Investigation of cytotoxic, genotoxic, and apoptotic effects of curcumin on glioma cells

Mehmet Hakan Seyithanoğlu1,6, Anas Abdallah1,6, Serkan Kitiş1, Eray Metin Guler2, Abdurrahim Koçyiğit2, Tolga Turan Dündar1, Meliha Gündağ Papaker1

1 Department of Neurosurgery, Bezmialem Vakif University, Istanbul, Turkey
2 Department of Biochemistry, Bezmialem Vakif University, Istanbul, Turkey

Abstract: Glioblastoma is a malignant tumor of the brain. The treatment of this tumor is still a challenge. Curcumin has been shown to have therapeutic effects when used to treat malignant diseases. However, the molecular mechanisms of its action are not fully elucidated. We hypothesized that reactive oxygen species (ROS) have a key role in curcumin-induced DNA damage, apoptosis, and cell death. To test our hypothesis, cytotoxic, genotoxic, apoptotic, and ROS-generating effects, as well as mitochondrial membrane potentials of curcumin on rat glioma cells (C-6) and normal fibroblastic cells (L-929) were investigated. We examined concentration-dependent cytotoxic, genotoxic, apoptotic, and ROS generating effects of curcumin at C-6 cells and L-929 cells. The cells were incubated with different doses of curcumin (10-100 µM) for 24 hours. Higher doses of curcumin resulted in greater cellular death of cancer than of normal cells at higher concentrations. Curcumin also induced ROS generation in cancer than normal cells in a concentration-dependent manner. Our results showed that curcumin-induced DNA damage in a dose-dependent manner (p < 0.001). At high curcumin concentration such as 80 µM, the proportions of live cells in cancer and normal cell lines were 11.5 and 44.3, respectively. The higher doses of curcumin resulted in greater apoptosis in cancer than normal cells. This in vitro study provided clear evidence that curcumin induced DNA damage and apoptosis. Cytotoxicity may be due to its pro-oxidant activity in a dose-dependent manner in cancer and normal cells. These activities were higher in cancer cells.

Key words: Glioblastoma; Curcumin; Antioxidants; Apoptosis; Cytotoxicity; Genotoxic Activity.

Introduction

Glioblastoma is one of the most malignant tumors which can affect the brain, characterized by high proliferation, migration, intracranial seeding metastasis and aggression. Although major improvements have been made in standardized treatment, including surgical resection, followed by radiotherapy and chemotherapy, the overall prognosis of glioblastoma multiforme remains very poor, with the median survival of approximately 1 year after diagnosis (1-3). The effectiveness of conventional chemotherapy is hindered severely, since chemotherapeutic drugs often are eliminated in circulation, and reaching a high local drug concentration at the tumor site is difficult. Moreover, overexpression of multidrug resistance proteins by tumor cells causes efflux of various chemotherapeutic drugs (4-6). Therefore, research for new chemotherapeutic agents are sorely needed.

Curcumin, derived from the rhizome of the plant Curcuma Longa, is the major pharmacologically-active component of the spice turmeric. This component potentially represents one of the effective therapeutic medications (7). Being the main ingredient of curries and, thus, part of the everyday diet of millions of people, curcumin is considered a safe agent in humans (7,8). Interest in this herb has grown in recent years depend on its reported putative beneficial pharmacologic effects, which include antioxidant, anti-inflammatory (9,10), and chemo-preventive properties (11,12), in addition to the actions of inhibiting cell proliferation and increasing apoptosis (13-15).

Therapeutic effects of curcumin on various cancer cells have been reported. Recently, more attention has been focused on complementary medicine as an alternative therapeutic modality for treatment of patients involved with malignant cancers such as aggressive glioblastoma multiforme. Despite the fact that the long-term studies in animals and humans are lacking, curcumin, being a natural compound and the main ingredient of turmeric, commonly known as “curry,” generally is regarded as a safe agent. Several recent published studies about curcumin aimed to elucidate the mechanism of the curcumin activities, in particular, anticancer activity (13-15). In current study, the authors evaluated the association between cytotoxic, apoptotic, DNA-damaging, and ROS-generating effects of curcumin on glioma cancer cells and compared them to the effects on normal fibroblastic cells in vitro.

Materials and Methods

Chemicals and reagents

Curcumin, fetal calf serum (FCS), Dulbecco’s modified Eagle medium (DMEM), 2,7-dichlorodihydro-
fluorescein-diacetate (H2DCF-DA), penicillin–streptomycin and ethidium bromide (EB), acridine orange (AO), and the Annexin-V fluorescein isothiocyanate (FITC) staining kit were obtained from EBioscience (San Diego, CA, USA). Unless mentioned otherwise, all reagents used in the study were of analytical grade. A 100 µM curcumin stock solution was prepared by solution in dimethyl sulfoxide (DMSO), and then diluted with DMEM (containing no fetal bovine serum) for obtaining required concentrations before use. The ultimate concentration of DMSO in the curcumin solution was < 0.1%. The level of DMSO and the serum-free media were confirmed not to induce DNA damage in the cells before beginning the experiments. Remaining reagents were prepared as fresh before each experiment.

**Cell culture and maintenance**

Rat glioma cells (C-6; ATCC® CCL-107™; as a standard cell line from Rattus norvegicus glial cells) and normal fibroblastic cells (L-929; NCTC clone 929; L cell; derivative of Strain L [ATCC® CCL-1™]; as a standard Mus musculus normal fibroblast cell line) were obtained from American Type Cell Culture Collection (ATCC; Manassas, VA, USA). The L-929 cells were cultured in Eagle’s minimal essential medium and C-6 cells were cultured in F12-K medium under 5% CO₂ atmosphere at 37°C equilibrium. The medium was supplemented with 10% FCS, 100 U/mL penicillin, and 100 ng/mL streptomycin. The trypan blue exclusion test was used for estimation of the number of viable cells.

**Cell viability toward cancer and normal cells**

Concentration-response cell viability assays were performed with C-6 and L-929 cells for 24 hours to evaluate the effect of curcumin on cell growth. Following incubation, the effects of curcumin were determined by the ATP cell viability assay. Cell viability in all cell cultures was > 95% before all experiments.

**Cytotoxicity assay**

Cytotoxic activity of curcumin on the cells was examined by adenosine triphosphate (ATP) levels measured with a luminescence test (Cell-Titer-Glo Luminescent Cell Viability Assay; Promega, Madison, WI, USA). Cells were seeded onto 96-well plates at a density of 5 x 10⁴ cells per well and incubated overnight at 37°C in 5% CO₂. The medium then was changed with fresh complete medium including varying concentrations of curcumin (10–100 µM). Control cells were treated with 0.1% DMSO. Cells were left for incubation under humidified 5% CO₂ and 95% O₂ at 37°C for 24 hours. Afterwards, the cells were rinsed with the culture medium and examined for ATP. Each sample was supplemented with 100 µL of the reagent (Cell Titer-Glo Luminescent Cell Viability Assay; Promega, USA), mixed for 2 minutes, and incubated for 10 minutes at room temperature. The results were evaluated with luminometry (Varioskan Flash Multimode Reader; Thermo Fisher Scientific, Waltham, MA, USA). The light emitted in the presence of ATP was measured as relative light units. The intensity of emitted light quants was related directly to ATP content in the tested sample. The cell viability was expressed as the percentage compared to the negative control group designated as 100%. Half-maximal growth inhibitory concentration (IC₅₀) values were calculated from the concentration-response curves by nonlinear regression analysis. All experiments were repeated three times and the standard deviation was within 5%.

**Measurement of ROS generation**

Generation of ROS was assessed using a cell-permeable fluorescent signal CM-H2DCF-DA (2,7-dichlorodihydrofluorescein) indicator of ROS. We evaluated intracellular reactive oxygen species (ROS) generation by using H2DCF-DA as a fluorescence probe. As described previously, H2DCF-DA is oxidized to a highly green fluorescent 2,7-dichlorofluorescein (DCF) by generation of ROS. C-6 and L-929 cells were pretreated with various concentrations of curcumin (10–100 µM) for 24 hours. After a 24-hours incubation period, the cells were rinsed with cold phosphate buffered saline (PBS) and incubated with 20 µM H2DCF-DA for another 30 minutes at 37°C. The fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, New Hampshire, USA) was used to measure DCF fluorescence intensity at Ex./Em. = 488/525 nm. The estimations were performed three times in triplicate, ensuring each time that the number of cells per treatment group were the same to ensure reproducibility. The values were expressed as percentage relative fluorescence compared to the control.

**Genotoxic activity assay**

Since ROS can cause damage to DNA, we measured the damage to DNA caused by curcumin treatment. To analyze genotoxic activity, cells were treated with different dosages of curcumin for 24 hours and DNA damage was detected by the comet assay. Alkaline single cell gel electrophoresis assay (comet assay) was performed for evaluation of genotoxic effects of curcumin on cell lines according to Singh et al (16) with slight modification. Two different cells were seeded onto 6-well cell culture plates (approximately 2 x 10⁵ cells per well) containing cell culture medium and incubated at 37°C in 5% CO₂ for 24 hours for determination of the genotoxic potential of curcumin. Following 24 hours, curcumin in 0.1% DMSO was added to the medium and incubated for another 24 hours at 37°C. DMSO (0.1%) was applied for negative control. The cells were rinsed with PBS following incubation, harvested using trypsin/ethylenediaminetetraacetic acid (EDTA), and collected for centrifugation at 400×g for 5 minutes at 4°C. The supernatant was unloaded and the cell density was adjusted to 2 x 10⁷ cells/mL using cold PBS. Then, 10 µL of resuspended cells were placed into centrifuge tubes for the comet assay as described below.

Then, 80 µL 0.6% low melting agarose and 10 µL cell suspension were mixed and placed onto 1% normal melting agarose precoated slides. The slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH 10) for 1 hour at 4°C following solidification of the agarose. The slides were removed from lysis solution, rinsed with cold PBS, and placed in a horizontal electrophoresis tank side by side. DNA was allowed to unwind for 40 minutes in freshly prepared alkaline electrophoresis buffer containing 300 mM NaOH and 10 mM Na₂EDTA (pH 13.0). After unwinding, electrophoresis was perfor-
med at 0.72 V/cm (26 V, 300 mA) for 25 minutes at 4°C under minimal illumination to prevent further DNA damage. The slides were rinsed with a neutralization buffer (0.4 M Tris, pH 7.5) for 5 minutes at 4°C three times and then dehydrated with ethanol for another 5 minutes before staining. Dried microscope slides were stained with EB (2 µg/mL in distilled H2O; 70 µL/slide), covered with a coverslip, and analyzed using a fluorescence microscope (Leica DM 1000; Leica, Solms, Germany) at 200 × magnification with epifluorescence equipped with a rhodamine filter (with an excitation wavelength of 546 nm and a barrier of 580 nm). A computerized image analysis system (Comet Assay IV; Perceptive Instruments Ltd, Bury St. Edmonds, UK) was used. The DNA percentage in tail was used as the primary measure of DNA damage according to the study of Hartmann et al. (17). All steps were performed three times.

Apoptosis

Apoptosis is critical in tumorigenesis and resistance to therapy. To clarify whether curcumin-induced cell apoptosis in cancer and normal cells, AO/EB double staining was done to confirm the morphologic characteristics of apoptosis. To quantify curcumin-induced apoptosis, we performed Annexin V/ FITC double staining experiments. Flow cytometry was used to detect positive cells after 24 hours of treatment. Annexin-V/FITC double staining represented early apoptosis, and later apoptosis or necrosis.

AO/EB double staining test

The AO/EB double staining test for evaluation of morphologic changes in cells was done as described by McGahon et al. (18). The cells undergoing apoptosis were differentiated from the viable cells by the morphologic changes of apoptotic nuclei. AO and EB are DNA intercalating dyes. AO is taken up by viable and dead cells and stains double-stranded (ds) and single-stranded (ss) nucleic acids. When AO diffuses into dsDNA, it emits green fluorescence upon excitation at 480–490 nm from viable cells (18). EB is taken up by dead cells and stains DNA orange. Briefly, the cells were cultured in six-well plates (2 × 10⁵ cells/well) and incubated for 24 hours. Following 24 hours, curcumin in 0.1% DMSO was added to the medium and incubated for another 24 hours at 37°C. DMSO (0.1%) was applied for negative control. The cells were rinsed with PBS following incubation, harvested using trypsin/EDTA, and collected for centrifugation at 400×g for 5 minutes at 4°C. The supernatant was unloaded and the cell density was adjusted to 2 × 10⁵ cells/mL using cold PBS. Finally, AO/EB solution was added to the cell suspension and the nuclear morphology was evaluated by fluorescence microscopy (Leica DM 1000; Leica). Multiple images were acquired at randomly-selected areas and a minimum of 100 cells were counted. Live cells had normal-appearing green nuclei, apoptotic cells had green nuclei with fragmented chromatin, and dead cells had orange/red nuclei according to the method. All experiments were performed three times. Determination of mitochondrial membrane potential (MMP)

To demonstrate mechanisms underlying apoptotic induction in C-6 cancer and L-929 normal cells, the mitochondrial apoptotic pathway was investigated. Since loss of ΔΨm leads to apoptosis via mitochondrial pathways, levels of ΔΨm were determined.

The MMP of cancer and normal cells during curcumin treatment was studied with 3,3-dihexyloxacarbocyanine iodide [DiOC6(3); λex max 484 nm, λem max 501 nm; Molecular Probes, Eugene, OR, USA] (19). In living cells, this cyanine dye can accumulate in the mitochondrial matrix under the influence of the mitochondrial transmembrane potential. Cells were collected and resuspended with culture medium to 10⁵ cells/mL and incubated at 37°C for a further 20 minutes, then, DiOC6(3) fluorescence was recorded immediately with flow cytometry (FACS Canto II; BD Biosciences, San Jose, CA, USA). For each sample, 10,000 cells were acquired for data analysis.

Statistical analysis

The results are presented as the mean ± standard deviation (SD) of three replicates. Data in all experiments were analyzed for statistical significance using one-way analyses of variance (ANOVA). The p value < 0.05 was considered statistically significant. The IC50 values of curcumin over the cell lines were calculated by nonlinear regression analysis. Associations between ROS generation and cell viability parameters were analyzed by Pearson correlation coefficient. All statistical analyses were performed using the SPSS package program for Windows (Version 20; SPS, Inc., Chicago, IL, USA).

Results

Cell viability toward cancer and normal cells

On the addition of curcumin, cell viabilities increased maximally (100%) up to 10 µM doses of curcumin. Cell viability level began to decrease at this concentration and, at the 10 µM dose, the cell viability level was the same as that of the controls. At upper levels of this concentration, the percentage of antiproliferative activity increased progressively and significantly (p < 0.001) in a concentration-dependent manner. Higher doses of curcumin resulted in greater cellular death of cancer cells than of normal cells at higher concentrations (Figure 1).

The IC50 of curcumin for C-6 and L-929 cells at 24 hours was calculated for a concentration-response curve and was 32 and 72 µM, respectively. These data indicated that curcumin has a proliferative effect at lower concentrations and an antiproliferative effect at higher concentrations, and these effects were increased in cancer cells than in normal cells.

Concentration-dependent reactive oxygen generating activity

As shown in Figure 2, treatment of cells with lower doses of curcumin (10–100 µM) for 24 hours decreased intracellular ROS production in cancer and normal cells (p < 0.001). However, ROS production increased progressively with higher doses of curcumin exposure (20–100 µM) in a concentration-dependent manner. Curcumin also induced ROS generation in cancer than normal cells in a concentration-dependent manner.
Curcumin induces DNA damage

To analyze genotoxic activity, cells were treated with different dosages of curcumin for 24 hours and DNA damage was detected by the comet assay. Nuclei with damaged DNA had a comet feature with a bright head and a tail, whereas nuclei with undamaged DNA appeared to be round without a tail (Figure 3).

Each figure represents a typical comet tail of the observed cells (at least 100 cells) from two slides in each experiment. Typical micrographs of comet assays are shown in Figure 3. After 24 hours of curcumin treatment, cancer cells showed different sizes, fragmentation, and comet structures with an increased tail percentage intensity compared to the normal cells. The results indicated that curcumin-induced DNA damage in a dose-dependent manner (p < 0.001), and there were significant changes in the tail percentage of DNA between the normal and cancer cells at higher doses of curcumin (Figure 4).

Curcumin increases apoptosis

C-6 and L-929 cells were exposed to different doses of curcumin for 24 hours, stained with AO/EB, and observed under fluorescence microscopy (Figure 5). As the concentration of curcumin increased, the number of uniformly green viable cells decreased and that of the yellow–orange apoptotic cells increased after 24 hours in cancer and normal cells. The higher doses of curcumin resulted in greater apoptosis in cancer than normal cells.

Higher concentration of curcumin induces apoptotic activity by annexin-V/FITC

Higher curcumin concentration resulted in detection of a higher proportion of Annexin-V/FITC staining in the C-6 glial cancer and L-929 cell lines. When cancer and normal cells were exposed to curcumin (10–80 µM), there was a significant increase in early and late apoptosis in cancer cells compared to normal cells in a dose-dependent manner. When the curcumin concentration was
80 µM, the proportions of live cells in cancer and normal cell lines were 11.5 and 44.3, respectively (Figure 6).

**Induction of loss of DΨm (Mitochondrial Membrane Potential) by curcumin in cancer and normal Cells**

Results from flow cytometric analysis indicated that curcumin increased loss of DΨm in a concentration-dependent manner by 24 hours after treatment with curcumin in cancer cells (Figure 7). However, loss of DΨm was not significantly higher in normal cells. Results indicated that curcumin increased loss of DΨm and apoptotic induction, which was supported by the linear correlation between cytotoxicity or apoptotic induction and DΨm in cancer cells.

**Discussion**

To date, glioblastoma multiforme has remained incurable malignant tumor which can progress after gross-total resection. Neither the implementation of multimodal therapies nor advances in surgical techniques have helped to increase mean survival of affected patients above the 48-month boundary (20-22). Therefore, new therapeutic strategies are constantly under investigation. Ideally, a chemotherapeutic drug would prove efficacious selectively against tumor cells without inducing unwanted side effects.

Therapeutic effects of curcumin on various cancer cells have been reported (23-25). Despite the fact that the long-term studies in animals and humans are lacking, curcumin, being a natural compound and the main ingredient of turmeric, commonly known as “curry,” generally is regarded as a safe agent (26). Curcumin shows an inherent cytotoxicity against cancer cells, it additionally has been shown to modulate radio- and chemosensitivity of malignant cells (27-30). Dietary constituents may exhibit promising chemopreventive agents or chemotherapeutic potential and, thus, alleviate the side effects associated with conventional chemotherapy (11). Recently, more attention has been focused on complementary medicine as an alternative therapeutic modality for treatment of patients involved with malignant cancers. Two pivotal signaling mechanisms are used to maintain normal condition in healthy tissues; cell cycle progression and apoptosis. As a dietary supplement or spicing agent, curcuminoids are used worldwide. Several recent published studies aimed to elucidate the mechanism of the curcumin activities, in particular, anticancer activity (24,25,30). Most anticancer and DNA-damaging agents arrest the cell cycle at the G0/G1 or G2/M phase and then induce cell apoptosis (12).

Wang et al (24) suggested that curcumin and quinacrine liposomes modified with p-aminophenyl-α-D-mannopyranoside is a potential preparation to treat brain glioma cells and brain glioma stem cells after their in vitro experiments revealed that the targeted liposomes could inhibit the growth of glioma cells. Maiti et al study (31) showed that curcumin has potent inhibitory properties of growth for glioblastoma multiforme cells. They found out that solid lipid curcumin particles can induce more DNA fragmentation and can rapidly kill more malignant glioma cells in vitro than curcumin itself. In the same study the authors supposed that solid lipid curcumin particles and curcumin induced cell death due to excess production of ROS (31). Very recently published Mukherjee et al study (32) postulated that by binding of phytosomal curcumin to peripheral M1-type macrophages and IL12-activated natural killer cells, the brain-released chemokine monocyte chemotactic protein-1 causes recruitment of peripheral immune cells into the glioblastoma multiforme, thereby causing destruction of the malignant glioma cells and glioblastoma stem cells. In our study, we observed that curcumin-induced apoptosis significantly in glial tumor cells, with flow cytometry and microscopic apoptosis, and apoptotic activities were significantly higher in C-6 glioma cells than in L-929 normal cells. Curcumin also induced DNA damage in a dose-dependent manner, and there were significant changes in the tail percentage of DNA between the normal and cancer cells at higher doses of curcumin. We identified effective concentrations for the cell death activity of curcumin using the most sensitive luminometric ATP cell viability assay and we found that cell viabilities increased maximally (%100) up to 10 µM doses of curcumin. Cell viability level began to decrease at this upper concentration and, at the 10 µM dose, the cell viability level was the same.
as in the controls. At upper levels of this concentration, the percentage of antiproliferative activity increased progressively and significantly in a concentration-dependent manner. Higher doses of curcumin resulted in greater cellular death of cancer cells than of normal cells at higher concentrations.

The most common cell death mode in curcuminoid treatment seems to be apoptosis (30). In our study, as the concentration of curcumin increased, the number of uniformly green viable cells decreased and that of yellow–orange apoptotic cells increased after 24 hours in cancer and normal cells. The higher doses of curcumin resulted in greater apoptosis in cancer than in normal cells apoptosis can be triggered by a large variety of different stimuli. Our results were in line with previously published studies about curcumin-induced apoptosis (24,30–32). To date, two major intracellular apoptosis signaling pathways have been identified. The intrinsic pathway involves an increase in outer MMP. The extrinsic pathway involves ligation of death (Fas) receptor, resulting in recruitment of the adaptor protein FADD through interaction between the death domains of both molecules (33). In both pathways, the stress-mediated apoptosis often is triggered by mitochondrial function loss. Accordingly, the same functional loss in curcumin-mediated apoptosis also was explored in MTT viability and MMP assays. Therefore, the apoptosis induced by curcumin was considered to be through the intrinsic pathway related to mitochondrial dysfunction. In our study, we found that ROS and MMP values were increased significantly in C-6 tumor cells given curcumin compared to the L-929 normal cells.

Previously published one study showed that curcumin can enhance chemotherapeutic agents such as Nitustine hydrochloride (ACNU) which is one of the widely used standard chemotherapeutic agents (34). Zhao et al study (34) supposed that curcumin can enhance the anti-proliferation, anti-migration, and proapoptotic activities of ACNU against glioblastoma multiforme cells, and provide strong evidence that combined treatment with curcumin and ACNU has the potential to be an effective therapeutic option for malignant gliomas. The same study showed that curcumin and ACNU acted synergistically in their antitumor effects by targeting N-cadherin/MMP2/9, PI3K/AKT, and NF-κB/COX-2 signaling (34). The ROS production increased progressively at upper doses of curcumin exposure (10–100 μM) in a concentration-dependent manner. Curcumin also induced ROS generation in malignant cells than normal cells in a concentration-dependent manner. This finding supports the fact that curcumin intrinsically causes apoptosis in malignant cells. The apoptotic effect of curcumin on malignant cells rather than normal cells is connected to the G1 phase of malignant cells. Studies also suggest that curcumin has an inhibitory role in the induction of apoptosis (31).

Recent studies showed that curcuminoids are well-established dietary antioxidants. These compounds are safe natural food coloring additive agents with lipid-lowering potency in vivo (31,35). Anticarcinogenic, hepatoprotective, and neuroprotective properties against heavy metal-induced neurotoxicity and Alzheimer’s disease are proven effects of curcuminoids (36-38). In addition, curcuminoids have shown a variety of biological activities for various human diseases in the preclinical setting. Their poor oral bioavailability poses significant pharmacologic barriers to their clinical application. Liposomal or nano-emulsion curcuminoids can conduct the pharmacokinetics of curcuminoids in vivo (34,39,40). Purkayastha et al (41) study showed that a soluble formulation of curcuminoids crosses the blood–brain barrier but does not suppress normal brain cell viability. Furthermore, tail vein injection, or more effectively intracerebral injection through a cannula, blocks brain tumor formation in mice or has a neuroprotective effect on focal cerebral ischemia in rats (41,42).

According to our results, curcuminoids, in a concentration-dependent manner, showed statistically significant apoptotic, cytotoxic, and genotoxic effects on C-6 glial cells compared to L-929 normal cells through ROS generation and MMP depletion.

Acknowledgments
Preparation for publication of this article is partly supported by Turkish Neurosurgical Society (Enago).

Conflict of interest
The authors declare that they have no conflict of interest.

Ethical approval and informed consent
This study does not involve any human or animal experiments. Hence, no ethical approval or informed consent forms were needed.

Author’s contribution
Conception and design: Seyithanoğlu, Abdallah, Güler. Acquisition of data: Seyithanoğlu, Abdallah, Kitiş, Koçyiğiş, Güler, Dündar, Gündaş Papaker. Analysis and interpretation of data: Seyithanoğlu, Abdallah, Koçyiğiş, Güler. Drafting the article: Seyithanoğlu, Abdallah. Critically revising the article: Abdallah. Reviewed submitted version of manuscript: Abdallah, Kitiş. Approved the final version of the manuscript on behalf of all authors: Abdallah. Statistical analysis: Abdallah, Güler. Administrative/technical/material support: Seyithanoğlu, Abdallah, Koçyiğiş, Güler. Study supervision: Seyithanoğlu, Abdallah, Koçyiğiş.

References


