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Original Article

# Apoptotic effect of epigallocatechin-gallate on C6 glioma cells

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***ABSTRACT***

**Objectives:** In the present study, epigallocatechin-gallate (EGCG) was investigated for its ability to induce programmed cell death in rat-derived C6 glioma cells.

**Materials and methods:** The C6 glioma cells were cultured. Then the cells were incubated with 0 (Control), 50, and 100 µg/mL EGCG for 24, 48, and 72 h. After the treatment of EGCG, the apoptotic cell death was assayed using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. The data obtained were analyzed using One-Way ANOVA.

**Results:** The study determined that EGCG, at concentrations of 50 and 100 µg/mL, induced apoptosis in C6 glioma cells after 24, 48, and 72 h. The most significant increase in apoptosis was observed in cells treated with 100 µg/mL EGCG for 72 h ($p<0.001$).

**Conclusion:** The present study demonstrates the apoptotic effect of EGCG on tumor cells, as assessed by TUNEL, supporting its potential as an alternative bioactive compound in cancer biology and therapeutic research. Given the molecular mechanisms of EGCG, combined treatment strategies for cancer therapies may be developed.

***Keywords:*** Apoptosis, C6 glioma, epigallocatechin-gallate, green tea, TUNEL.

Glioblastoma multiforme (GBM) is the most malignant and frequent type of primary tumor in the adult cerebrum and constitutes a substantial proportion of malignant central nervous system tumors.[1-3] Despite optimized therapeutic modalities that combine maximal surgery, radiation, and chemotherapy, survival rates remain limited, and tumor recurrence is common.[2,4] The highly diffuse infiltrative nature, pronounced cellular heterogeneity, and elevated proliferative capacity of GBM cells are key biological barriers to effective treatment.[4] Furthermore, the blood-brain barrier (BBB) restricts the penetration of many pharmacological agents into the central nervous system, and the development of resistance to chemoradiation over time further limits the efficacy of current therapies.[5]

Polyphenols from plants have attracted significant attention in cancer research due to their antioxidant activity, radical-scavenging properties, and effects on multiple cellular signaling pathways.[6,7] Epigallocatechin-gallate (EGCG), one of the main polyphenolic fractions of green tea *(Camellia sinensis)*, exerts its effects not only through free radical scavenging but also through its influence on key stages of cell proliferation, angiogenesis, and metastasis.[6,8,9] The EGCG has been found to cause cell cycle arrest, increase caspase activation, and modulate B cell lymphoma 2 (Bcl-2) family proteins in a pro-apoptotic manner in various studies.[10-12] There is also evidence to indicate that it may inhibit telomerase activity and matrix metalloproteinases responsible for tumor invasion.[13-15]

Research studies on brain tumors demonstrated decreased cell viability and

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** Prof. Dr. Tuncay Altuğ made substantial contributions to the conception and design of the study and to the interpretation of the data. Sadly, he passed away prior to the submission of this manuscript.*

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increased apoptotic cell death in glioma cell lines at specific EGCG concentrations.[16,17] Apoptosis, a process of programmed cell death, represents a basic mechanism for maintaining a homeostatic balance of cellular content.[18] Such a process is activated upon encountering a set of defined cellular or extracellular signals, including deoxyribonucleic acid (DNA) damage, growth factor deprivation, cytokine signaling, or oxidative stress. It is characterized by a set of defined morphological and biochemical alterations, such as cell shrinkage, chromatin condensation, internucleosomal fragmentation of DNA, cellular membrane changes, and the formation of apoptotic bodies.[19,20]

Apoptosis control is a complex process that involves key molecular components, including caspases, Bcl-2 family members, and the tumor suppressor p53. While caspases are involved in the regulated degradation of cellular and nuclear proteins, Bcl-2 family members are essential in regulating the levels of antiapoptotic and proapoptotic signals that control a cell's fate of survival versus apoptosis. The tumor suppressor p53 acts as an important regulatory molecule that triggers an apoptotic signal following DNA damage and also regulates cell cycle events.[21,22] Imbalance in these apoptotic mechanisms is a characteristic of cancer and the cause of tumor formation and treatment insensitivity.[23]

Deoxyribonucleic acid strand breaks occurring at the final stage of apoptosis can be specifically detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. The TUNEL assay targets the final step of apoptosis by labeling free 3′-OH ends of single- and double-stranded DNA breaks via terminal deoxynucleotidyl transferase (TdT). These labeled sites are visualized as brown signals under light microscopy using peroxidase-conjugates and a chromogenic substrate 3,3'-Diaminobenzidine (DAB). This method is widely used for cultured cells and for frozen and paraffin-embedded tissue sections.[24]

This study aimed to investigate the dose- and time-dependent apoptotic effects of EGCG, the principal catechin of green tea, on C6 glioma cells using the TUNEL method.

## MATERIALS AND METHODS

This study was conducted at the Cell Culture Laboratory of Demiroğlu Science University.

### C6 glioma cell culture

The rat C6 glioma cell line, derived from a chemically induced glioma, is a well-characterized and widely used experimental model in brain tumor research.[25] The C6 glioma multiforme cell line (CCL-107; ATCC, Rockville, Maryland, USA) was obtained from a cell bank and used to examine the dose- and time-dependent effects of EGCG. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma D5546) with nutrient mixture F-12 (Sigma N6658), 5% fetal bovine serum (FBS, Seromed S0115), and antibiotics (100 U/mL penicillin G and 100 µg/mL streptomycin; Biological Industries 03-031-1C). Cultures were maintained in a humidified incubator at 37 °C with 5% $CO_2$.

Cultured C6 glioma cells were trypsinized and seeded onto round coverslips at a density of $1\times10^4$ cells/coverslip, then incubated overnight in an incubator (at 37 °C in a 5% $CO_2$). Following, culture media were supplemented with EGCG (Sigma-Aldrich E4143) at concentrations of 0 µg/mL (Control), 50 µg/mL, and 100 µg/mL, and cells were incubated for 24, 48, and 72 h.

### Determination of apoptosis by the TUNEL method

Deoxyribonucleic acid fragmentation resulting from apoptotic cell death was detected using the in situ Apoptag® Plus Peroxidase (TUNEL) kit (Chemicon; S7101, Millipore).[24] Cells cultured on round coverslips and treated with 0 (Control), 50, and 100 µg/mL EGCG for 24, 48, and 72 h were washed with 1×PBS for 5 min. Cells were fixed in methanol at –20 °C for 5 min and subsequently rinsed in 1×PBS for 5 min. Following fixation, cells were incubated with Equilibration Buffer for 5 min. After removal of the buffer, the TdT reaction mixture was applied, and cells were incubated at 37 °C for 1 h.

The cells, which were then washed with PBS, were incubated with anti-digoxigenin for 30 min at room temperature. Following additional PBS washes and rinsing with distilled water, DAB peroxidase substrate

was applied, and the reaction was monitored under a microscope. After the reaction was complete, it was stopped with distilled water and counterstained. Cells were counterstained with hematoxylin-eosin for 5 min, rinsed with tap water, mounted using mounting medium, and examined under an Olympus BX-50 light microscope. Fifty random fields were analyzed, and brown-stained cells were identified as apoptotic cells.

### Statistical analysis

Statistical analysis was performed using the PASW version 18.0 software (SPSS Inc., Chicago, IL, USA). The data are presented as means ± standard error (SE) of three separate experiments, each repeated in triplicate. Apoptotic cell counts were analyzed using One-Way ANOVA (analysis of variance). Statistical significance was defined as $p<0.001$.

## RESULTS

In the control group of C6 glioma cells not treated with EGCG, only a limited number of apoptotic cells were observed at 24, 48, and 72 h, with no statistically significant change in apoptotic cell ratio over time. In contrast, EGCG-treated C6 glioma cells exhibited a marked increase in apoptotic cell ratio with increasing dose and incubation time, as shown in Figure 1.

In the group administered 50 µg/mL EGCG, a limited increase in apoptotic cells was observed at 24 h compared with the control group; however, statistically significant

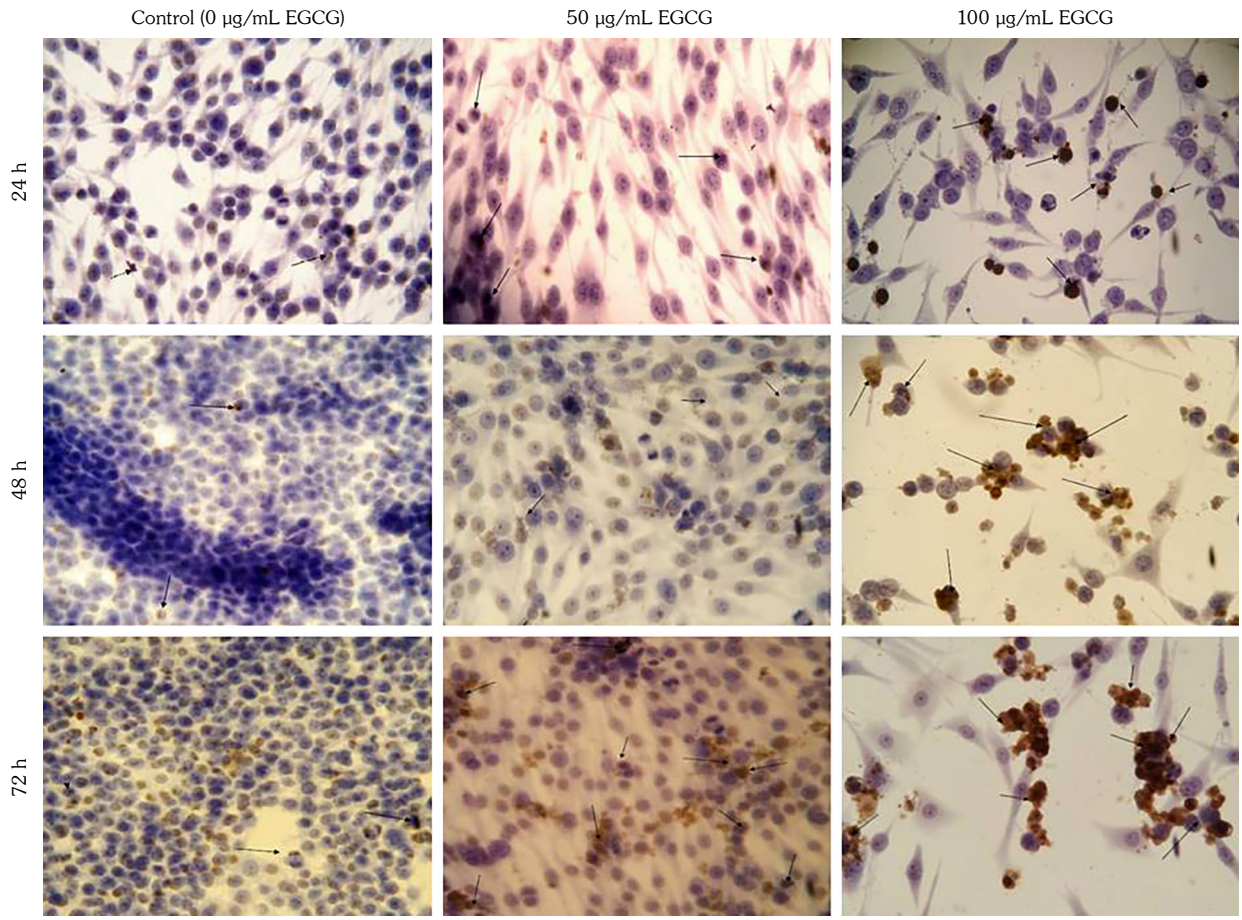


**Figure 1.** Detection of apoptotic cell death in C6 glioma cells by TUNEL staining following EGCG treatment. C6 cells were exposed to EGCG at concentrations of 0 (control), 50, and 100 µg/mL for 24, 48, and 72 h. TUNEL-positive cells, indicative of DNA fragmentation associated with apoptosis, are shown as dark-stained nuclei. Representative micrographs were captured at 600× magnification.

EGCG: Epigallocatechin-gallate; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

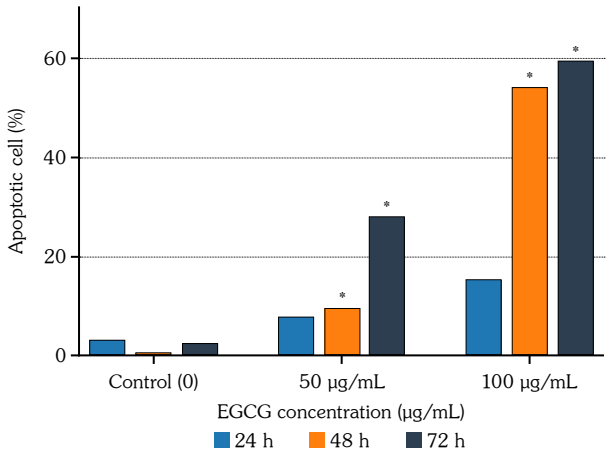


**Figure 2.** Quantitative analysis of apoptosis in C6 glioma cells following EGCG treatment. Cells were treated with EGCG at concentrations of 0 (control), 50, and 100 µg/mL for 24, 48, and 72 h. The percentage of apoptotic cells was determined based on TUNEL staining. EGCG induced a significant, dose- and time-dependent increase in apoptosis compared with the control group. Data are shown as mean±SE of three separate experiments (n=3).

EGCG: Epigallocatechin-gallate; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; SE: Standard error; * p<0.001 versus control.

increases in apoptosis were detected at 48 and 72 h (p<0.001) with the same dose, indicating a time-dependent enhancement of the apoptotic effect. Cells treated with 100 µg/mL EGCG showed significantly higher apoptotic rates than the control group at 48 and 72 h (p<0.001). The highest apoptotic index was observed in C6 glioma cells treated with 100 µg/mL EGCG for 72 h, as shown in Figure 2.

Morphological analysis results obtained using the TUNEL assay confirmed that EGCG increased the apoptotic response in C6 glioma cells in a dose- and time-dependent manner.

## DISCUSSION

One practical approach in cancer therapy is to direct tumor cells toward programmed cell death, specifically apoptosis, using biologically active compounds.[15,23] Tea *(Camellia sinensis)* is a beverage widely consumed worldwide and has been extensively studied for its health-promoting and chemopreventive properties.[10,26,27] Epigallocatechin-gallate, a polyphenolic compound found in green tea, is identified as a potent apoptosis inducer, and it has been demonstrated to have regulatory functions at different stages of apoptosis as well as on the expression of key apoptosis signaling proteins.[8-10,28] Epigallocatechin-gallate, because of its antioxidant and chemopreventive potential, has been intensively studied for different types of cancers, including brain cancers, and it has been linked closely to the suppression of metastasis, invasions, angiogenesis, and development of cancers.[8,16,17] It has further been reported that EGCG induces cell cycle arrest and triggers apoptosis in cancer cells while exerting minimal or negligible effects on normal cells.[10,29]

Catechins exert their antitumor effects in different cancer cell models by regulating the cell cycle and activating apoptotic signaling pathways. Green tea catechin extract and EGCG have been shown to induce apoptosis and inhibit cell growth in human gastric cancer KATO III cells.[30] In experimental carcinogenesis models, green tea catechins were demonstrated to inhibit small intestinal carcinogenesis. However, they have also been reported to slightly increase hepatocarcinogenesis in a dose-dependent manner when administered during and after carcinogen exposure.[31] Ahmad et al.[10] have investigated the effect of EGCG and green tea polyphenols on the induction of programmed cell death (apoptosis) and regulation of cell cycle in mouse lymphoma cells (L5178Y), normal human epidermal keratinocytes (NHEKs), human prostate carcinoma cells (DU145), human epidermoid carcinoma cells (A431), and human carcinoma keratinocyte (HaCaT). Treatment of A431 cells with green tea polyphenols and EGCG resulted in apoptosis. Apoptosis was observed in DU145, HaCaT, and L5178Y cells treated with EGCG, but not in NHEK cells. In their study, EGCG was reported to trigger apoptotic responses by inducing cell cycle arrest at the G0/G1 phase in A431 epidermoid carcinoma cells. These studies demonstrate that EGCG and related catechins suppress tumor cell proliferation while exerting minimal effects on normal cells.[29] Consistent with this activity, EGCG has been shown to exert no significant growth-inhibitory impact on normal human fibroblast W138 cells, whereas it markedly inhibits the proliferation of malignant W138VA cells.[32]

In U-373 MG, C6 glioma, and U-87 MG cell lines, treatment with EGCG has been shown to inhibit cell viability and promote apoptosis using various experimental methods, including the MTT assay and flow cytometric analysis.[33] Pervin et al.[34] investigated the pharmacokinetics of orally administered EGCG in a mouse model. Through LC-MS-based analyses, they reported that catechins were absorbed into the systemic circulation and distributed to brain tissue, indicating that catechins can cross the BBB. In addition, the potential role of EGCG in combination strategies with conventional chemotherapy drugs has been highlighted, further increasing its importance in glioblastoma studies.[35]

This study has some limitations. The primary limitation of this study is the use of only a single glioma cell line (C6). Additional studies involving human GBM cell lines (U87MG, T98G) and primary patient-derived samples are required to confirm the generalizability of these findings.

In conclusion, the results obtained using the TUNEL method demonstrate that EGCG, the main polyphenolic compound in green tea, increases apoptosis in C6 glioma cells in a dose- and time-dependent manner. These findings align with previous studies reporting proapoptotic effects of EGCG in various malignant tumor cells and support its experimental application in glioblastoma research. Epigallocatechin-gallate therefore, emerges as a promising agent in cancer research and as a potential therapeutic approach.

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**Data Sharing Statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Author Contributions:** H.A., M.E., T.A.: Idea, concept, design, interpretation, methodology, formal analysis; M.E.: Literature review, visulization, writing the article; H.A., M.E.: Investigation, references, editing.

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