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Apoptotic effect of antioxidants with silver and titanium dioxide nanoparticles on glioblastoma cancer cells in BALB/C mice

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Glioblastoma is one of the most aggressive cancers affecting people globally. Numerous studies have demonstrated that nanoparticles possess potential anti-cancer properties. Nanoparticles have shown promising results in delivering drugs to the brain, although they can have side effects. One possible solution to these issues is the development of nanoparticles combined with antioxidants to improve their efficacy. This study aimed to investigate the impact of curcumin, vitamin C, and vitamin E, in combination with silver and titanium dioxide nanoparticles, on cytotoxicity and their effects on TFAM and miR-455 expression. In this study, the glioma model was induced in female BALB/C mice by implanted Gl261 cells. After two weeks, the animals were sacrificed, and tumor tissue samples were collected to evaluate apoptosis levels using MTT and flow cytometry. In addition, RNA was extracted to examine the expression of Bax, Bcl-2, miR-455, and TFAM genes in the tumor cells. Our study reveals the positive impact of combining vitamin C, vitamin E, and curcumin with Ag and TiO2 NPs on altering Bax and Bcl-2 gene expression, thereby enhancing apoptosis. The data indicated that using nanoparticles in conjunction with antioxidants decreased the levels of TFAM and miR-455, which could potentially reduce the growth of glioma tumor cells. These results suggest that combining antioxidants with Ag and TiO2 nanoparticles can significantly enhance their apoptosis-inducing effects. This combination therapy can prevent metastasis by reducing the expression of miR-455 and TFAM. Overall, this strategy improves glioma cancer therapy by targeting genes involved in tumorigenesis.

Keyword: Curcumin, Glioblastoma, miR-455, Nanoparticles, TFAM, Vitamin C, Vitamin E

Glioblastoma multiforme (GBM) accounts for an estimated 48.6% of malignant central nervous system (CNS) tumors, with an incidence of over 0.59 to 5 per 100, 000 cases in a year[1,2]. Malignant brain tumors are highly lethal diseases with a grim outlook, as the average survival rate of patients after medical treatment is less than two years[3,4]. The major obstacles to drug efficacy in treating brain tumors are the difficulty in transporting drugs across the blood-brain barrier, optimal distribution of drugs in cancer, and minimizing the destruction of healthy cells in addition to tumor cells by drug delivery. Recently, nanotechnology has gained significant attention for addressing these challenges in brain tumor treatment. Nanoparticles can cross the blood-brain barrier, target cancer cells with high specificity, and deliver therapeutic agents directly to the tumor. The potential use of nanoparticles for delivering proteins and other macromolecules across the blood-brain barrier suggests that this technology holds significant promise for the non-invasive treatment of CNS disorders, including neoplasms[3].

A wide range of nanoparticles have been developed to facilitate the transport of therapeutic drugs across the blood-brain barrier. Biodegradable polymers and metal nanoparticles can be employed to deliver drugs for treating human gliomas by crossing this barrier[5,6]. Recent research has shown that silver nanoparticles (Ag NPs) offer a unique approach to tumor treatment, particularly for tumors of neuroepithelial origin[27]. Ag NPs inhibit the proliferation of GBM cells and exhibit proapoptotic properties[7,8]. These nanoparticles exert potent anti-tumor effects through mechanisms such as oxidative stress, DNA damage, cell cycle arrest, and apoptosis induction[6,9]. Mitochondria are the powerhouses of cells and control the critical metabolic functions required for cellular survival. During cancer growth, development, and apoptosis, various mitochondrial metabolic pathways, including oxidative phosphorylation and reactive oxygen

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species (ROS) production, become disrupted[10]. ROS a class of highly bioactive molecules, have been extensively studied in various types of cancer. Typically, cancer cells have higher basal levels of ROS compared to normal cells due to an imbalance between oxidants and antioxidants. ROS play a dual role in cell metabolism: at low to moderate concentrations, ROS act as signal transducers and activate cell proliferation, migration, invasion and angiogenesis. In contrast, high concentrations of ROS can damage proteins, nucleic acids, lipids, membranes and organelles and lead to cell death. Extensive studies have shown that cancer therapies that manipulate ROS levels show promising *in vitro* and *in vivo* results[10,11]. Several studies have shown that nanomaterials cause alterations in mitochondrial morphology and respiratory function and promote cytochrome C release. Nanoparticles induce apoptosis by translocating cytochrome C into the cytoplasm and Bax into the mitochondria[12, 13]. Silver nanoparticles (Ag NPs) can be combined with chemotherapeutic agents for treating glioblastoma multiforme (GBM). This combination enables the use of lower doses of anticancer drugs while reducing the cytotoxic impact on normal cells[7].

Some nanocarriers are toxic to tumor cells[14]. TiO2 or metallic nanoparticles (≤100 nm) can pass the blood-brain barrier, so they could work well for delivering drugs to brain tumors. The results of Glaser *et al*. suggest that TiO2 would trigger an increase in free radical production and cell death in glioblastoma following irradiation. Titanium dioxide causes membrane damage and DNA fragmentation, which is characteristic of apoptosis[15,16]. The main mechanism underlining the potential toxicity induced by TiO2 NPs seems to be related to the production of reactive oxygen species (ROS) leading to oxidative stress, inflammation, genotoxicity, metabolic alterations, and potential carcinogenic[17]. In recent years, there have been several publications on the toxicities caused by nanoparticles and their harmful mechanisms of action, but also on the beneficial effects of NPs synthesized with antioxidant compounds such as vitamins, minerals, natural substances, or plant extracts[18]. However, there are only a few studies investigating combination therapies involving nanoparticles and antioxidants in the treatment of glioblastoma cancer. The objective of this study was to evaluate the effects of curcumin, vitamin C, and vitamin E on apoptotic genes in mitochondria by co-administering silver and titanium dioxide nanoparticles.

Materials and Methods

Ethical Statement

Female BALB/c mice (5-7 weeks old and weighing approximately 25-30 g) were purchased from the Pasteur Institute of Iran. The animals were housed in the animal house of the molecular biology laboratory under standard laboratory conditions at a temperature (22 ± 1°C, humidity 65.5%) and a 12 h light cycle with unrestricted access to both food and water. The protocol of this study was reviewed and approved by the IAU Ethics Committee (IR.IAU.PS. REC.1399.024).

Preparation of GL261 cells

The GL261 is a mouse glioma cell line obtained from the Pasteur Institute of Iran and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C and 5% CO2. The cell lines were maintained and passaged to 70% confluence, then the media were changed, and the cells were grown in serum-free media. For tumor implantation, cells were washed with 0.05% trypsin–EDTA and resuspended in phosphate-buffered saline (PBS) at a final concentration of 5×10^4 cells/ 5 μL. The GL261 cells were injected into female BALB/C mice.

Stereotaxic intracranial tumor implantation

All procedures involving mice were conducted according to the Guidelines of Animal Experiments of Royan Institute. GL261 cells were injected into the brains of BALB/c mice. Mice were positioned in a stereotactic frame and anesthesia mask. Surgical anesthesia was maintained with 2% isoflurane mixed with oxygen. After cleaning the surgical area, an incision was made in the midline of the calvarium extending from the bregma to the lambda suture. The coordinates of the target site for the underlying right striatum were: 2 mm posterior to the bregma, 2 mm lateral to the coronal suture, and 4 mm dorsal ventral to the exposed dura. Using a 10 μL gas-tight Hamilton syringe, a GL261 cell line was stereotactically injected through a 1.2 mm burrhole in the calvarium into the underlying target site[19]. On day 14 after surgery, three mice from different cages were sacrificed. The tumor was analyzed by a pathologist to confirm the cancer diagnosis.

Evaluation of cytotoxicity activity on GL261 cells line (MTT)

The cytotoxicity of Ag NPs and TiO2 NPs was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Approximately 1×10^5 cell/mL GL261 cells in the exponential growth phase were seeded in a 96-well flat-bottom polystyrene-coated plate and incubated at 37°C in a 5% CO2 incubator for 24 h. The GL261 cell line were treated with a series of concentrations Ag NPs, TiO2 nanoparticles, vitamin E, vitamin C and curcumin dilutions (20, 50, 80, 110, and 140 μg/mL) to investigate the cytotoxicity. After 24 h of incubation, 10 μL of MTT reagent was added to each well and incubated for another 4 h. Formazan crystals formed in eachwellafter 4 h weredissolved in DMSO (50 mL), and the plateswereimmediatelyread in a microplatereader (BioTek-ELx800, USA) at 570 nm. Wellswithcompletemedium, nanoparticles, and MTT reagent, withoutcells, wereusedas blanks[20].

Study design

BALB/c mice were used in this study, The mice were randomly divided into two groups healthy and tumoral mice, and the tumoral group received the GL261 cell line. After two weeks, when the tumor volume in mice reached 600–800 mm^3 in diameter, the tumoral mice were divided into nine groups (2-4 mice per group): 1) vehicle, injected with PBS; 2) Ag NPs; 3) Ag NPs and curcumin; 4) Ag NPs and vitamin E; 5) Ag NPs and vitamin C; 6) TiO2NP; 7) TiO2 NP and curcumin; 8) TiO2 NP and vitamin E; 9) TiO2 NP and vitamin C. This extract was injected (1 mg/kg) into the experimental mice's brains, The concentration of injection was determined based on inhibitory concentration associated with 50%[20] effect (IC_{50}), then the mice were rested for ten days. In addition, as with the experimental groups, we had control groups with healthy mice. After ten days, all mice were then sacrificed, and tumors were harvested for further examination by flow cytometry, MTT, and real-time PCR.

Preparation of cell suspensions from brain tumor

Brain tumor samples were obtained from BALB/c mice through surgery. Brain samples were transferred to the laboratory, and the tumor biopsy was washed with phosphate buffer saline containing 100 U/mL penicillin, 100 μg/mL streptomycin. Cells were done in sterile DMEM/F-12 1:1 (V: V) and Pen/Strep under sanitary conditions in the cell culture room. The preparation of single-cell suspensions of the tumor was carried out with the Enzymatic method.

To obtain single-cell suspensions, samples of tumor tissues were mixed with collagenase III to a final concentration of 0.2 mg/mL in a serum-free medium, DMEM/F-12, and Pen/Strep and incubated at 37°C overnight. Afterward, the enzymatically digested tissue was filtered through a 45-μl nylon mesh, washed with DMEM/F-12, and washed twice with PBS. Obtained cells from the last step were filtered again through sterile 0.2 μM nylon mesh too mitbacterial contaminations. The cellswerewashed in DMEM/F-12 and Pen/Strep and centrifuged at 1000×g; resuspension and centrifugation were repeated three times. Then, the tumor-cell cultured in standard tissue culture flasks.

Cytotoxicity assay

The MTT assay was used to analyze the toxicity of nanoparticles in tumor cells. In our assays, 200 μL of tumor cells at $5 \times 10^4 – 1 \times 10^5$ cells/mL were added in triplicate to a 96-well plate and incubated for 24 h. After 24 h, the cell solution was removed, replaced with 200 μL medium (vehicle control) or test substances, and incubated for 24 h. After incubation, the medium was removed and replacedwith the MTT reagent, and cells were incubated for another 4 h. The cells were then washed and add 100 μL DMSO to dissolve the formazan. After 10 min, the plates were analyzed using a BioTek-ELx800 Plus micro platereader at 570 nm.

Annexin V-FITC/propidium iodide apoptosis assay

Tumor cells isolated from mice brains were placed in a six-well culture plate and treated with 160μg/mL and 320μg/mL of AgNPs, TiO2 NPs, and antioxidants for 24 h. Accordingto the manufacturer'sinstructions, normal, necroticcells, and apoptotic were distinguished using an Annexin V-FITC/propidium iodide assay kit (TreeStar Inc., version 9.6.3). Then, cells (1.0×10^6) were washed with PBS, resuspended in 400 μL of bindingbuffer, and added 5 μL of Annexin V-FITC to the samples. Afterincubation for 15 minat 4°C in the dark, 10 μL of propidiumiodidewasadded and incubated for fiveminat 4°C[21]. The flow cytometry analysis was performed.

Extraction of RNA and Reverse Transcription

The RNeasy Kit (GeneAll Biotechnology, South Korea) was used to extract total RNA from the brain tissue of experimental mice, and the amount and integrity of the extracted RNA were analyzed using the Nano Drop® ND-1000 spectrophotometer. The cDNA synthesis kit (Daejeon, South Korea) was used to transcribe the RNA from each sample into cDNA according to the manufacturer's instructions. The cDNA samples were then stored at −20C until required.

Quantitative Real-Time PCR

The TFAM, BCL2, BAX and mir455 levels were quantified using the real-time PCR thermal cycler (Gene All Biotechnology, South Korea). The specific forward and reverse primers and their corresponding NCBI Gene Bank accession numbers for each gene are listed in (Table 1). Real-time PCR amplifications were performed in 25 µL of total reaction mixtures containing 2 µL of reverse cDNA, 1 µL of each specific primer for each gene (forward and reverse), 12.5 µL of 1xSYBR Green PCR Master, and 8.5 µL of RNase-free H_2O. The thermal cycling protocol consisted of an initial denaturation at 95°C for approximately 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec, with a final elongation step at 72°C for 30 sec. At the conclusion of the qRT-PCR cycles, melting curves were generated to confirm the precise amplification of the target gene products. The relative expression of the gene in each sample was normalized compared to the GAPDH gene and calculated using the $2^{-\Delta\Delta Ct}$ method[22].

Table 1 — Primer sequences used in real-time quantitative PCR reaction

Target Gene	Primer Sequence
ACTB	F: CATTGCTGACAGGATGCAGAAGG
	R: TGCTGGAAGGTGGACAGTGAGG
Bcl2	F: CCTGTGGATGACTGAGTACCTG
	R: AGCCAGGAGAAATCAAACAGAGG
Bax	F: AGGATGCGTC2CACCAAGAAGCT
	R: TCCGTGTCCACGTCAGCAATCA
TFAM	F: GAGGCAAAGGATGATTCGGCTC
	R: CGAATCCTATCATCTTTAGCAAGC
mir-455	F: GCAGTCCATGGGCATATACAC
	R: GCTGTCAACGATACGCTACCTA

Statistical analysis

Two-way analysis of variance (ANOVA) was used to identify significant differences in the studied traits between groups. In addition, SPSS software version 22 and GraphPad Prism software Version 9 were used for data analysis. A p-value less than 0.05 was regarded as statistically significant.

Result

Nanoparticles decrease GL261 cell viability

To determine the cytotoxic activity, experiments were performed *in vitro* at different concentrations by MTT assay on GL261 cells. As shown in (Fig. 1), the nanoparticles and antioxidants showed significant cytotoxicity to GL261 cells. The GL261 cell line was assessed for cytotoxic activity at various concentrations of the vehicle control over 24, 48, and 72 h periods (Table 2). The treatments mentioned above resulted in a decrease in cell viability. The Ag

Table 2 — IC_{50} values evaluated after 24, 48 and 72 h exposure of GL261 cells to nanoparticles (Ag and TiO2), antioxidant (vitamin C, vitamin E and curcumin)

Samples	IC_{50}(µg/mL)		
	24 h	48 h	72 h
Ag NPs	-	45.49	34.94
TiO2 NPs	-	8.73	6.31
curcumin	-	1.2	0.85
vitamin E	-	3.48	2.7
vitamin C	-	81.25	68.7

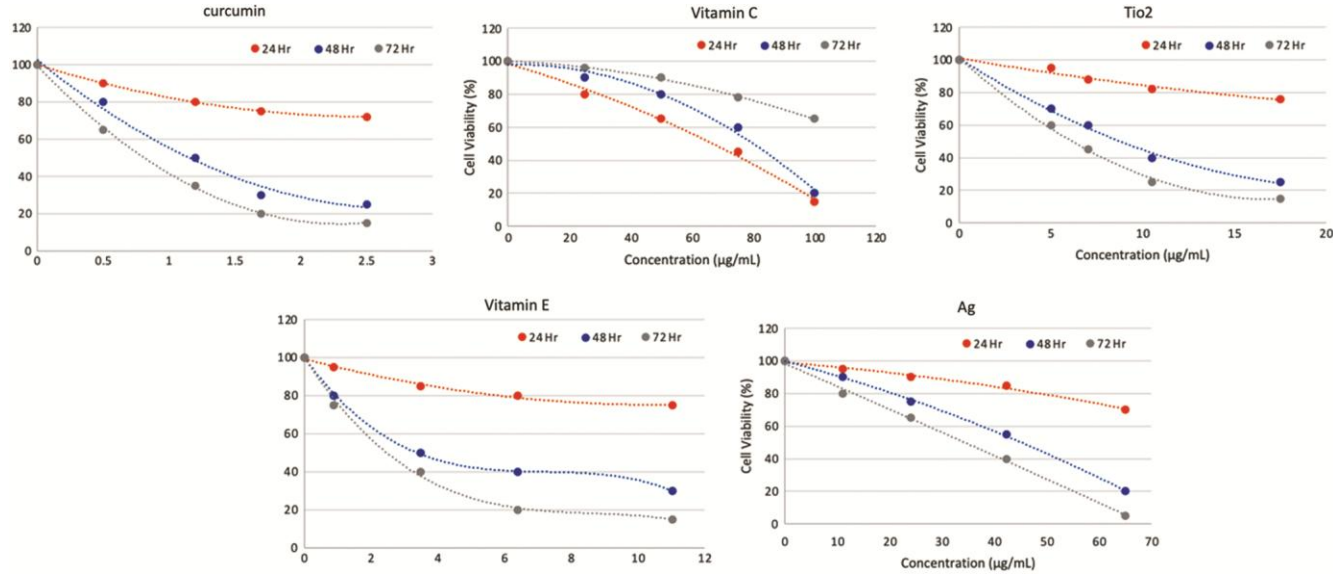

Fig. 1 — Nanoparticles and antioxidant show cytotoxicity activity in a time and dose dependent manner. Cell viability evaluated after 24, 48 and 72 h exposure of cells to nanoparticles (Ag and TiO2 NPs) and antioxidant (vitamin C, vitamin E and curcumin), n = 3

NPs showed a stronger toxic effect at 40 µg/mL and the TiO2-NPs showed a stronger toxic effect at 140 µg/mL. Vitamin C showed a stronger toxic effect at 100 µg/mL, vitamin E showed a stronger toxic effect at 200 µg/mL and curcumin showed a stronger toxic effect at 2 µg/mL.

Anticancer activity

To evaluate the potential of this treatment to enhance the antitumor activity of nanoparticles, a series of experiments were conducted using an MTT assay on glioma tumor cells. As shown in Figure 2, the nanoparticles exhibited significant cytotoxicity to glioma cells. ($P< 0.05$). Results demonstrated Curcumin (50%) has a more toxic effect than vitamin C (59%) and vitamin E (60%). And TiO2 nanoparticles also showed higher toxicity than Ag-NP (57% and 59%, respectively). The study discovered that combining curcumin with TiO2 and Ag-NP nanoparticles led to a stronger antitumor effect (24% and 32% increase, respectively).

Flow cytometry analysis of glioma cell apoptosis

To further confirm and detect the apoptosis effect on tumor cells induced by the nanoparticles and antioxidants, Annexin V-FITC /propidium iodide (PI) double-staining technique was used. It's important to identify cells in the early stage of apoptosis using distinct double staining patterns: viable cells (Annexin V- and PI-, lower left square), early apoptotic cells (Annexin V+ and PI-, lower right square), and late apoptotic cells (Annexin V+ and PI+, upper right square). Figure 3 results indicate that curcumin, vitamin C, and vitamin E treatments cause greater early apoptosis (24%, 17.5%, and 15.3%, respectively) compared to late apoptosis (20.7%, 14.8%, and 10%, respectively) in cancer cells, and there is no significant difference in rates between early and late apoptosis. Ag NPs increased apoptosis in tumor groups, while TiO2 nanoparticles did not significantly increase apoptosis in glioma cells. These results indicate that curcumin, vitamin C, and vitamin E can promote early cell death in glioma cells and that silver nanoparticles have the potential to induce cell death in both early and late stages. On the other hand, the TiO2 nanoparticles did not demonstrate a significant impact on apoptosis in glioma cells.

In this study, Ag NPs and TiO2 nanoparticles were used, and discovered that some treatments led to an increase in apoptosis. Using nanoparticles with curcumin or vitamin E resulted in a significant change in mortality rate. The balance shifted towards apoptosis. When TiO2 nanoparticles were used with curcumin, vitamin C, and vitamin E, there was a significant improvement in their effects (45%, 38%, and 40%, respectively). A significant difference was also observed when using Ag-NP nanoparticles with the antioxidants curcumin, vitamin C, and vitamin E, resulting in reductions of 48%, 28%, and 42%, respectively. The present study has revealed the significant impact of curcumin on enhancing the apoptosis of tumor cells when combined with nanoparticles. The results of this figure are remarkable, in (Fig. 4).

Effect of Ag NPs and TiO2 nanoparticles and antioxidant on mRNA Expression Patterns of BCL2, and BAX

We studied how silver nanoparticles, titanium dioxide nanoparticles, vitamin C, vitamin E, and curcumin affect the expression of BCL2 and BAX genes in female mice with glioma. The BCL2 proteins are important for controlling cell death and are known to be expressed differently in various types of cancer. The results of treatment with nanoparticles and antioxidants show an increase in BCL2 gene

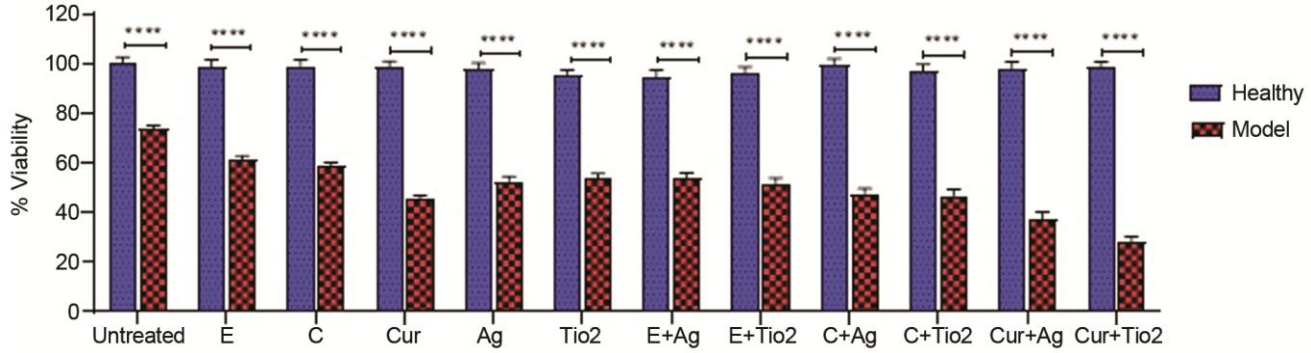

Fig. 2 — Glioma cell viability evaluation treated by nanoparticles and antioxidant. The data are expressed as a percentage of cell viability and represent the average ± standard error mean values (n=3). In all the individual experiments, control (without treatment) was taken as 100% viable. ($P< 0.05$)

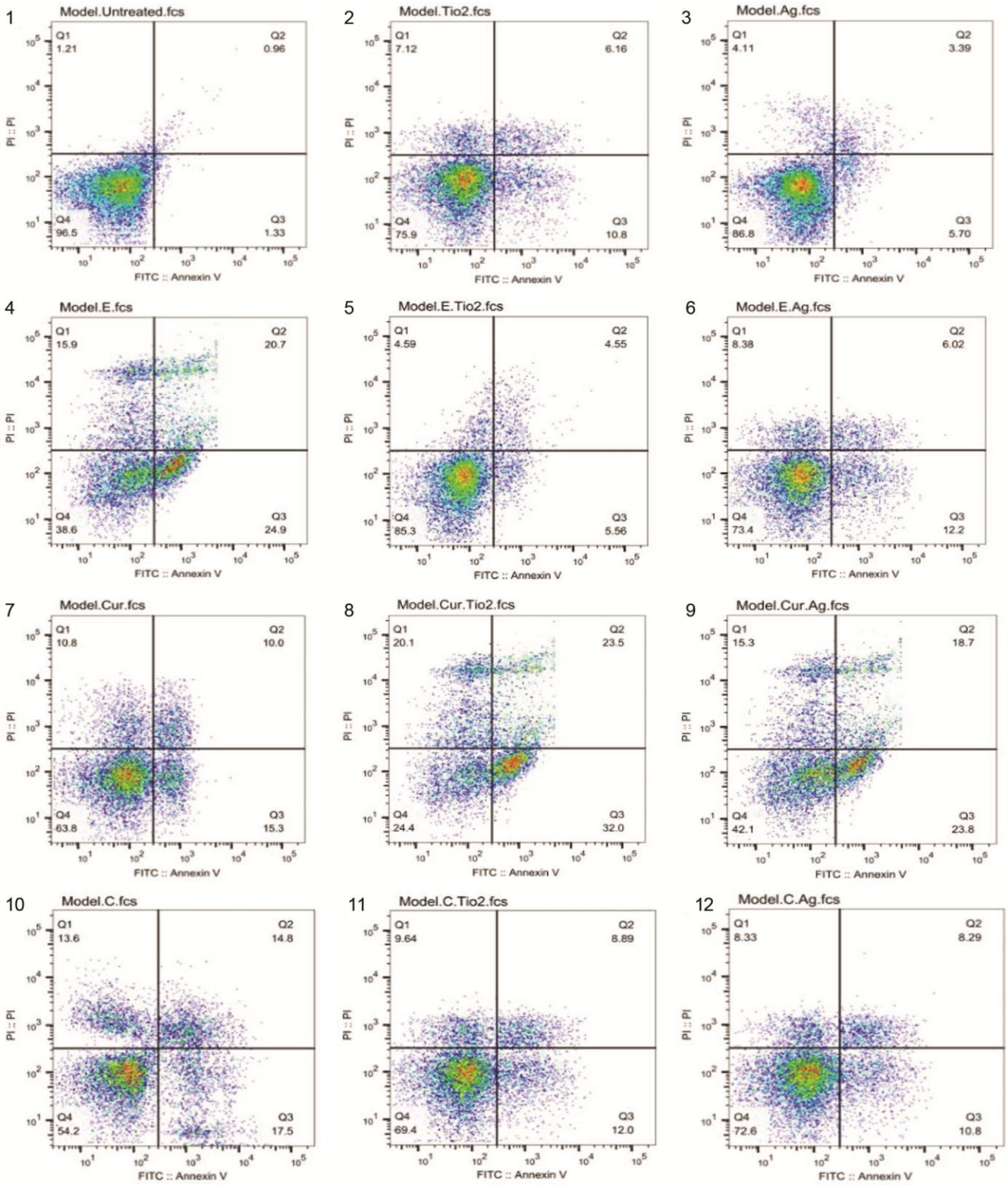

Fig. 3 — Annexin V/PI double-staining assay of glioma tumor cells. Flow cytometric analysis results of nanoparticles (Ag NPs and TiO2 NPs), antioxidant (vitamin C, vitamin E and curcumin) and nanoparticles+ antioxidant are shown

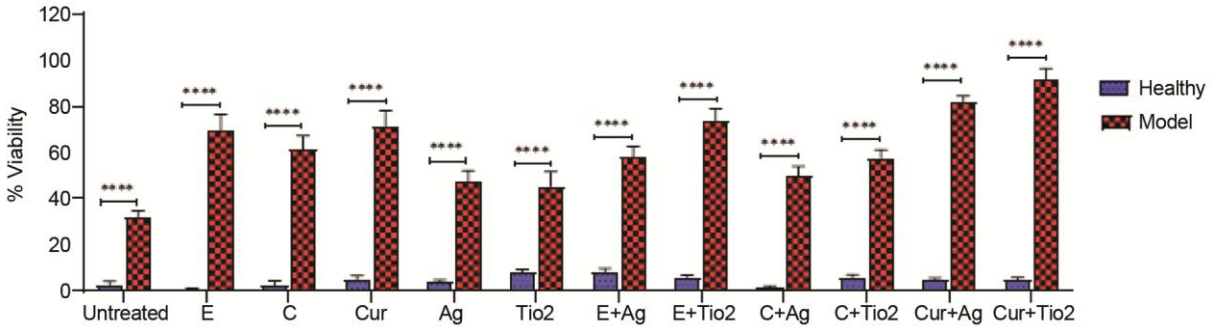

Fig. 4 — A statistical graph of annexin V-FITC/PI staining is shown. Concentration-apoptotic effects of nanoparticles and antioxidant against glioma cells. The results are expressed as the means± standard Apoptotic cells included the Annexin V + /PI − cells and the Annexin V + /PI + cells. *** $P< 0.05$

expression in all groups, with the highest increase observed in the curcumin + Tio2 group (0.156-fold) compared to the control group ($P< 0.05$). In the other group, it was noticed that combining vitamin C and vitamin E with nanoparticles increased BCL2 mRNA expression more than using nanoparticles alone. These values are reported for the, Ag NPs +vitamin E (0.744-fold), Ag NPs +vitamin C (0.696-fold), and Ag NPs +curcumin (0.517-fold). These values are given for the TiO2 +vitamin E (0.77-fold), TiO2 +vitamin C (0.718-fold), and TiO2+Curcumin (0.433-fold) groups (Fig. 5A).

In contrast, the expression of Bax was found to be significantly reduced in all groups. The curcumin +Ag NPs (2.343-fold) and the group with curcumin+Tio2 (1.24-fold) showed the greatest reduction in Bax expression. In another group, it was found that combining vitamin E, and vitamin C in nanoparticles reduced the expression of the Bax gene more than when using nanoparticles alone. These values are reported for, Ag NPs +vitamin E (1.428-fold), and Ag NPs +vitamin C (1.665-fold). These values are reported for the TiO2 + vitamin E (1.427-fold), and TiO2 + vitamin C (1.78-fold) groups. (Fig. 5B).

The Bax to Bcl-2 ratio determines if cells live or die when exposed to a signal for cell death. The results showed that Bax gene expression and Bax/Bcl2 ratio were significantly higher ($P< 0.05$) in the trained group than in the control group. The most ratio was in curcumin+Tio2 (19.36-fold) group (Fig. 5C).

Effect of Ag NPs and TiO2 nanoparticles and antioxidant on mRNA Expression Patterns of TFAM and mir455

Mitochondrial transcription factor A (TFAM) is a novel diagnostic marker and therapeutic target for gliomas and other cancers. The impact of Ag NPs and TiO2 nanoparticles and antioxidants on TFAM mRNA expression patterns in glioma tissues of female mice is depicted in (Fig. 6A). The results of nanoparticle and antioxidant treatment show a decrease in TFAM gene expression in some tumoral groups compared to control. The strongest decrease was observed in the curcumin + Ag NPs group in the tumor group compared to the control group (0.342-fold) ($P< 0.05$). Furthermore, it has been observed in various groups that the combination of vitamin C and vitamin E with nanoparticles resulted in a greater reduction of TFAM expression compared to groups using nanoparticles alone. The reported values show significant reductions for the Ag NPs + vitamin C (0.71-fold), and Ag NPs + curcumin (0.386-fold). These values are given for the TiO2 +vitamin C (1.497-fold) and TiO2+Curcumin (0.746-fold) groups. The reported values show nonsignificant reductions for the Ag NPs + vitamin E (0.438-fold) and TiO2 +vitamin E (1.124-fold) in compared tumoral group with control.

A recent study suggests that miR-455 is significantly increased in glioma cell lines and may regulate tumor cell progression. The impact of Ag NPs and TiO2 nanoparticles and antioxidants on miR-455 expression patterns in glioma tissues from female mice is depicted in (Fig. 6B). The results of treatment with nanoparticles and antioxidants show a decrease in miR-455 gene expression in the Ag tumoral compared to the control. These values are reported for the Ag NPs +vitamin E (1.79-fold) and Ag NPs +curcumin (1.875-fold). The provided values represent the fold increase for the TiO2 + vitamin E (1.85-fold), TiO2 + vitamin C (2.48-fold), and TiO2 + curcumin (1.785-fold) groups. In addition, the strongest decrease was observed in the combination of nanoparticles with curcumin in the tumor group compared to the control group, as it showed a significant down-regulation twofold ($P< 0.05$).

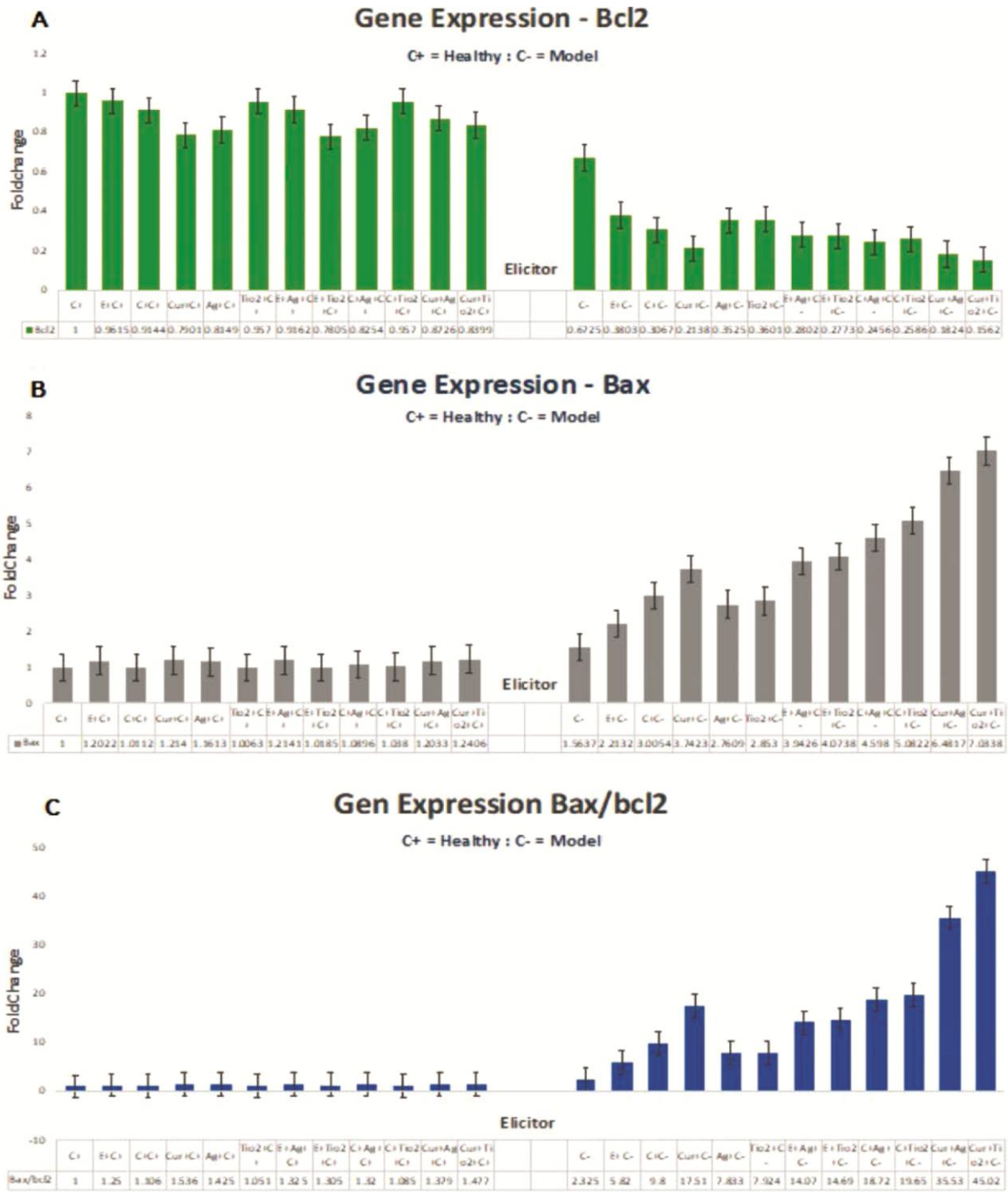

Fig. 5 — Expression of Bax and Bcl-2 in glioma cancer cells, analyzed with real-time PCR. (A) Relative Bcl2 gene levels; (B) Relative Bax gene levels; and (C) Bax: Bcl-2 ratios of cells. The healthy cell was applied for control. (n=3; $P< 0.05$)

Discussion

Nanoparticles have been used in various therapeutic areas in recent years[23, 24]. Several nanomaterials have been investigated as carriers of therapeutic agents for the treatment of GBMs. Utilizing brain-specific drug delivery systems in combination with multimodal therapies offers a promising strategy for treating glioblastoma. This study aimed to discover ainnovativeapproach for cancer treatment by using nanoparticles combined

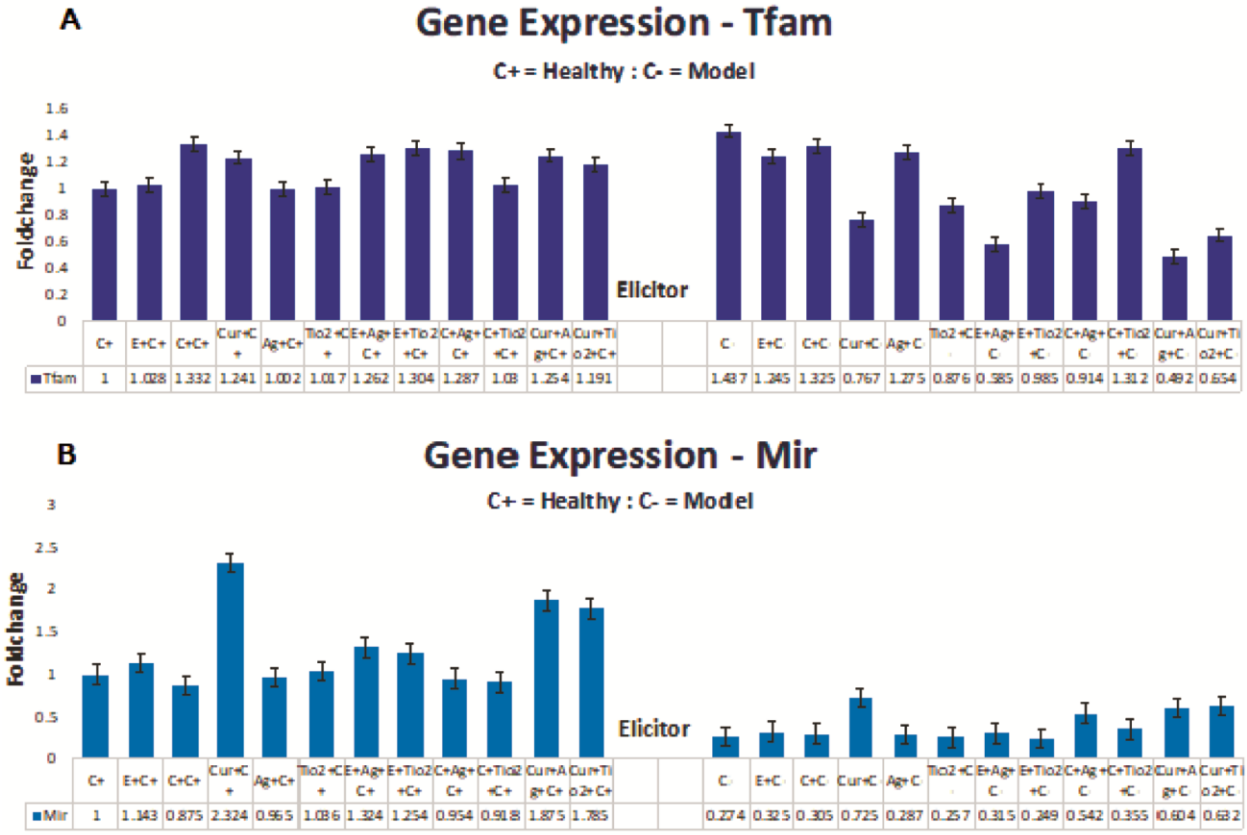

Fig. 6 — Expression of TFAM and miR-455 in glioma cancer cells, analyzed with real-time PCR. (A) Relative TFAM gene levels; and (B) Relative miR-455 gene levels. The healthy cell was applied for control (n=3; $P < 0.05$)

with antioxidants. The research demonstrated that this combination has significantly enhanced cytotoxic effects against glioma tumors compared to nanoparticles alone. This combination therapy demonstrated potent antitumor activity through its ability to increase apoptosis and induce changes in the expression patterns of TFAM, BCL2, BAX, and miR-455 genes in response to glioma tumor treatment.

Silver nanoparticles (Ag NPs) are among the most commonly utilized nanomaterials, renowned for their exceptional catalytic activity as well as their remarkable antitumor and antibacterial properties[25,26]. Ag NPs have also been found to exert cytotoxic effects[27]. The use of this nanotool against human GBM stem cells *in vitro* or Ehrlich ascites carcinoma cells *in vivo* showed that it triggered cell cycle arrest in the G2/M phase and reduced the growth rate of GBM cells[28]. Other studies have also confirmed that Ag NPs interfere with certain molecular signaling pathways associated with inflammation or cellular repair processes, such as the mitogen-activated protein kinase (MAPK) cascade, in human glial cells[29]. The results of our study confirm the antiproliferative properties of Ag NPs in GBM cells and suggest that Ag NPs could potentially benefit GBM patients in their cancer treatment. These findings align with previously published research on the subject[30,31]. We know the intensity of apoptosis also has a major impact on tumor growth. Studies have shown a high incidence of apoptosis in spontaneously regressing tumors and tumors treated with cytotoxic agents. Therefore, the estimation of apoptosis in tumor cells, together with cytotoxicity is an important marker for tumor development and has prognostic significance[32]. In our study, the mean apoptosis of tumoral cells cultured *in vivo* was detected by flow cytometry, and Ag NPs increased early and late apoptosis in tumor groups. Additionally, Ag NPs promoted apoptosis, as revealed by an enhancement in Bax /Bcl-2 expression ratio.

Recently, the biological properties of these nanoparticles, particularly their anticancer activity, have been investigated. TiO2 NPs have been studied in different types of cancer cells, such as mesenchymal stem cells, lymphoblastoid breast[33,34], lung[35], epidermal[36], and colon[37] cancer cells.

Biological assays have revealed the promising potential of TiO2 NPs to induce apoptosis and arrest cells at the sub-G1 phase of the cell cycle in cancer cell lines[32]. In this study, TiO2 NPs showed a strong cytotoxic effect on glioma tumor cell populations. Apoptosis and cell cycle assays showed that the TiO2 NPs induced apoptosis. Gene expression analysis confirmed the up-regulation of Bcl2 and down-regulation of Bax, emphasizing the effects of TiO2 NPs on the mitochondrial apoptosis mechanism.

Previous researchers have proven that curcumin improves apoptosis parameters in tumor cells like prostate cancer[39,40]. Curcumin is a bioactive compound that has been demonstrated to effectively reduce cell proliferation in specific colon cancer cells, even in the presence of increased insulin (a potent proliferation)[40,41]. Notably, the combination of curcumin with nanoparticles can effectively regulate the expression of apoptosis-related proteins and induce apoptosis *in vitro*. Treatment with curcumin combined with TiO2 NP nanoparticles and Ag NPs showed significant effects on Bax: Bcl-2 ratios, higher than curcumin alone.

Vitamin C and vitamin E are widely recognized antioxidants, commonly utilized as supportive treatments in cancer therapy. Clinical drug combined with vitamins C and E inhibits GBM cell growth via the caspase-3 death pathway[38]. Numerous studies have investigated the use of Vitamin C and E are commonly used as adjunct treatments in cancer therapy. Clinical drug combined with vitamins C and E inhibits GBM cell growth via the caspase-3 death pathway[42]. Yun *et al* showed that high doses of vitamin C can selectively kill colorectal cancer cells with mutations in KRAS or BRAF[43]. Research has shown that vitamin C triggers cell death in various cancer types, including mesothelioma, pancreatic cancer, leukemia, and renal cell carcinoma[44-46]. Collectively, these findings suggest that high doses of vitamin C may inhibit tumor cell proliferation. Additionally, vitamin C treatment has been associated with an increase in the pro-apoptotic protein Bax, while levels of the anti-apoptotic protein Bcl-2 decreased. In the present study, vitamin C has a cytotoxic effect at high concentrations, and the combination of vitamin C with TiO2 NP nanoparticles and Ag NPs leads to an increase in cytotoxic potency. Treatment of vitamin C with TiO2 NP nanoparticles and Ag NPs has significant effects on Bax: Bcl-2 ratios, which are higher than vitamin C alone.

One of the most interesting therapeutic applications of vitamin E currently being researched is its use as an anti-cancer agent. Certain forms of vitamin E show potent apoptotic activity against a variety of cancer cell types. In addition, previous studies have shown that vitamin E can reduce cancer risk and inhibit cancer growth[47-49]. Studies have also shown that vitamin E can reduce the risk of GBM and improve the quality of life of GBM patients[50]. Vitamin E increases Bcl-2 levels in endothelial cells, which may provide increased protection against apoptosis. In our study, vitamin E caused an increase in Bcl-2 ($P< 0.05$). The Bcl-2: Bax ratio was increased by vitamin E compared to the healthy group.

Cancers are closely associated with mitochondrial dysfunction. TFAM, the mitochondrial transcription factor A, plays a prominent role in the transcription and replication of mt-DNA to synthesize various mitochondrial proteins[51]. It is known that TFAM is crucial for the regulation of mitochondrial DNA and is upregulated in glioma cell lines and glioma tissue samples. Therefore, TFAM could be a new diagnostic marker and therapeutic target for gliomas and other cancers[52]. Our analysis showed that TiO2 NP and Ag NPs nanoparticles have a potential to inhibit TFAM. In addition, combination of antioxidants with nanoparticles can enhance this function.

MicroRNAs have been implicated in the development of various cancers, including gliomas[53]. In patients with glioma, miR-455-3p was found to be significantly upregulated. This particular microRNA has emerged as a novel prognostic biomarker for glioma, with patients exhibiting elevated levels of miR-455-3p showing a significantly reduced 5-year overall survival rate compared to those with lower expression levels. Recent studies have found that nanoparticles combined with curcumin can reduce the mir-455 in breast cancer[54]. Our finding revealed the expression level of MiR-455 decreased in all treated tumoral groups compared with that in the healthy group. In addition, treatment with NPs alone and combined treatment with antioxidants decreased the expression of MiR-455 in the tumoral cells.

Conclusion

GBM is a highly intricate cancer characterized by a network of complex molecular pathways, gene mutations, and diverse tumor microenvironments. We report on the antiproliferative effects of combination therapies of nanoparticles and antioxidants on glioma

cells. The combination of Ag NPs and Tio2 NPs with antioxidants significantly enhanced cell cytotoxicity compared to control group. We hypothesized that each nanoparticle and antioxidant would decrease cell viability in a dose-dependent manner. Our results confirmed our hypothesis, with increasing drug concentrations leading to a corresponding decrease in cell viability. Cell-based analysis showed that the nanoparticles were able to induce apoptosis. In addition, the antitumor effect on glioma cells and the modification with target molecules by antioxidants should be further investigated.

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Conflicts of interest

All authors declare no conflict of interest.

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