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Journal of Scientific Reports-A

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E-ISSN: 2687-6167 ***Number 62, September 2025***

RESEARCH ARTICLE

Receive Date: 27.06.2025 *Accepted Date: 19.08.2025*

Betaine induces apoptosis via ROS-independent mechanisms in U87 glioblastoma cells: a potential metabolic anticancer strategy

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Abstract

Betaine (trimethylglycine), a naturally occurring osmolyte and methyl donor, has attracted attention for its potential anticancer properties through its role in cellular stress responses and epigenetic regulation. Glioblastoma multiforme (GBM) represents one of the most aggressive forms of primary brain cancer characterized by rapid progression, poor prognosis, and resistance to conventional therapies. In this study, we aimed to investigate the dose- and time-dependent cytotoxic and pro-apoptotic effects of betaine on U87 glioblastoma cells, along with its influence on oxidative stress, gene expression, and protein-level markers. U87 cells were treated with increasing concentrations of betaine, and cell viability was assessed using the MTS assay. Apoptosis was evaluated via Annexin V/PI flow cytometry, while ROS levels were measured with DCFDA staining. Quantitative RT-PCR and ELISA tests were conducted to assess gene and protein expression patterns associated with apoptosis, oxidative stress, and inflammatory signaling. Our findings demonstrated that betaine reduced U87 cell viability in a concentration-dependent fashion, triggered late apoptosis and necrotic cell death, and markedly lowered intracellular reactive oxygen species (ROS) levels. Furthermore, betaine modulated the expression of key signaling molecules including PTEN, BCL-2, AKT1, and NF-κB, while increasing mitochondrial apoptotic markers such as CASP3 and cytochrome C. Interestingly, the anticancer effects of betaine appeared to occur through ROS-independent mechanisms. The results indicate that betaine may serve as a promising anticancer agent for glioblastoma, warranting further investigation in preclinical models.

Keywords: Betaine; Glioblastoma; Apoptosis; Oxidative Stress

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1. Introduction

Betaine (trimethylglycine) is a naturally occurring quaternary ammonium compound found in plants, animals, and microorganisms (Figure 1) [1]**.** It was first isolated from sugar beets in the 19th century and is now obtained through dietary sources such as wheat bran, spinach, seafood, and beets, or synthesized endogenously via choline metabolism [2]. By participating in methylation pathways, betaine contributes to epigenetic regulation through DNA methylation. Betaine functions via the enzyme betaine-homocysteine methyltransferase (BHMT) to convert homocysteine into methionine, supporting the maintenance of S-adenosylmethionine (SAM) levels. This process impacts various biological events including DNA, RNA, and histone methylation [3], thereby facilitating activation of tumor suppressor genes and repression of proto-oncogenes.

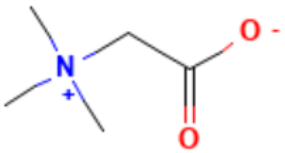

Fig. 1. Chemical structure of the betaine (trimethylglycine) molecule. The molecule contains a quaternary ammonium structure ($–N^+(CH_3)_3$) with three methyl groups and a carboxylic acid group (–COOH) [1]

A wide range of experimental studies conducted both in vitro and in vivo has provided evidence of significant biological responses associated with the anticancer effects of betaine. In a study involving the DU-145 prostate cancer cell line, betaine was shown to inhibit cell proliferation in a dose-dependent manner, increase oxidative stress levels, induce apoptosis, and elevate proinflammatory cytokines such as TNF-\u03b1 and IL-6 [4]. Similarly, in HeLa cervical cancer cells, high doses of betaine inhibited cell growth and triggered apoptosis via caspase-3 activation, upregulation of p53, and downregulation of Bcl-2 expression [5]. A comprehensive study in oral squamous cell carcinoma (OSCC) cells showed that betaine suppresses invasion and migration by downregulating the expression of MMP1, MMP2, and MMP9 genes, as well as epithelial-mesenchymal transition (EMT) markers such as FN1 [6]. In animal models, betaine has also shown anticancer efficacy. In a colitis-associated colorectal cancer model (AOM/DSS-induced), betaine reduced tumor incidence, suppressed reactive oxygen species (ROS) production, and downregulated proinflammatory gene expression such as COX-2, TNF-\u03b1, and IL-6 [7]. The results imply that betaine has the potential to modulate the tumor microenvironment by reducing DNA damage and inflammation. Another study in the same model demonstrated that betaine improves glutathione metabolism, mitigates oxidative stress, and reduces inflammatory damage to the colonic mucosa [8]. Epidemiological evidence in humans also supports the protective role of betaine against cancer. According to the Long Island Breast Cancer Study Project, high dietary intake of betaine and choline was significantly associated with reduced breast cancer mortality. Furthermore, the rs3733890 polymorphism in the BHMT gene was reported to confer a protective effect on survival [9]. The anticancer potential of betaine is supported by its multifaceted biological activities including apoptosis induction, cell cycle regulation, epigenetic modification, inflammation suppression, and inhibition of cell invasion. However, the magnitude of these effects may vary depending on factors such as dose, duration, and cell type, indicating the need for further research into its underlying molecular mechanisms. Accordingly, this research set out to assess the dose- and time-dependent cytotoxic, apoptotic, and oxidative stress-related effects of betaine on U87 glioblastoma cells, using molecular, biochemical, and flow cytometric approaches to better understand its underlying anticancer processes.

2. Materials and Methods

2.1. Propagation of glioblastoma cells and HEK-293

HEK-293 cells, immortalized human embryonic kidney epithelial cells, were used as a non-cancerous control group to compare the cytotoxic effects of betaine on tumor and non-tumor cells. These cells are frequently used as a reference in cancer studies due to their robust growth, human origin, and well-characterized molecular profile. Although not strictly "normal" cells, their lack of malignancy contrasted with the aggressive phenotype of glioblastoma, allowing us to better assess selective cytotoxicity.

2.2. Assessment of betaine-induced cytotoxicity in human glioblastoma cells

A stock solution of Betaine Hydrochloride was initially prepared by dissolving the compound in distilled water with continuous mixing at ambient temperature. This mixture was subsequently diluted with DMEM to obtain the required treatment levels**.** U87 glioblastoma cells were maintained in 96-well culture plates at a cell concentration of 5×10^3 per well, each containing 200 µL of DMEM supplemented with 10% fetal bovine serum (FBS) and 1% PSA (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin B). After cell attachment, they were exposed to increasing doses of betaine (high-dose: 0.5, 2.5, 5, 10, and 20 mg/mL; low-dose: 0.5 to 6 mg/mL, at 1 mg/mL intervals). Control wells received no treatment. Cell survival was analyzed after 24 and 48 h using the MTS-based CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Cat. No: G3580), according to the instructions supplied by the manufacturer. Absorbance was determined at 490 nm following a 3-h incubation at 37 °C in the dark. The half-maximal inhibitory concentration (IC_{50}) of betaine was calculated by comparing absorbance values with those from untreated controls.

2.3. Evaluating the effect of betaine on cancer cell proliferation and survival

2.3.1. Analysis of apoptosis

U87 cells were cultured into 6-well plates with a density of 500,000 cells per well. The cells received treatment with betaine at previously identified IC50 levels, conducted in triplicates for each tested condition. The cells were maintained in a humid environment at 37°C and 5% CO_2 for three days. After the incubation period, cells were harvested and pelleted by spinning at 1500 rpm for 5 minutes. Apoptotic activity was then assessed by suspending the obtained cell pellet in a 1X binding buffer, followed by labeling the cells with Annexin V conjugated to FITC and propidium iodide (PI) through a commercially available apoptosis assay kit (ABP, Cat. No: A026) according to the manufacturer's recommended protocol. Subsequently, the labeled cells underwent analysis using flow cytometry on a Cytoflex S analyzer (Beckman Coulter, USA; Cat. No: B47903). Data collection and gating methodologies followed standard practices and were consistent with previously published research [10, 11].

2.3.2. Assessment of oxidative stress via ROS detection

To evaluate ROS levels, cells were plated into wells at a concentration of 500×10^3 cells per well and subsequently exposed to effective concentrations of betaine. After a 48-hour incubation period, the cellular ROS levels were assessed utilizing a commercially provided DCFDA/H2DCFDA assay kit (Abcam; Catalog No: ab113851), performed according to the supplier's guidelines. Cells were incubated with the fluorescent dye DCFDA at a final concentration of 20 µM for 30 minutes without any washing steps afterward. Fluorescent intensity was quantified via flow cytometry, utilizing excitation at 485 nm and emission detection at 535 nm. Tert-butyl hydrogen peroxide (TBHP) at a concentration of 50 µM, prepared from a 55 mM initial stock, served as the positive experimental control [9,11].

2.3.3. Flow cytometric evaluation of sema 3A expression

After 48 h of treatment with effective dose of betaine, cells were collected and transferred to 1.5 ml tubes. Cells were labeled using SEMA3A Antibody (A-12) PE (Cat No: sc-74554 PE) by intracellular staining following the supplier's instructions and protocols outlined in previous studies. The specificity of SEMA3A staining was validated by using an unstained (UNS) control to assess background fluorescence and an isotype-matched PE-conjugated antibody as an isotype control in flow cytometry analyses. Labeled cells were then analyzed by Cytoflex S flow cytometry according to methods previously described in our experiments.

2.3.4 Gene expression analysis (RT-qPCR)

U87 cells were maintained in 6-well culture plates at a seeding density of 5×10^5 cells per well and then treated with defined concentrations of betaine. After 24 h of incubation, cells were collected to extract total RNA. RNA purification was carried out with the PureLink RNA Mini Kit (Invitrogen, Thermo Fisher Scientific, Cat. No: 12183025), following the manufacturer's protocol. Complementary DNA (cDNA) was subsequently produced using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat. No: 4368814). Quantitative real-time PCR (qRT-PCR) experiments were conducted employing the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Cat. No: K0221), together with primers obtained from Oligomer (Turkey) (see Table 1). PCR amplifications were performed on the Bio-Rad CFX96 Touch™ Real-Time PCR platform. Gene expression analysis was carried out using the ΔΔCt approach, with GAPDH chosen as the internal control gene to ensure reliable and consistent quantification [10, 12].

Table 1. Oligonucleotide primers applied in qRT-PCR assays

Gene	Forward (5' to 3')	Reverse (5' to 3')	Function
GAPDH	TTTTGCGTCGCCAGCC	ATGGAATTTGCCATGGGTGGA	Serves as a control gene to standardize the measurement of expression levels
VEGF	TAAGTCCTGGAGCGTTCCCT	ACGCGAGTCTGTGTTTTTGC	Promotes the formation of new vasculature
p53	AAGTCTAGAGCCACCGTCCA	ACCATCGCTATCTGAGCAGC	Mutations in the p53 gene are associated with tumor advancement and reduced sensitivity to chemotherapy
PTEN	AGCTGGAAAGGGACGAACTG	ACACACAGGTAACGGCTGAG	Functions by blocking pathways that promote tumor cell growth and resistance to treatment.
BCL-2	AAAAATACAACATCACAGAGGAAGT	TCCCGGTTATCGTACCCTGT	Supports cancer cell survival by inhibiting programmed cell death.
AKT1	CCAGGATCCATGGGTAGGAAC	CTCCTCCTCCTCCTGCTTCT	Participates in signaling pathways that help cancer cells evade apoptosis and continue proliferating.
BAX	AGGGGCCCTTTTGCTTCAG	TGTCCAGCCCATGATGGTTC	Plays a key role in initiating cell death processes.
BAK	GCAGGCTGATCCCGTCC	GGCTAAGGAGGTCCCAGAGA	Facilitates apoptosis by promoting mitochondrial membrane permeabilization.
NFKB	AACAGCAGATGGCCCATACC	AATAGGCAAGGTCAGGGTGC	Supports tumor development and dissemination, contributes to resistance against chemotherapy, heightens inflammatory signaling, and may also inhibit programmed cell death.

CAS-3	TGCTATTGTGAGGCGGTTGT	TCTGTTGCCACCTTTCGGTT	Induces cell death in tumor cells through the activation of apoptosis mechanisms.

2.3.5. Analysis of protein levels using ELISA

U87 cells were exposed to effective concentrations of betaine. After 48 hours, the expression levels of SEMA3A (E2078Hu), SEMA3E (E1562Hu), CASP3 (E4811Hu), Cytochrome C (E7110Hu), GPX-4 (E6887Hu), ACSL4 (E7227Hu), IL-6 (E0090Hu), TNF-α (E0099Hu), and IL-10 (E0102Hu) were quantified in the experimental groups using ELISA kits provided by BT LAB (China). Total antioxidant status (TAS) and total oxidant status (TOS) were determined using commercial kits from Rel Assay Diagnostics (Gaziantep, Turkey). TAS levels were expressed in mmol Trolox equivalents per liter, while TOS values were reported in μmol H_2O_2 equivalents per liter. All kits used were specifically tailored for human (Hu) samples. Before performing ELISA, total protein concentrations in the samples were assessed using the Bradford assay to ensure precision. All procedures were performed following the manufacturer's guidelines, utilizing a Thermo Scientific Multiscan SkyHigh ELISA reader along with a Thermo Scientific Wellwash microplate washer [4]**.** All data are presented as mean ± standard deviation, and experiments were repeated in triplicate. All procedures were performed following the manufacturer's guidelines, utilizing a Thermo Scientific Multiscan SkyHigh ELISA reader along with a Thermo Scientific Wellwash microplate washer.

2.4. Evaluation of statistical data

All analytical evaluations were performed using GraphPad Prism software (version 8.4.2; GraphPad Software, San Diego, CA, USA). Results were reported as the mean ± standard deviation (SD) from a minimum of three separate biological experiments. For analyzing data involving more than two groups, one-way ANOVA followed by Tukey's post-hoc test was conducted. For comparisons involving only two groups, the unpaired two-tailed Student's t-test was applied. A p-value below 0.05 was interpreted as statistically meaningful. Significance levels were defined as follows: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.

3. Results

3.1. Evaluation of growth dynamics and survival rates in U87 and HEK-293 cells

Figure 2 shows the dose and time-dependent cytotoxic effects of betaine on U87 glioblastoma cells (Figure 2A and C) and non-tumorous HEK-293 embryonic kidney cells (Figure 2B and D) after 24 and 48 hours of application. In U87 cells, betaine was observed to significantly reduce cell viability at concentrations of 5 mg/ml and above. Especially at concentrations of 20, 10, 6 and 5 mg/ml, viability decreased well below the 50% threshold value at both time points. The calculated IC_{50} value at the end of 48 hours of application was determined as 4.1 mg/ml and IC_{25} value as 2.7 mg/ml. These findings demonstrate that betaine has a strong cytotoxic effect on glioblastoma cells. However, no significant decrease in viability was observed at doses of 2.5 mg/ml and lower; This shows that the dose-response relationship has a clear threshold (Figure 2A and C). In contrast, a dose-dependent decrease in viability was also observed in HEK-293 cells (Figure 2B and D), but this effect was milder compared to U87 cells. Although significant cytotoxicity was observed at high doses (20, 10, 6 and 5 mg/ml), cell viability remained above 50% at 2.5 and 0.5 mg/ml doses for 24 h. Even after 48 h, only a limited cytotoxic effect was observed at these low concentrations. This suggests that HEK-293 cells are more resistant to betaine.

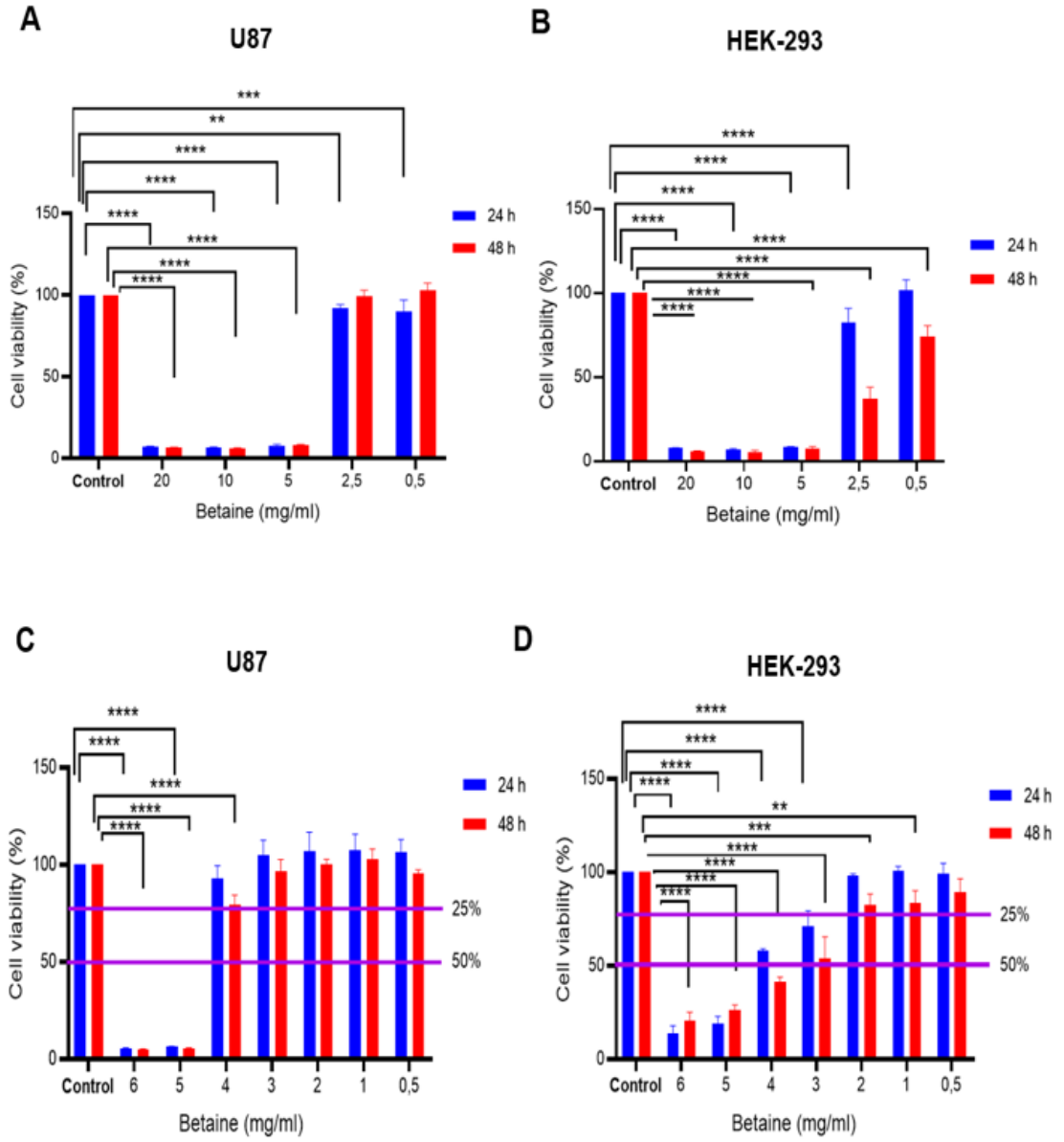

Fig. 2. Effect of betaine on the viability of U87 glioblastoma (A, C) and HEK-293 kidney epithelial cells (B, D) at 24 h and 48 h post-treatment. The bar graphs illustrate cell viability (%) after exposure to both high (A, B) and low (C, D) concentrations of betaine, assessed via MTS assay. Betaine treatment significantly reduced cell viability in both U87 glioblastoma (A, C) and HEK-293 normal kidney epithelial cells (B, D) in a dose- and time-related manner. In high-dose conditions (0.5–20 mg/mL), U87 cells (A) showed a pronounced loss of viability at ≥5 mg/mL, while HEK-293 cells (B) were less sensitive at lower doses but exhibited significant reductions at 5 mg/mL and above. In the low-dose range (0.5–6 mg/mL), U87 cells (C) maintained high viability up to 4 mg/mL, but showed substantial cytotoxicity at 5 and 6 mg/mL, aligning with IC25 and IC50 levels (indicated by purple lines). HEK-293 cells (D) retained higher viability overall but showed significant decline starting from 3 mg/mL. Results are expressed as mean ± SD of three independent replicates. Data-driven evaluation was performed using one-way ANOVA with Tukey's post hoc test (** $p \leq 0.01$, ***$p \leq 0.001$, ***$p \leq 0.0001$).

3.2. Assessing the cytotoxic and anti-proliferative effects of betaine on U87 glioblastoma cells

3.2.1. Assessment of apoptosis in cancer cells

The effects of betaine on apoptosis in U87 glioblastoma cells showed significant changes depending on the dose and time (Figure 3). In Figure 3A, which shows the 48-hour application, the live cell ratio decreased significantly at IC25 (2.7 mg/ml) and especially at IC50 (4.1 mg/ml) doses; this decrease was found to be strongly significant based on statistical analysis at the high dose (****p <0.0001). Under the same conditions, the late apoptosis ratio increased significantly and it was observed that the majority of the cells passed to the advanced stages of the apoptotic process (****p <0.0001). In addition, a significant increase was observed in the necrotic cell ratio at the 48-hour high dose application, indicating that advanced cellular damage is not only limited to apoptosis but can also trigger the necrosis mechanism characterized by the disruption of cell membrane integrity. In contrast, in Figure 3B, which shows 24-hour betaine application, a limited but significant decrease in the rate of live cells was observed at lower doses (1 and 2 mg/ml) (**p < 0.01, *p < 0.05); and the rate of early apoptosis also showed a notable rise with statistical relevance (*p < 0.05). However, no significant change was detected in the rates of late apoptosis and necrosis during this period. The data obtained show that betaine creates a cytotoxic effect that progresses over time and increases depending on the dose in U87 cells, and that it activates more widespread cell death mechanisms, including early stages of apoptosis at low doses and short periods, and late apoptosis and necrosis at high doses and long periods.

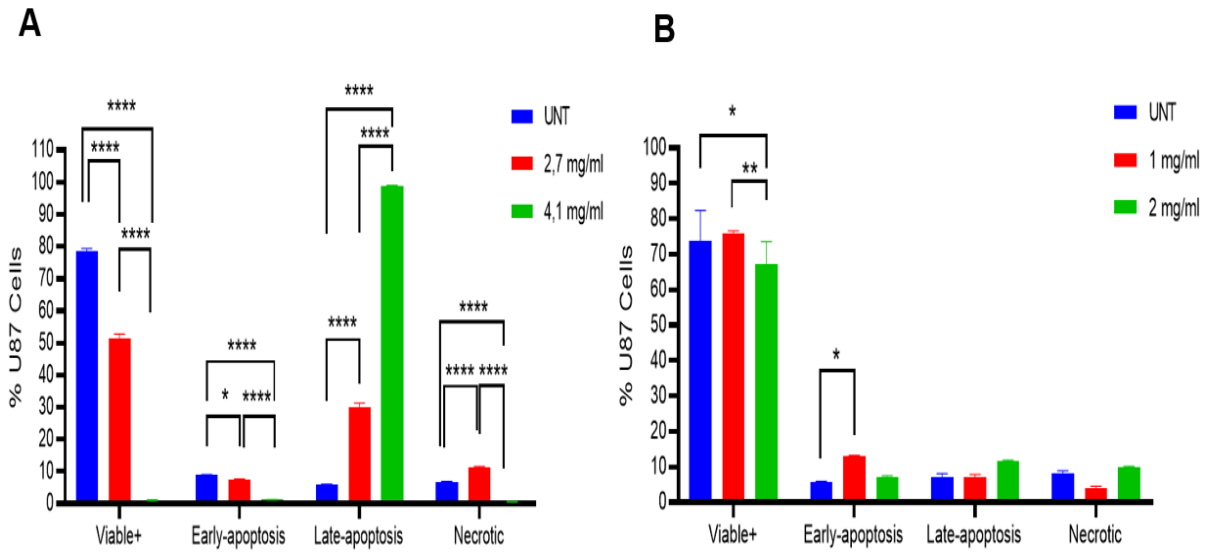

Fig.3. Flow cytometry analysis of betaine-treated U87 cells. (A) After 48 h, doses of 2.7 and 4.1 mg/mL significantly decreased viability and increased late apoptosis (****p < 0.0001). (B) At 24 h, doses of 1 and 2 mg/mL caused a slight but significant decrease in viability and increased early apoptosis (*p < 0.05, **p < 0.01). Values represent mean ± SD (n = 3). Comparisons among groups were carried out through one-way ANOVA with Tukey's multiple comparison test.

Morphological changes caused by betaine in U87 glioblastoma cells showed significant differences depending on the dose and time (Figure 4). In the control group (UNT), cells maintained their regular monolayer structure, while cell morphology was uniform and their distribution was homogeneous. In the 24-hour betaine application, while a slight change in shape and cytoplasmic thickening were noted in the cells at a dose of 1 mg/ml, significant changes

indicating early and late apoptosis such as decrease in cell density, rounding and clustering were observed at a dose of 2 mg/ml. In the 48-hour applications, it was observed that the borders of the cells disappeared after the 2.7 mg/ml betaine treatment, intense cytoplasmic changes and cell shrinkage began. In the IC50 dose of 4.1 mg/ml application, it was observed that most of the cells became round, separated from the monolayer structure, and detachment and membrane disruptions suggesting advanced apoptosis and necrosis occurred.

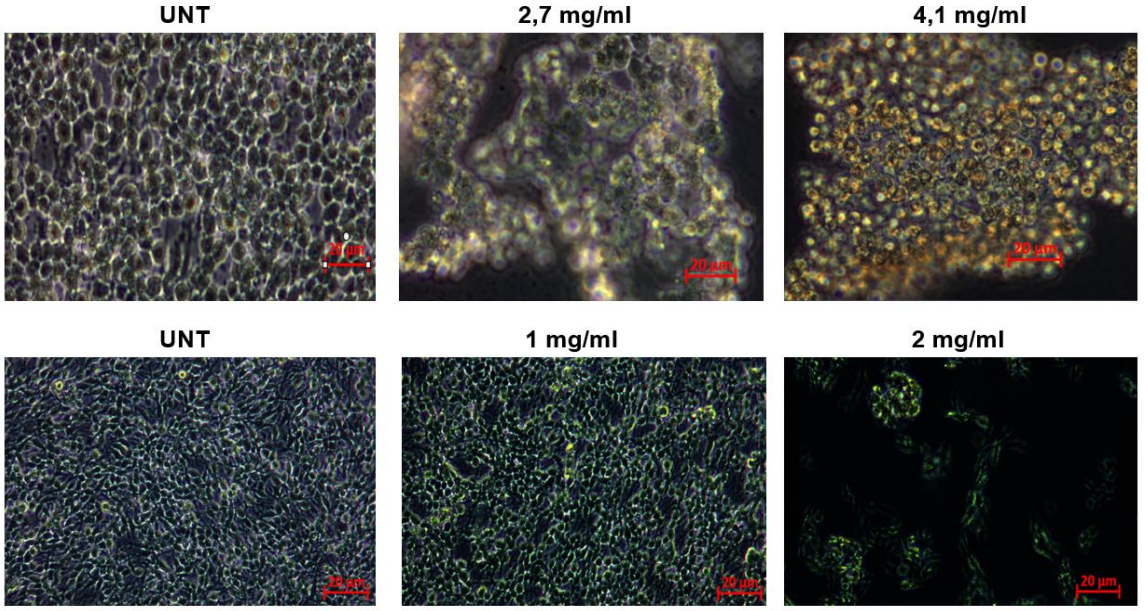

Fig.4. Representative microscopic images of U87 glioblastoma cells after 24 h (bottom) and 48 h (top) of betaine treatment at indicated concentrations. Control (UNT) cells show normal morphology and confluency, while betaine-treated cells exhibit dose-dependent morphological changes, including rounding, shrinkage, and detachment—indicative of apoptosis. Microscopic images were obtained using a ZEISS imaging system at 10× magnification. Scale bar = 20 µm.

3.2.2. *Analysis of ROS activity*

DCFDA based ROS analyses presented in Figure 5 reveal that betaine significantly suppressed reactive oxygen species (ROS) levels in U87 glioblastoma cells in a dose- and time-dependent manner. While the DCFDA positive cell ratio was high in the control (UNT) and TBHP-treated positive control groups, this ratio was significantly decreased in the betaine-treated groups. Especially, the IC50 dose (4.1 mg/ml) at 48 h administration caused a notable reduction confirmed by statistical analysis in ROS levels (****$p < 0.0001$), and this decrease was supported by the simultaneous increase in the DCFDA negative cell ratio (Figure 5A). Similarly, significant ROS suppression was observed at 1 and 2 mg/ml doses at 24 h administration (****$p < 0.0001$) (Figure 5B). These findings indicate that the anticancer effect of betaine occurs by reducing intracellular oxidative stress, in contrast to the induction of pro-oxidative stress observed in most classical chemotherapeutics. Despite this decrease in ROS levels, the significant apoptosis and necrosis rates observed in previous analyses indicate that betaine can effectively trigger cell death through alternative mechanisms independent of ROS. Therefore, betaine is considered a unique anticancer agent that can not only have a strong antioxidant capacity but also can induce cytotoxic responses by targeting non-ROS signaling pathways.

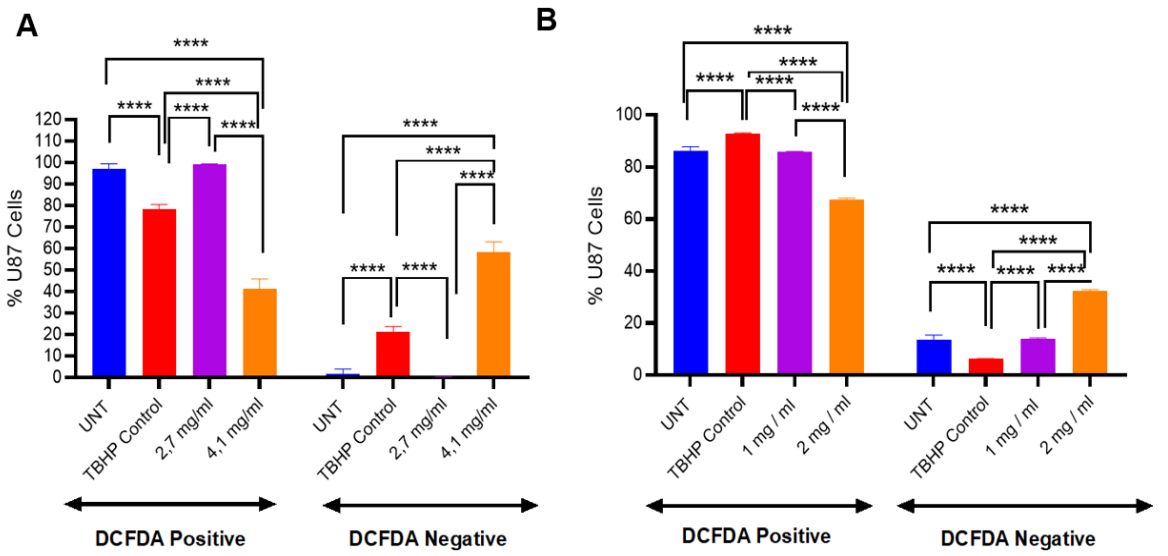

Fig. 5. Quantification of intracellular ROS levels in U87 cells after betaine treatment using DCFDA staining. (A) After 48 h of exposure, betaine significantly reduced the percentage of DCFDA positive (ROS+) cells in a manner proportional to the applied dose when evaluated against the untreated (UNT) and TBHP-induced oxidative stress control groups (****$p < 0.0001$). A concomitant increase in DCFDA negative (ROS−) cells was observed, especially at 4.1 mg/mL. (B) A similar but milder trend was detected after 24 h; 1 and 2 mg/mL betaine treatment led to a significant decrease in ROS+ cells and an increase in the ROS− population (****$p < 0.0001$). The findings are presented as mean ± SD ($n = 3$), and differences between groups were analyzed through one-way ANOVA with Tukey's post hoc comparison.

3.2.3. Quantification of sema 3A expression using flow cytometry

According to the flow cytometry data presented in the graph (Figure 6), approximately 100% of U87 glioblastoma cells were detected as Sema3A positive before betaine treatment. This high expression rate indicates that Sema3A is endogenously expressed intensively in U87 cells. Sema3A (Semaphorin 3A) is a signaling molecule that plays a role in processes such as cell invasion, migration and angiogenesis in cancer biology, apart from neural development. Therefore, such high expression of Sema3A in U87 cells suggests that this molecule may be associated with glioblastoma progression and aggressive phenotype. This finding indicates that changes in Sema3A expression following betaine treatment may be of biological and therapeutic importance.

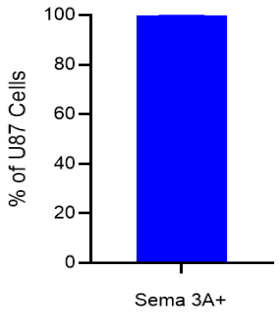

Fig.6. Flow cytometric analysis of Sema3A surface expression in U87 glioblastoma cells before betaine treatment. Almost 100% of the cell population was Sema3A positive (Sema3A^{+}), indicating high basal expression of Sema3A in untreated cells before any experimental intervention.

3.2.4. Analysis of RT-PCR

The RT-qPCR results presented in Figure 7 show that betaine significantly modulated the gene expressions related to apoptosis and oxidative stress in U87 glioblastoma cells. Betaine treatment significantly increased the expression of the tumor suppressor gene PTEN, especially at the dose of 1 mg/ml (***$p < 0.001$), while it caused a meaningful decline with statistical validation in BCL-2, AKT-1 and NF-κB genes that promote cell survival and proliferation (*$p < 0.05$–***$p < 0.001$). These findings suggest that betaine promotes apoptosis by suppressing anti-apoptotic pathways such as PI3K/AKT and NF-κB. In addition, when evaluated together with ROS analyses, it was observed that betaine can induce apoptotic cell death despite reducing intracellular oxidative stress levels. This suggests that betaine may trigger apoptosis through ROS-independent pathways—particularly through the PTEN/AKT signaling axis and BCL-2 family members. The decrease observed in pro-apoptotic genes, particularly CASP3 and BAX, suggests that the classical effector phase of apoptosis may be regulated differently depending on cell type, timing, or decreased ROS levels. According to these findings, betaine both suppresses ROS through its antioxidant effects and induces apoptosis through a ROS-independent mechanism by targeting intracellular proliferative and survival signals.

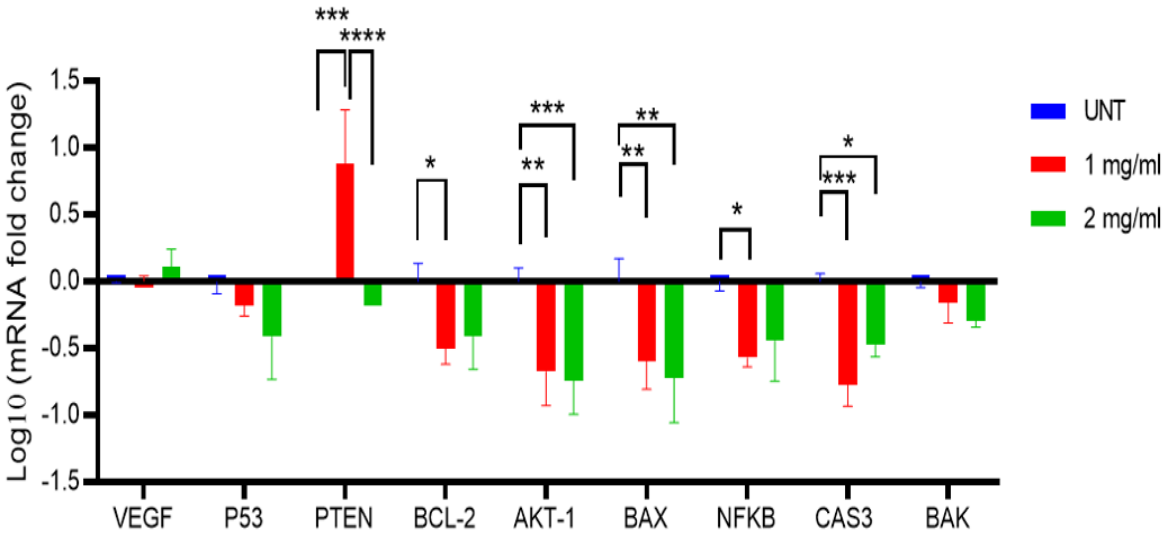

Fig 7. RT-qPCR analysis of gene expression levels in U87 glioblastoma cells treated with betaine (1 mg/ml and 2 mg/ml) for 24 h. Betaine treatment resulted in significant up-regulation of the tumor suppressor gene PTEN and down-regulation of important pro-apoptotic and anti-apoptotic genes including BCL-2, AKT-1, and NFKB. Pro-apoptotic markers such as BAX and CASP3 were also significantly down-regulated in a dose-dependent manner. Results are shown as $\log_{10}$ fold changes compared to the untreated control (UNT); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.2.5. Analysis of protein levels using ELISA

Data obtained by ELISA show that betaine causes significant biochemical changes on apoptosis, oxidative stress and antioxidant defense systems in U87 glioblastoma cells. CASP3 **(**Figure 8F) and CYCS (Figure 8G) protein levels showed significant increases, especially at 1 mg/ml betaine dose (**p < 0.01), indicating that apoptosis is activated through the mitochondrial pathway. However, no significant change was observed in IL-6, IL-10 and TNF-α levels (Figure 8C, D, E), which represent the inflammatory response, indicating that betaine acts through intracellular signaling pathways rather than a direct inflammatory suppression effect on cytokine levels. There was no significant change in SEMA3A and SEMA3E levels (Figure 8A, B), indicating that betaine's anticancer effects may be independent of semaphorin-mediated signaling. In the evaluations made on antioxidant system, while a significant increase was observed in total antioxidant capacity (TAS) level at 1 mg/ml dose in 1 mg/ml betaine application (Figure 8I, **p < 0.01), this value decreased at 2 mg/ml dose. In contrast, total oxidant level (TOS) decreased significantly at 2 mg/ml dose (Figure 8İ, *p < 0.05). These results show that betaine at low dose strengthens intracellular antioxidant defense, while at high dose it directly suppresses ROS production and reduces net oxidative stress. In addition, the significant decrease observed in GPX4 level (Figure 8H, ****p < 0.0001) suggests that lipid peroxidation-sensitive cell death forms such as ferroptosis may also be involved in the process.

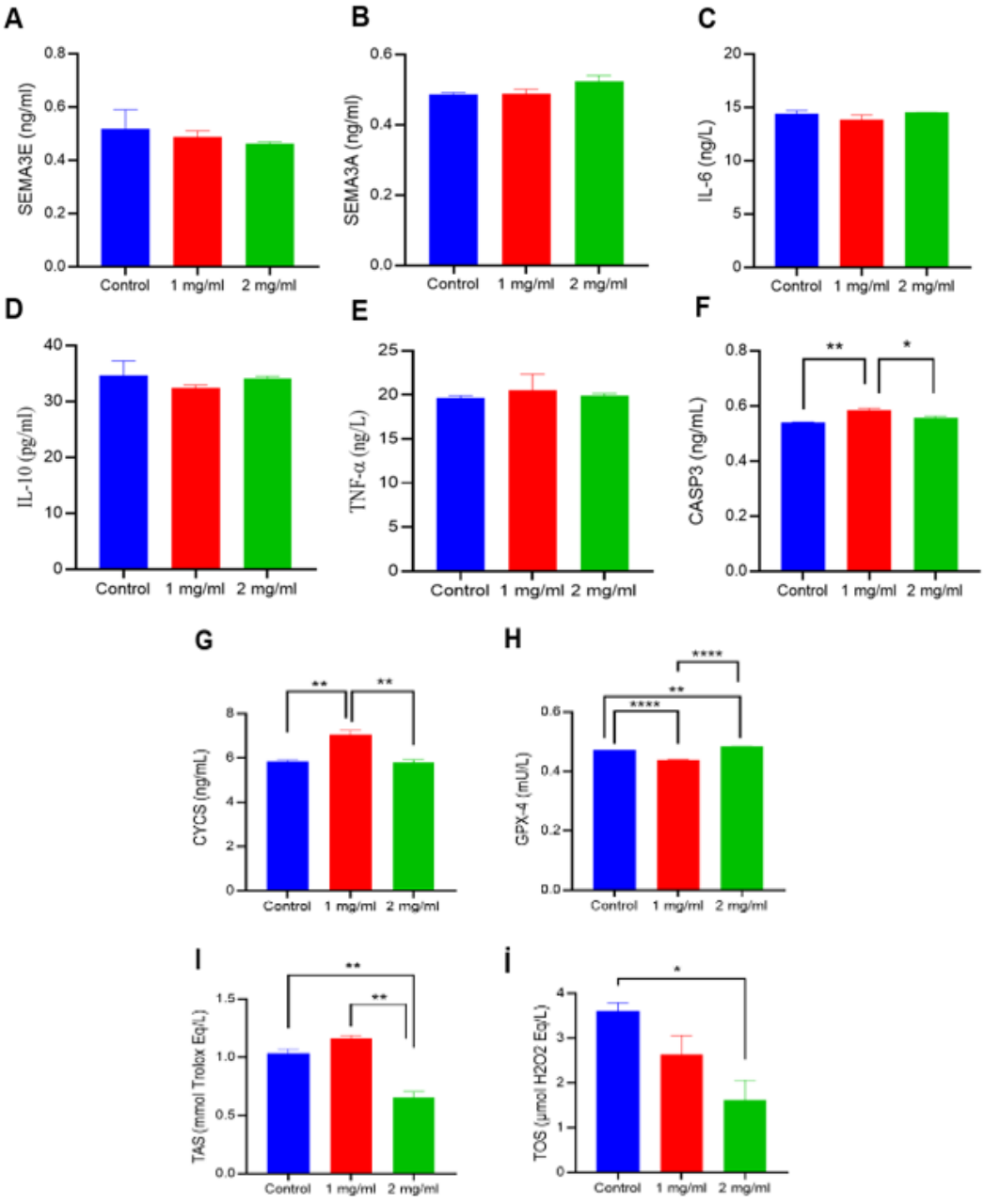

Fig.8. Quantification of multiple protein and oxidative stress markers in betaine-treated U87 cells. (A–B) ELISA analysis, no significant changes were observed in the levels of SEMA3E and SEMA3A after treatment among the groups. (C–E) The levels of proinflammatory cytokine IL-6, anti-inflammatory cytokine IL-10, and TNF-α also remained stable. (F) A significant increase in CASP3 levels was observed at both 1 mg/mL (**p < 0.01) and 2 mg/mL (*p < 0.05) betaine doses. (G) Cytochrome c (CYCS) was significantly elevated at 1 mg/mL (**p < 0.01), indicating mitochondrial pathway activation. (H) GPX4 levels were significantly decreased in both treatment groups (**p < 0.01, ****p < 0.0001), supporting ROS-independent apoptosis (I–İ) Total antioxidant capacity (TAC) was significantly decreased upon 2 mg/mL betaine administration (**p < 0.01), while total oxidant levels (TOS) decreased in a dose-dependent manner upon treatment (*p < 0.05).

4. Discussion

The outcomes of the present investigation provide compelling evidence that betaine exerts a significant cytotoxic effect on U87 glioblastoma cells, primarily through the induction of apoptosis via mitochondrial pathways. This is underscored by increased Annexin V/PI staining and decreased mitochondrial membrane potential following betaine treatment, consistent with the activation of the intrinsic apoptotic cascade. Notably, these changes were observed alongside a marked suppression of ROS levels, suggesting a redox-independent mechanism of apoptosis induction—an observation that contrasts with traditional views of oxidative stress as a pro-apoptotic trigger.

This paradoxical relationship between ROS suppression and increased apoptosis in glioblastoma cells offers an intriguing contrast to earlier reports in other cancer models. For instance, in studies involving colon cancer models, Kim et al. [7] observed that betaine reduced tumor formation by decreasing ROS and inflammatory cytokines such as TNF-α, IL-6, and COX-2. These effects were interpreted as protective, preventing DNA damage and tumor initiation. In our context, however, the suppression of ROS by betaine appears to disrupt redox signaling homeostasis in glioblastoma cells, potentially interfering with ROS-mediated survival signals and thereby facilitating apoptosis. This interpretation aligns with the growing recognition that cancer cell dependency on basal ROS levels for proliferation creates a therapeutic window that can be exploited by either increasing or depleting ROS.

Our gene expression results further support this mechanism, with significant upregulation of mitochondrial apoptotic markers such as CASP3 and CYCS, along with a reduction in anti-apoptotic BCL-2 levels. These findings are consistent with previous work by Guo et al. [5], who reported that betaine induced apoptosis in HeLa cells via increased caspase-3 and p53 expression while suppressing BCL-2. Additionally, we observed a downregulation of NF-κB gene expression, suggesting a broader suppression of survival and inflammatory signaling. Kar et al. [4] similarly demonstrated that betaine inhibited NF-κB and promoted apoptosis in prostate cancer cells.

The comparison between U87 glioblastoma cells and HEK-293 epithelial cells revealed that betaine exerts a more pronounced cytotoxic effect on cancer cells, as demonstrated by a sharper decline in viability at lower concentrations and earlier time points. While HEK-293 cells also exhibited reduced viability at higher doses, their overall resistance to betaine supports its preferential targeting of tumorigenic cells. These findings underscore the potential therapeutic window where cancer cells can be effectively targeted while minimizing toxicity to normal tissue analogs.

A unique aspect of our study is its focus on glioblastoma, a malignancy known for its high resistance to conventional therapies and limited responsiveness to immune modulation. The findings suggest that betaine's pro-apoptotic effects are not limited to gastrointestinal or epithelial-origin cancers but extend to neuroepithelial tumors such as glioblastoma. This opens the door for future investigations into whether betaine might enhance the efficacy of existing treatments like temozolomide or radiotherapy through mitochondrial sensitization.

Epidemiological data also lend support to the anticancer potential of betaine. Xu et al.[9] demonstrated that higher dietary intake of choline and betaine was associated with improved survival in breast cancer patients and highlighted the role of one-carbon metabolism and DNA methylation as potential mediators. Although our study did not explore epigenetic markers, it is plausible that betaine's function as a methyl donor may contribute to gene regulation in glioblastoma cells. Given the known epigenetic alterations in GBM, including DNA hypermethylation and histone modifications, further work is warranted to explore this dimension.

Furthermore, the inclusion of HEK-293 cells as a comparative control allowed for the evaluation of betaine's selectivity toward glioblastoma cells over non-tumorigenic counterparts, which is essential in assessing its therapeutic potential.

In conclusion, our study adds to the growing body of evidence that betaine possesses significant anticancer properties and may induce apoptosis in glioblastoma cells through mitochondrial and redox-independent mechanisms. This distinguishes its action in brain tumors from its well-documented anti-inflammatory and antioxidative roles in other cancer types. Future research should focus on in vivo models to validate these effects and on combinatorial studies integrating betaine with standard-of-care treatments to evaluate its translational potential in glioblastoma therapy.

Conflict of Interest

The authors declare that there are no conflicts of interest related to this study.

Acknowledgments

OpenAI and GoogleAI were utilized to enhance the wording used in the text.

Author Contributions

NM was responsible for designing the study, planning the experiments, collecting the data, and performing the data analysis. EK and FK contributed to the experimental work and aided in analyzing the data.

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