of FA and TMZ significantly reduced Cyclin D1 expression in U87-MG cells compared to the control group (Figure 3C). This decrease in expression directly indicates an extraordinarily strong arrest in the cell cycle. Additionally, we observed a significant decrease in PARP expression levels in the combination groups compared to the control group (Figure 3B). Given the DNA damage caused by TMZ, this decrease in PARP levels is particularly important, as it indicates that ferulic acid impairs the ability of GBM cells to repair damage and renders them much more sensitive to TMZ-induced apoptosis. In summary, the simultaneous inhibition of both proteins confirms that the combination of FA and TMZ targets glioblastoma cells for both proliferation and apoptosis simultaneously.

Ferulic Acid and Temozolomide Combination Triggers Apoptotic Changes in GBM Cells

To demonstrate changes in nuclear morphology and for apoptosis analysis, we performed Hoechst 33258 staining following a 96-hour sequential treatment regimen. Our data showed that TMZ (40 and 80 μM) administered alone for 72 hours did not cause a radical change in nuclear structure compared to the control group. However, the intense chromatin staining and structural disruptions characteristic of cells undergoing apoptosis revealed a profile markedly different from that of healthy cells (Figure 4A).

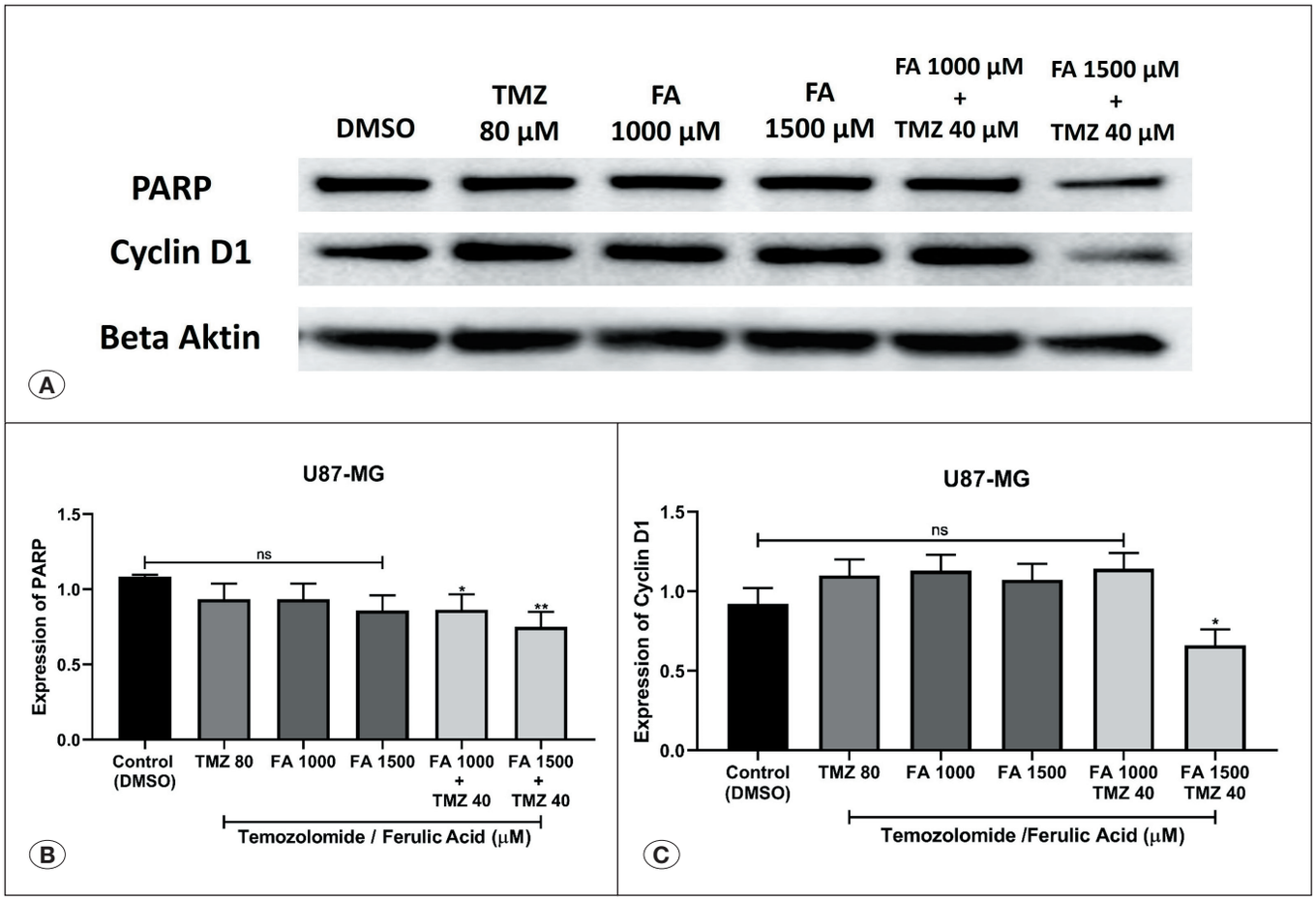

Figure 3: Molecular analysis of PARP and Cyclin D1 expression in U87-MG cells following 96-hour sequential treatment. **A)** Representative Western blot images showing the expression levels of PARP (a key BER pathway enzyme) and Cyclin D1 (a cell cycle regulator). **B)** Quantitative analysis of PARP expression, showing significant downregulation in combination groups. **C)** Quantitative analysis of Cyclin D1 expression, demonstrating a significant decrease compared to the control group. Beta-actin was used as the loading control (ns: not significant; $p>0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$).

In the final 24 hours of the process, we observed that nuclear morphological changes increased in a dose-dependent manner in cells treated only with FA (1000 and 1500 µM) (Figure 4B). However, the most striking results emerged in the combination groups where TMZ and FA were administered together. A substantial proportion of cells in this group exhibited intense bright blue fluorescence, a characteristic sign of chromatin condensation and nuclear fragmentation.

The combination groups most clearly observed this transformation, where fragmented apoptotic bodies replaced the normal nuclear structure. These morphological indicators are definitive evidence of apoptosis. In conclusion, the microscopic images we obtained confirm that ferulic acid strongly supports and enhances TMZ-induced programmed cell death (apoptosis) in GBM cells.

DISCUSSION

GBM poses a significant clinical challenge due to its high malignancy, rapid progression, and resistance to current treatment approaches. Traditional treatments, including surgery, radiotherapy, and chemotherapy, can only extend the average survival time of GBM patients to a limited extent (22,25). Therefore, the development of more effective treatment strategies is crucial.

TMZ is a basic alkylating agent used in the standard treatment of GBM. Although it is known that TMZ triggers cell death through methylation on DNA (1), at least 50% of treated glioblastoma patients do not respond adequately to treatment. The most important reasons for this treatment resistance include overexpression of the O6-methylguanine-DNA methyltransferase (MGMT) gene and/or defects in DNA repair mechanisms in tumor cells. In this context, combined therapies that may increase the efficacy of TMZ in GBM treatment should be investigated (17). In our study, the effects of TMZ combination with ferulic acid (FA), a natural phenolic compound, on GBM cell line (U87-MG) were investigated, and promising results were obtained.

The researchers strategically selected the 96-hour sequential treatment protocol (48 hours of TMZ followed by 24 hours of FA) to suppress the DNA repair mechanism. This timing ensures that the phytochemical reaches cells that are in their most vulnerable state as they attempt to recover from the initial chemical attack.

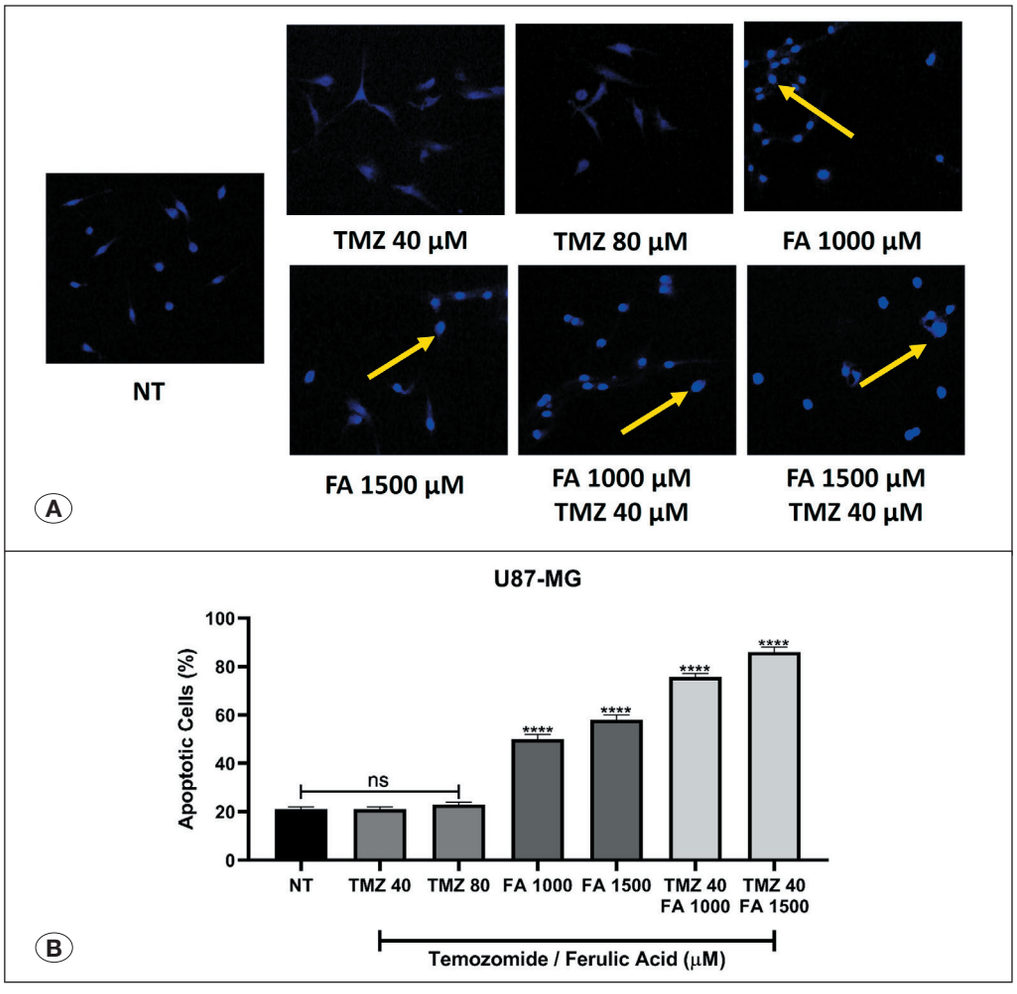

Figure 4: Morphological assessment of apoptotic cell death in U87-MG cells via Hoechst 33258 staining following sequential treatment.
A) Fluorescence microscopy images representing nuclear morphology; untreated control cells show uniform nuclei, while treated cells exhibit bright blue fluorescence. **B)** Statistical representation showing the significant increase in apoptotic cells (exhibiting nuclear fragmentation and chromatin condensation) in FA and TMZ-treated groups compared to the control (***p<0.001; ****p<0.0001).

Previous studies have reported that FA increases DNA damage in GBM cells and exhibits anticancer effects by activating apoptotic pathways (11). Similarly, Dell'Albani et al. reported that FA encapsulated in nanoparticles suppressed glioma cell proliferation four times more than free FA (4). Naumowicz et al. reported that FA and cinnamic acid compounds reduce cell viability by causing changes in the membrane surface charges of glioblastoma cells (19). Studies conducted by Morin et al. on Hs683 and LN319 cell lines indicate that FA and caffeic acid phenethyl ester compounds have therapeutic potential (18).

In this study, we demonstrated that the combination of Ferulic Acid (FA) and Temozolomide (TMZ) significantly suppressed the proliferation of U87-MG cells, and that this effect was much more pronounced compared to the use of FA alone. We determined that this effect is achieved by inhibiting the expression of Cyclin D1 and PARP, which play a critical role in DNA repair. This indicates that FA essentially disrupts the cell's DNA repair mechanism, renders the damage caused by TMZ permanent, and ultimately leads to the cell entering an irreversible death process.

DNA damage caused by TMZ is primarily repaired via Base Excision Repair (BER). The significant decrease in PARP levels observed in our analyses indicates that ferulic acid effectively suppresses this repair mechanism. Silencing PARP-1, an indispensable sensor of the BER pathway, is a known strategy in literature to enhance the effect of alkylating agents such as TMZ, but it takes on a new dimension with this combination. FA prevents the repair of DNA damage caused by TMZ, leading to the accumulation of lethal double-strand breaks in the cell and ultimately triggering apoptosis. This mechanism is clearly supported by our Hoechst 33258 staining results. The nuclear fragmentation and chromatin condensation observed in the combination groups demonstrate that this is programmed cell death, not suppression of proliferation, random tissue death (necrosis), or simple metabolic slowing.

Although our study only includes vitro experiments, ferulic acid stands out as a promising natural compound for GBM treatment. The combination of TMZ and FA could form the basis for current treatment strategies. Of course, the results shown in this study using the U87-MG cell line are not sufficient, but the genetic diversity of glioblastoma, especially factors such as TMZ resistance, is decisive for clinical success. Therefore, to generalize our findings, it is vital to validate this synergy in resistant lines such as T98G and in primary cultures obtained from patients in the next step.

CONCLUSION

In conclusion, consistent responses obtained from various analytical methods, such as MTS, colony formation, Western blot, and Hoechst staining, emphasize that FA may be a potent adjuvant in GBM treatment. However, more comprehensive in vitro and in vivo studies are needed to understand fully the clinical value of this combination. A detailed analysis of the mechanisms is required.

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Declarations

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Availability of data and materials: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure: The authors declare that they have no competing financial or non-financial interests related to this study.

Ethics Statement: This study included only in vitro cell line experiments. No human participants or animals were involved. Therefore, ethics committee approval and informed consent were not required.

AUTHORSHIP CONTRIBUTION

Study conception and design: HU, ZH

Data collection: SA, AG, VC, NN

Analysis and interpretation of results: HU, SA, AG, VC, NN, ZH

Draft manuscript preparation: HU, SA, ZH

Critical revision of the article: HU, SA, ZH

Other (study supervision, fundings, materials, etc...): HU, SA, AG, VC, NN, ZH

All authors (HU, SA, AG, VC, NN, ZH) reviewed the results and approved the final version of the manuscript.

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