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Research Article

Turkish coffee suppresses the progression of C6 glioma cells via the activation of apoptosis

Melike ERSÖZ[1,2], Zeynep Mine COŞKUN YAZICI[1,2,*], Berfin ÜLGEN BAĞIRGAN[1], Hamit Baturalp SAYINBATUR[1], Aynur ACAR[1]

[1]Department of Molecular Biology and Genetics, Demiroglu Bilim University, Istanbul, 34394, Türkiye
[2]Authors contributed equally to this study

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ABSTRACT

Glioma is the most invasive form of brain tumor and usually results in death within months of diagnosis. C6 glioma cells are frequently used in glioblastoma multiform studies because they are cells with different malignant glioblastoma features. Coffee is one of the most popular beverages consumed in large quantities. Recent research has shown the functional and protective potential properties of coffee as well as its stimulatory effect. Coffee blending and grinding processes change the antioxidant composition of coffee. The main characteristic separating Turkish Coffee (TC), which is formed from freshly roasted pulverized coffee beans, from the other coffee types is the brewing method. Our aim in this study is to investigate the antioxidant and apoptotic effects of TC prepared with the traditional method on C6 glioma cells, which are glial cells derived from rat brain with glioma. Cell viability in C6 glioma cells treated with TC at different concentrations (0-8000 µg/ml) was analyzed by the MTT method. According to MTT results, three doses (10000, 15000, and 20000 µg/ml) of TC were applied to the cells and the untreated cells were considered the control. Total oxidant and antioxidant statuses (TOS, TAS) and oxidative stress markers were determined. Caspase-3, caspase-8, and caspase-9 mRNA expressions were detected by using quantitative real-time PCR. It was determined that the application of TC at concentrations of 4000 µg/ml and above to C6 glioma cells inhibited cell proliferation depending on the concentration. Caspase 3, caspase-8, and caspase-9 mRNA expression levels increased in C6 glioma cells treated with TC at concentrations of 10000 and 15000 µg/ml as compared to control cells. TAS and TOS levels were unchanged, while protein carbonyl levels increased in TC treated C6 glioma cells compared to the control group. These findings suggested that TC may induce apoptosis by changing caspases expressions and inducing protein oxidation. Thus, it can be thought that TC may prevent the proliferation of C6 glioma cells.

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***Corresponding author.**
*E-mail address: zeynepmine.coskun@demiroglu.bilim.edu.tr

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INTRODUCTION

Glioma is one of the most common and aggressive types of the tumor with high morbidity and poor prognosis in the central nervous system. In addition, gliomas are highly proliferative and invasive primary intracranial tumors [1]. Liu et al. [2] emphasized that there is an urgent need for research that enables the development of innovative drugs as effective treatment is still lacking. Studies have shown that apoptosis, a programmed cell death, is a target for many cancers, including glioma, and may be promising for therapeutic development [2-4].

Programmed cell death, also called apoptosis that regulates cell proliferation and maintains tissue homeostasis is a crucial mechanism for multicellular organisms. Most anti-cancer drugs used in clinical oncology exploit apoptotic signaling pathways to induce cancer cell death [5]. In addition, studies on glioma cells have suggested that apoptosis promoters may be target therapy [6,7]. On the other hand, researchers stated that agents with anti-cancer activity cause oxidative stress and lead to the death of cancer cells [8,9].

A study with coffee, which is among the most consumed beverages, have suggested that consumption of roasted coffee extracts is associated with a wide range of beneficial health effects [10]. Furthermore, it is supported by a cohort study that coffee consumption may be associated with a decrease in deaths due to diseases such as chronic liver disease and certain types of cancer [11]. Choi et al. [12] reported that coffee and its components have antioxidant and anti-inflammatory activities, and these activities may vary according to the degree of roasting.

In this paper, we investigated the effect of Turkish coffee (TC), which has a unique brewing method, on glioma cells in terms of programmed cell death and oxidative stress.

MATERIALS AND METHODS

Turkish Coffee Brewing

Traditional TC was taken from a local market in Turkey. To prepare traditional TC, 20 grams of coffee and 100 ml of cold water were added to the coffee pot and stirring over low heat. The TC was boiled twice in succession. The prepared TC was filtered through membrane filters, 0.22 μm pore size (Millipore, USA) before use.

Culture of Rat Brain Tumor Cell Line C6 Glioma

All culture materials and cells were obtained from Sigma-Aldrich (St. Louis, MO, USA) and American Type Culture Collection (ATCC). C6 cells, glial cells derived from rat brain with glioma, were cultured in the complete medium [Dulbecco's Modified Eagles Medium/F-12 medium, supplemented with 5% fetal bovine serum, 0.2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 μg/mL streptomycin)], at 37 °C in an atmosphere containing 5% CO_2. The morphology of C6 glioma cells was evaluated using Olympus BX-50 brightfield microscope microscopy.

Proliferation Assay

To assess cell proliferation after the addition of TC, C6 glioma cells were first seeded in a 96-well plate at a density of $1x10^4$cells/well. Cells were treated with TC (12 different doses from 0 to 8000 μg/ml) for 24 h. After the incubation, cell proliferation rates were detected by using a tetrazolium compound, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent] kit (Promega, WI, USA). The absorbance at 490 nm was recorded using an Epoch™ Microplate Spectrophotometer (BioTek, VT, USA).

Apoptosis Measurement by Quantitative Real-Time PCR (qRT-PCR)

C6 glioma cells were seeded in a 6-well plate at a density of $1x10^5$ cells/well. Control cells are C6 glioma cells without TC. Treatment group cells were incubated with TC at 10 000, 15 000, and 20 000 μg/ml concentrations for 24 h. Total RNA was extracted from cells with Direct-zol Miniprep Plus Kit (Zymo Research, CA, USA). The obtained RNA (200 ng) was reverse-transcribed using SensiFAST cDNA Synthesis Kit (Bioline Reagents Limited, UK). qRT-PCR analyses were performed using SYBR Green Real-Time PCR Kit (Hybrigen, Turkey) by a CFX96 Touch Real-Time PCR Detection Systems (Bio-rad Laboratories Inc., CA, USA). Target genes (caspase-3, caspase-8, and caspase-9) were normalized with β-actin (Table 1).

Oxidative Stress Detection

Cells were cultivated in four flasks at a density of $2x10^5$ cells/ml with complete fresh medium and treated with 10000, 15000, and 20000 μg/ml concentrations of traditional TC for 24 h. Following incubation, C6 glioma cells were washed with PBS. Cell lysates were collected, and total antioxidant status (TAS, Rel Assay Kit Diagnostics, Turkey), total oxidant status (TOS, Rel Assay Kit Diagnostics, Turkey), malondialdehyde (MDA), protein carbonyl (PCO), 8-hydroxy-2´-deoxyguanosine (8-OHdG, YLbiont, Shanghai, China) and glutathione (GSH) levels and superoxide dismutase (SOD) activity were measured [13-16]. MDA was detected by thiobarbituric acid reacting substances assay. Absorbance values were recorded at 532 nm. PCO reacts with 2, 4-dinitrophenyl-hydrazine to

Table 1. Primers used for the mRNA expression studies

Genes	Primer Sequence
Caspase-3	F 5'- ACT GGA AAG CCG AAA CTC TTC-3'
	R 5'-AGT TCC ACT GTC TGT CTC AAT A-3'
Caspase-8	F 5'- TCT GCT GGG GAT GGC TAC T -3'
	R 5'- CAT GTT CCT CGG GTT GTC TT -3'
Caspase-9	F 5'- CTC CTG GAG AGA CAA GAA GAG C -3'
	R 5'- AAA ACA GCC AGG AAT CTG CT -3'
β-actin	F 5'- CTA AGG CCA ACC GTG AAA AG -3'
	R 5'- TCT CCG GAG TCC ATC ACA AT -3'

generate chromophoric dinitrophenylhydrazones. Sample absorbances were read at 360 nm. GSH level was analyzed by following the rate of decline of 5,5'-dithiobis-2-nitrobenzoic acid. The absorbance read at 412 nm, spectrophotometrically. SOD enzyme activity involves the inhibition of nitroblue tetrazolium reduction with xanthine/xanthine oxidase and the activity was measured at 560 nm.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism software version 5. Comparison between groups was evaluated via the non-parametric Kruskal-Wallis and Mann-Whitney tests. Results were expressed as the mean ± SEM, and statistical significance was considered at $P < 0.05$. All experiments were repeated at least three times.

RESULTS AND DISCUSSION

Coffee brewing methods vary around the world. These different brewing methods greatly affect the coffee's content, polyphenolic properties, taste, and aroma [17]. For this reason, the effect of coffee, which is frequently consumed all over the world, on human health continues to be a matter of curiosity. In our study, we observed that TC had a cytotoxic effect on C6 glioma cells due to oxidative stress.

According to the MTT test results, the treatment of C6 glioma cells with TC at the concentrations lower than 4000 µg/ml did not affect cell viability (Figure 1). Morphological examination was also similar to our MTT test result. When C6 glioma cells were examined morphologically under a phase contrast microscope; at concentrations of 4000 µg/ml and above, TC inhibited the proliferation of cells, caused damage and disruptions in cell morphology (Figure 1). According to the study by Derossi et al. [18], the cooking method affects the coffee quality and coffee content properties more than the grinding level. In a study, the antioxidant activity of espresso, filter coffee, and TC was measured and compared with spectrophotometric tests. As a result of the measurements, it was determined that the antioxidant activity of TC is higher than that of filter and espresso coffees [19].

Administration of C6 glioma cells with 10000 µg/ml of TC for 24 hour caused the significant increase in the levels of caspase-8, caspase-9, and caspase-3 mRNA expressions compared with TC-untreated control, respectively ($P < 0.05$, $P < 0.05$, and $P > 0.05$). Similarly, 15000 and 20000 µg/ml of TC increased remarkably the levels of caspase-8, caspase-9 and caspase-3 mRNA expressions compared to control cells ($P < 0.05$ for all) (Figure 2). In the present study, we found that TC application increased caspase-3, caspase-8, and caspase-9 expression levels, leading to apoptosis of C6 glioma cells from both the extrinsic and intrinsic pathways. In the meta-analysis study by Yu et al. [20] on coffee consumption and cancer risk, individuals with cancer who drink coffee in 24 h periods or rarely drink coffee were compared. According to the results, an increase in the overall consumption of 1 cup of coffee per day was associated with a 3% reduced risk of cancer. In addition, coffee consumption has been reported to be associated with a reduced risk of bladder, breast, pharyngeal, colorectal, endometrial, esophageal, hepatocellular, leukemic, pancreatic, and prostate cancers. Moreover, Barnung et al. [21] determined that there is a potential association between gene expression profiles and coffee consumption. According to the results of blood analysis, they suggested that high and low coffee consumption affected the expression of 297 genes thought to be associated with both metabolic and inflammatory processes. In a study on MCF-7 cells, it was suggested that the combined administration of coffee extract and vitamin C plays a role in the anticancer pathway by changing the expression levels

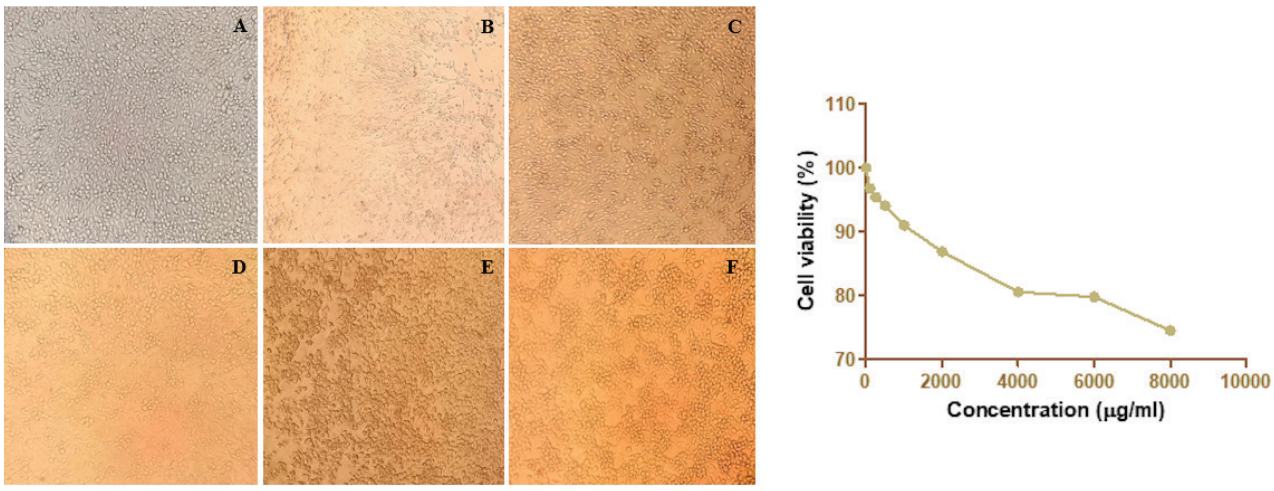

Figure 1. Phase-contrast microscope images and the cell viability of Turkish coffee (TC)-treated C6 glioma cells. Control group (A), C6 glioma cells treated with TC at a concentration of 4000 µg/ml (B), 8000 µg/ml (C), 10000 µg/ml (D), 15000 µg/ml (E), 20000 µg/ml (F). (Magnification: X20).

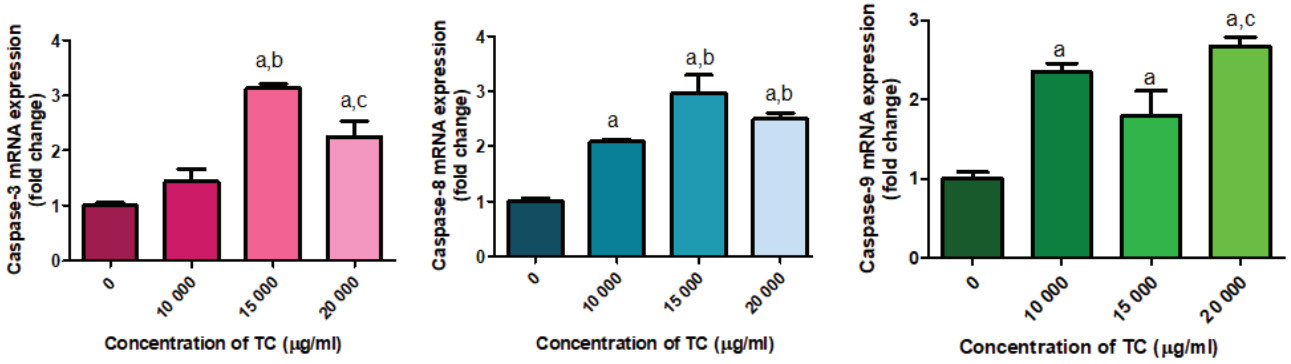

Figure 2. Changes in mRNA expression of caspase-3, -8, and 9 in C6 glioma cell treated with Turkish coffee (TC). Data are expressed as means ± SEM. [a]P < 0.05 vs control group, [b]P < 0.05 vs TC at concentrations of 10000 µg/ml, [c]P < 0.05 vs TC at concentrations of 15000 µg/ml.

of caspase-3 and p53 [22]. According to the observations by Kolberg et al. [23] coffee modulates the transcription of some genes related to prostate cancer and inflammation by inhibiting Tumor necrosis factor-α (TNFα)-induced nuclear factor kappa B (NF-κB) activity in prostate cancer cell cultures and PC3 xenografts in mice [23].

To detect the effect of traditional TC on the cellular redox status in C6 glioma cells, antioxidant defense system capabilities, lipid peroxidation, protein oxidation, and oxidative DNA damage were evaluated (Figures 3 and 4). No significant changes in the levels of TAS and TOS were detected in the cells applied traditional TC at concentrations of 10000, 15000, and 20 000 µg/ml (P > 0.05). SOD and GSH play a role in the cellular antioxidant defense mechanism. SOD activity in C6 glioma cells applied TC 15 000 µg/ml was increased significantly compared to control (P < 0.05). Also, GSH levels showed an insignificant increase in cells treated with TC 15000 and 20000 µg/ml compared with control cells (P > 0.05). Our results indicated that the LPO level increased insignificantly in the cells treated with TC 15000 and 20000 µg/ml compared with controls (P > 0.05). Similarly, TC 15000 and 20000 µg/ml elevated significantly PCO levels (P < 0.05). 8-OHdG, a marker of oxidative DNA damage, was unchanged in C6 glioma cells treated with all concentrations of TC (P > 0.05). In a study conducted with healthy individuals consuming coffee prepared at a concentration of 50 g/L, it was reported that coffee consumed up to 5 cups a day did not affect lipid and DNA damage related to oxidative stress, and also did not prevent inflammation. Therefore, no beneficial or harmful effects of coffee on human health have been detected [24]. On the other hand, Morii et al. [25] showed that instant coffee consumption had little effect on mouse liver cancer due to oxidative stress. Also, they found that coffee consumption did not show significant differences in liver SOD activities and LPO levels. In a recent study, it was reported that the aryl hydrocarbon receptor (AHR), which causes a protective response against oxidative stress in epithelial cells, and the erythroid 2 p45-related factor 2 (Nrf2) pathway genes, which are the main regulators of antioxidant responses, are up-regulated by coffee. According to the results of this in vitro study, coffee may have effects on gene expression as well as contribute to antioxidant defense and detoxification processes [26]. In our findings, TC did not significantly change the total antioxidant and oxidant status, as did the GSH, LPO, and 8-OHdG levels. However, TC induced protein damage and increase SOD activity in C6

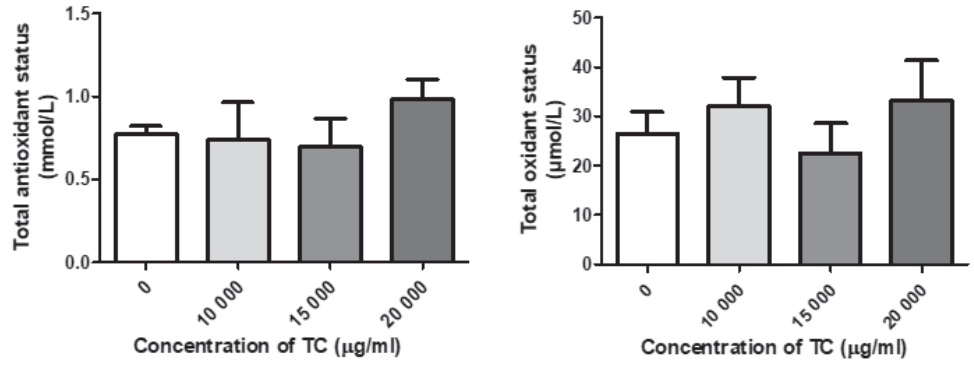

Figure 3. Total antioxidant and oxidant statuses in C6 glioma cells treated Turkish coffee (TC). Data are expressed as means ± SEM.

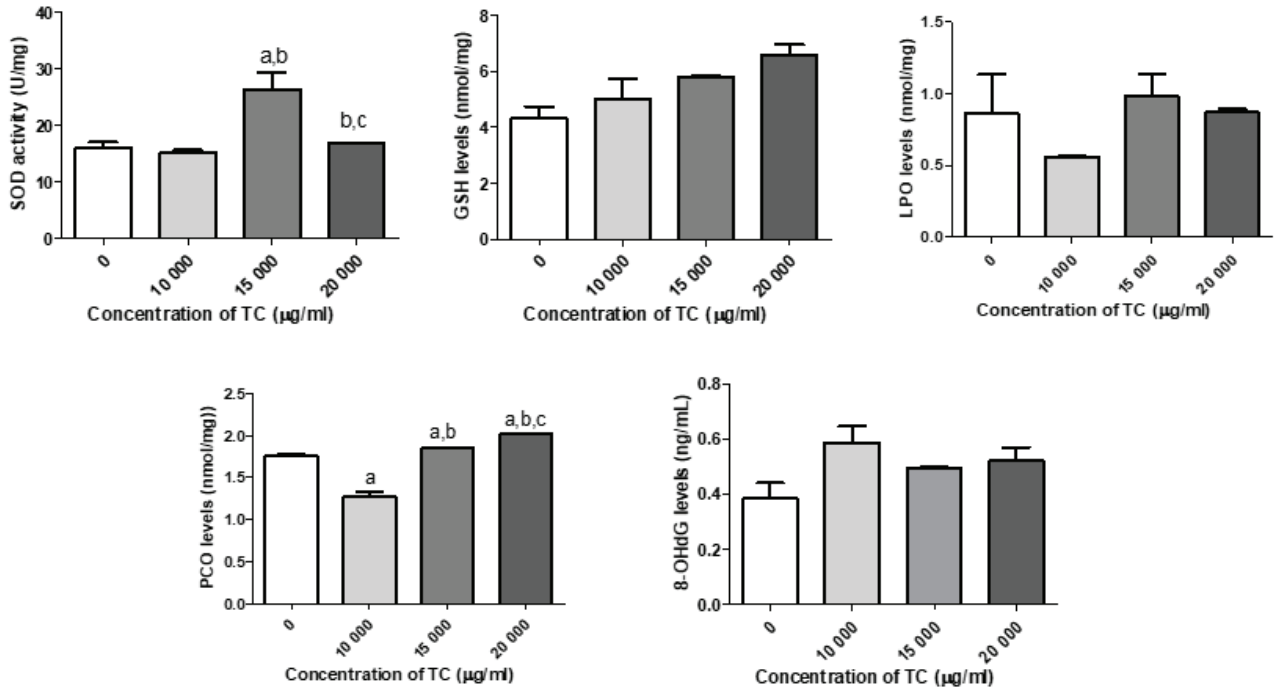

Figure 4. Antioxidant defense and oxidative stress biomarker levels in C6 glioma cells treated Turkish coffee (TC). Data are expressed as means ± SEM. [a]P < 0.05 vs control group, [b]P < 0.05 vs TC at concentrations of 10 000 µg/ml, [c]P < 0.05 vs TC at concentrations of 15 000 µg/ml.

glioma cells. Oxidative damage has been observed in cancer cells in treatments showing anticancer activity. Li et al. [27] showed that β-mangostine treatment inhibited cell proliferation due to oxidative stress induction. Similarly, thymoquinone showed cytotoxic and anti-proliferative effects depending on oxidative stress [28]. Similar to the aforementioned studies, it is thought that TC has a cytotoxic effect on cells due to protein oxidation in C6 glioma cells.

CONCLUSION

As a result, in the present study, it was determined that TC can cause apoptosis in both extrinsic and intrinsic pathways by changing the caspase-3, caspase-8, and caspase-9 expression levels. In addition, TC may have a cytotoxic effect by increasing protein oxidation, which is a marker of oxidative stress in C6 glioma cells. In line with these results, it can be thought that TC may cause apoptosis in C6 glioma cells due to protein oxidation. Thus, we can predict that TC consumption in the daily diet may contribute to prevent the cell proliferation in patients with glioblastoma multiforme.

AUTHORSHIP CONTRIBUTIONS

Research design was made by ME, ZMCY and AA. Data collection in the field was performed by ME, OU, HBS and ZMCY. Manuscript was written by ZMCY. Manuscript was critically revised by ME and ZMCY and finally approved by all the authors for further publication.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

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