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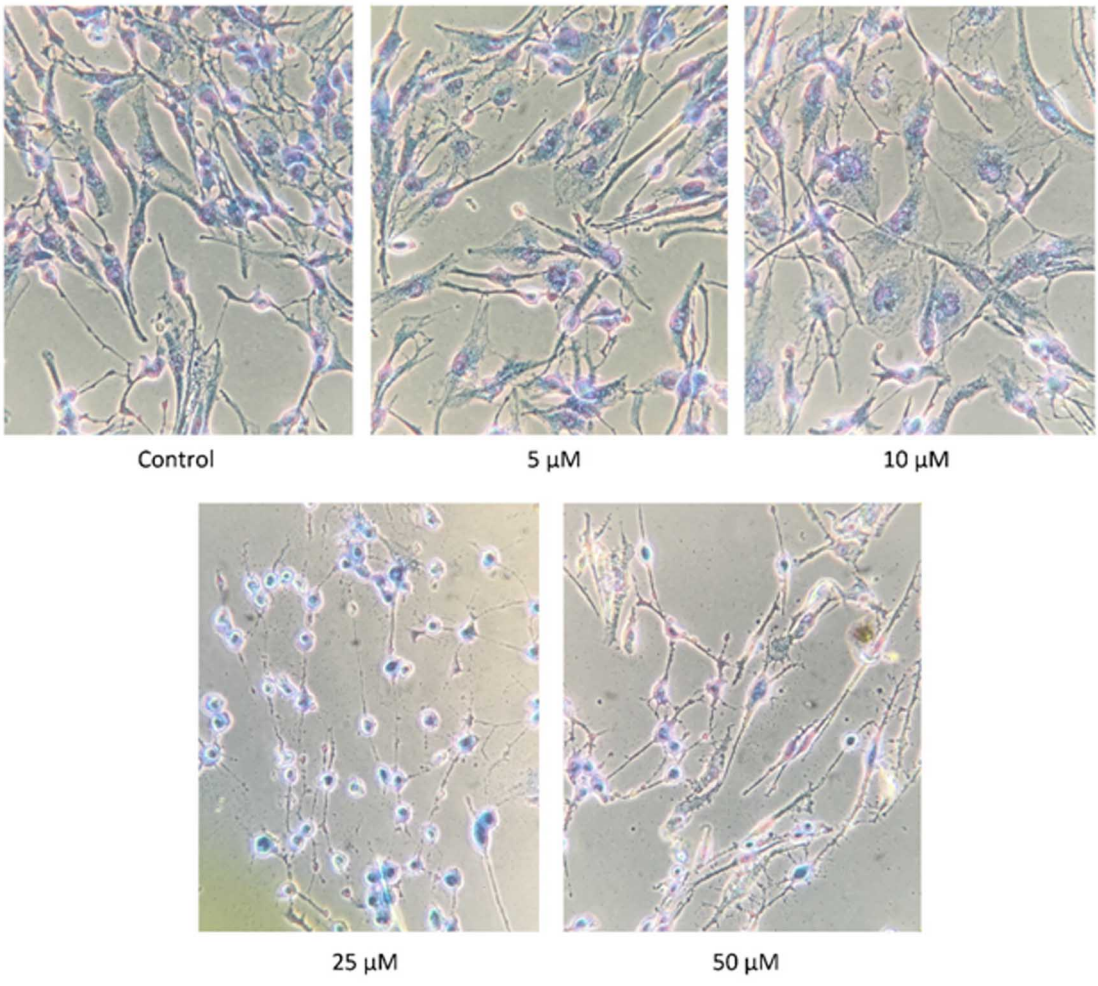

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AIM AND SCOPES
Journal of Cellular Neuroscience and Oxidative Stress is an online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

Areas of particular interest are four topics. They are;

A- Ion Channels (Na^+- K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

D- Gene and Oxidative Stress
(Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

READERSHIP

Biophysics	Biochemistry
Biology	Biomedical Engineering
Pharmacology	PhysiologyGenetics
Cardiology	Neurology
Oncology	Psychiatry
Neuroscience	Neuropharmacology

Keywords
Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide, ageing, antioxidants, neuropathy, traumatic brain injury, pain, spinal cord injury, Alzheimer's Disease, Parkinson's Disease.

RESEARCH ARTICLE

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Effects of different doses of curcumin on apoptosis, mitochondrial oxidative stress and calcium influx in DBTRG glioblastoma cells

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Abstract

Transient Receptor Potential (TRP) channels superfamily has mostly calcium ion (Ca^{2+}) permeable non-selective cation channels. Transient receptor potential melastatin subfamily 2 (TRPM2) is widely expressed in central nervous system. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) may change depend on TRPM2 and TRPM8 activations from extracellular liquid to cytosol. Curcumin as natural antioxidant shows phenolic structure, synthesized by *Curcuma longa* L, has powerful antioxidant effect. Low dose curcumin treatment can decrease calcium signaling *via* cation channel inhibition and prevent elevation of $[Ca^{2+}]_i$ levels. Hence, we investigated effects of four different concentrations (5, 10, 25 and 50 μM) of curcumin on apoptosis and cell viability (MTT), reactive oxygen species (ROS) production, mitochondrial membrane potential levels, caspase 3 and caspase 9 values in DBTRG glioblastoma cells.

We found that curcumin reduces cell viability by concentration dependent manner. It was also observed that high dose of curcumin induces apoptosis via caspase 3 and 9 related pathways. However, it was not found any direct relationship between the effect of increased concentrations of curcumin and inhibition or activation of Ca^{2+} signaling in the DBTRG cells. The $[Ca^{2+}]_i$ concentration was lower in 5 μM group as compare to control group. Curcumin acted important role on decrease of mitochondrial membrane potential and ROS production in the cells. Moreover, curcumin treatment markedly supported reduced glutathione concentration levels in the cells.

In conclusion, it was firstly assessed the effects of different doses of curcumin on calcium signaling and interaction with various apoptosis parameters in DBTRG glioblastoma cells.

Keywords: Curcumin; Cell viability; Ca^{2+} signaling; TRPM2; DBTRG cells.

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List of Abbreviations;

ΔΨ, Mitochondrial membrane potential; **$[Ca^{2+}]_i$,** Intracellular calcium concentration; **CHPx,** Cumene hydroperoxide; **DHR-123,** Dihydrorhodamine 123; **Fura-2 AM,** Fura-2 acetoxymethyl ester; **GSH,** Reduced glutathione; **GM,** Glioblastoma multiforme; **GSH-Px,** Glutathione peroxidase; **H_2O_2,** Hydrogen peroxide; **JC-1,** 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetramethyl benzimidazol carbocyanine iodide; **LP,** Lipid peroxidation; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **ROS,** Reactive oxygen species; **TRPM2,** Transient receptor potential melastatin subtype 2

Introduction

Harmful effects of increased reactive oxygen species (ROS) production on cellular metabolism and organic essential substances such as nucleic acids, proteins, lipids and carbohydrates are called as oxidative stress. Central nervous system and neurons are highly vulnerable to oxidative stress, because of lipid rich chemical composition of neural tissues. Furthermore, it is showed that oxidative stress has key player on pathogenesis of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases and also neuronal cancer cell proliferation (Floyd, 1999; Klaunig et al., 2010). Prevention of destructive effects of ROS and to keep under control the oxidant / antioxidant balance, cells mainly use intracellular powerful defense mechanisms including reduced glutathione (GSH) and glutathione peroxidase (GSH-Px) enzyme. Cells get into metabolic reactions and use several kind of non-enzymatic antioxidants with foods such as vitamins, phenolic, flavonoid and carotenoid compounds of plants. Curcumin (diferuloylmethane) is lipid soluble active polyphenolic compound of *Curcuma longa* L. (Indian saffron, turmeric), chemical formula was firstly described in 1910 (Hatcher et al., 2008). Initial studies focused on anti-inflammatory activities of curcumin, however recent investigations have shown that it has eight fold more potent antioxidant capacity compare to vitamin E (Hatcher et al., 2008). Powerful antioxidant properties of curcumin on intracellular antioxidant defense system studied and observed with both *in vitro* and *in vivo* researches (Khopde et al., 1999; Jayaprakasha et al., 2006). Calcium ion (Ca^{2+}) has key roles in neuronal cells including proliferation, excitation and neurotransmitter secretion, cellular signal transduction and amplification processes. Excessive increase of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) have been related with neurological diseases and also initiation of cellular apoptosis signal pathways *via* cysteine dependent aspartate directed proteases (caspases) (Nazıroğlu, 2011). The difference of $[Ca^{2+}]_i$ between intracellular and extracellular media is under controlled by ion channels including *Transient Receptor Potential* (TRP) channels. TRP channel superfamily has 28 members and mostly non-selective cation channels, divided into seven different subfamilies according to similarity of amino acid sequences. TRPM subfamily has characteristic melastatin homology region in N-terminal part of the channel structure and classified to four subgroups. TRPM2 is a Ca^{2+} permeable non-selective cation channel that can be activated by adenosine diphosphate ribose (ADPR) and its derivate cyclic adenosine diphosphate ribose (cADPR) on unique ADPR pyrophosphatase activity region in the C-terminal nudix domain (Wehage et al., 2002; Clapham, 2003). It could act as a cellular oxidant sensor and also independently activated by experimental oxidative stress agent, hydrogen peroxide (H_2O_2) (Kaneko et al., 2006, Naziroğlu and Lückhoff 2008a). Despite the fact that numerous channel inhibitors of these channels have been discovered, none of them can completely block the ionic influx. Since a decade, a novel approach has come out to ROS sensitive activation of TRP channels can inhibit by antioxidants. However, we have less data about whether different doses of antioxidant administration have inhibitor or activator effects on TRPM2 channel gating manner. In fact, high concentrations of antioxidant administration may cause reduction of cell viability and it can be resulted with ionic concentration changing. Denver brain tumor research group (DBTRG) cell line is an *in vitro* model of glioblastoma multiforme (GM). Recent studies emphasized that TRP channel mediated Ca^{2+} signaling is related with migration and proliferation in the glioblastoma cells (Lepannetier et al., 2006; Wondergem and Bartley, 2009). In several studies, researchers also have shown the strong restrictor effects of curcumin on cell proliferation and emphasized it can be a therapeutic agent for different type of glioblastoma cell lines (Tsunoda et al. 2005; Zanotto-Filho et al., 2012). However, we have less data about how curcumin acts as an reducer on DBTRG cell viability and targets which molecular pathways including TRPM2 related Ca^{2+} signaling. Thus, the main aim of the current study is to investigate different concentrations of curcumin administration on cell viability, ROS production, mitochondrial membrane potential, caspase 3 and 9 activity levels and some oxidant/antioxidant parameters. It was also evaluated that concentration dependent effects of curcumin on TRPM2 channel mediated calcium signaling in DBTRG glioblastoma cells.

Materials and Methods

Chemicals, cell culture and dose determination

The DBTRG human glioblastoma cell line was puchased from Şap Institute, Ankara, Turkey. RPMI-1640 medium, fetal bovine serum (FBS), sodium pyruvate, antibiotic mixture penicillin+streptomycin and phosphate buffered saline (PBS) were purchased from Biochrom, Berlin, Germany. Curcumin, dimethyl sulfoxide (DMSO), L-glutamine, Trypsin-EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, cumene hydroperoxide (CHPx) and all other reagents and salts were purchased from Sigma Aldrich, St. Louis, MO, USA. Fura-2 acetoxymethyl ester (Fura-2 AM) dye was purchased from Invitrogen, Carlsbad, CA, USA. Dihydrorhodamine-123 (DHR-123) dye was obtained from Molecular Probes, USA and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1) dye from Santa Cruz Biotechnology, USA. AC-DEVD-AMC substrate of caspase 3 and AC-LEHDAMC substrate of caspase 9 were purchased from Bachem, Switzerland. The APOPercentage was provided from Biocolor Ltd., Belfast, Northern Ireland. Histological stains wright-giemsa and methylene blue were purchased from GBL Ltd., İstanbul and Biyans BÜ Ltd., Ankara, Turkey, respectively.

The DBTRG cells were cultured in RPMI-1640 medium containi<ng 10% FBS, 1% sodium pyruvate, 1% L-glutamine and 100 U/ml penicillin+100 µg/ml streptomycin at 37 °C in 5% CO_2 humidified incubator (Heal Force HF90, Smart Cell, Japan). After cells have reached 80-85% confluence, washed with fresh 1X PBS, detached with 0.25% Trypsin-EDTA and splited into the new culture flasks. 7-13 number of passages were used in the experiments. Curcumin dissolved in a DMSO stock solution, kept under dark condition at +4 ^{o}C in a fridge, freshly diluted in cell culture medium before doing incubation. To exert time and dose dependent manner of curcumin on DBTRG cell viability, cells were seeded in a 96 well plates (~10.000 cells/well), incubated for 24 h in culture conditions to attachment bottom of the well. Control (curcumin free medium) and ten different doses of curcumin (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 µM) were administered to wells and incubated through three different times as 24, 48, 72 h. The 20 µl of MTT dye (5 mg/ml) added into wells and incubated for 3 h after defined time courses end. Then, wells discarded carefully, 150 µl of DMSO added, and gently shaked in the dark for 5 min, finally read in a multiplate reader (Tecan Infinite 200 Pro, Austria) at 490 nm excitation, 630 nm emission wavelengths. Results expressed as the fold of experimental/control (Mahalingaiah and Singh, 2014).

Methylene blue and Wright-giemsa staining

The DBTRG cells were plated and cultured at a density of $4x10^5$ cells/well in 6 well plates in order to staining with methylene blue and wright-giemsa modified stains for identification of morphological features, alterations and apoptotic morphology. At the end of the 24 h curcumin administrations, the culture media were aspirated from wells. Cells were washed two times with ice cold 1x PBS for in situ methylene blue staining. Each plate was placed on ice, and ice cold ethanol 50% (v/v) was added to fixation for 5 min; ethanol was aspirated followed by the addition of 2 ml of ice cold 0.2% (v/v) methylene blue solution which prepared in 50% ethanol. Cells were stained for 1 min and washed twice with ice cold water. Cells were examined under a light microscope (Axiostar, Zeiss, Germany) at 200x magnification (Zhang et al., 2009).

To wright-giemsa staining, after removing the culture media, wells were washed twice with 1x PBS and fixed with methanol for 10 min at room temperature. The methanol was removed and wells were washed twice with 1x PBS again and added in wright-giemsa modified solution for staining. After 5 min incubation at room temperature, dishes were washed with PBS then examined under the light microscope at 100x magnification and photographed (Karmakar et al., 2007).

Determination of intracellular free calcium ($[Ca^{2+}]_i$) concentration

In order to evaluate the effects of different doses of curcumin on TRPM2 related calcium signaling, cells were incubated in culture media containing 5, 10, 25, 50, 100 µM of curcumin for 24 h before analyses. For the determination of $[Ca^{2+}]_i$, ~2 × 10^6 cells/ml were washed and gently re-suspended in loading buffer, Na -4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Na-HEPES) solution (at pH 7.4 containing; NaCl 140

mM, D-glucose 10 mM, HEPES 10 mM, KCl 4.7 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 1.1 mM) and loaded with 4 μM of Fura-2 acetoxymethyl ester (Fura-2 AM) dye for 45 min at 37 °C shaker bath in the dark (Pariente et al., 2001; Uğuz and Nazıroğlu, 2012). The study groups were exposed to CHPx to evoke Ca^{2+} signals. Fluorescence was recorded from 2 ml aliquots of magnetically stirred cellular suspension at 37 °C using a spectrofluorometer (Varian Cary Eclipse, Sydney, Australia) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_i$ were monitored by using the Fura-2 AM 340/380 nm fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (1985). Ca^{2+} concentrations in the DBTRG cells were estimated using the integral of the rise in $[Ca^{2+}]_i$ for 150 sec. after addition of CHPx (0.1 mM). Ca^{2+} concentrations are expressed as nM quantities, taking a sample every second as previously described (Grynkiewicz et al., 1985).

Measurement of intracellular ROS production

The DHR-123 probe was used to determination of intracellular ROS production. Cells were loaded with 2 μM DHR-123 at 37 °C for 30 min as previously described (Uguz et al., 2012). The fluorescence intensity of rhodamine-123 was measured in a fluorescence multiplate reader (Tecan Infinite 200 Pro, Austria). Excitation was set at 488 nm and emission at 543 nm. Treatments were carried out in triplicate. Data were calculated as fold increase experimental to control.

Measurement of GSH, GSH-Px and protein assays

The GSH content of the DBTRG cells after curcumin administrations were measured at 412 nm using the method of Sedlak and Lindsay (25). GSH-Px activities in DBTRG cells were measured spectrophotometrically at 37 °C and 412 nm according to the method published by Lawrence and Burk (1976). The protein content in the homolyzed DBTRG cells were measured by the method of Lowry et al. with bovine serum albumin as the standard (Lowry et al., 1951). Values are expressed as μM/g protein.

Measurement of lipid peroxidation level (LP)

LP levels in the DBTRG cells were measured with the thiobarbituric acid reactive substances assay (TBARS) by the method of Placer et al. (Placer et al., 1966). The quantification of TBARS were determined by comparing the absorption with the standard curve of malondialdehyde (MDA) equivalents generated by acid catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. Briefly, the principle of the method depends on the determination of the pink color that is produced by the interaction of thiobarbituric acid with MDA to form a colored MDA–TBA adduct. Levels of lipid peroxidation as MDA were determined spectrophotometrically (Shimadzu UV-1800, Kyoto, Japan) at a wavelength of 532 nm. The values of LP in the cell homogenate expressed as μM/gram protein.

Measurement of mitochondrial membrane potential (ΔΨm) levels

The JC-1 probe was used to determination of membrane potential of mitochondria. The green JC-1 signal was measured at the excitation wavelength of 488 nm and the emission wavelength of 525 nm for the green signal, and the red signal, at the excitation wavelength of 540 nm and the emission wavelength of 590 nm. Fluorescence changes were analyzed using a fluorescence multiplate reader (Tecan Infinite 200 Pro, Austria). Treatments were carried out in triplicate. Data are presented as green to red emission ratios (525/590). Changes in mitochondrial membrane potential were quantified as the integral of the decrease in JC-1 fluorescence ratio of experimental to control (Uguz et al., 2012).

Assay for caspase 3 and 9 activity

The activities of caspase 3 and 9 were assessed from cleavage of the specific fluorogenic substrates AC-DEVD-AMC and AC-LEHDAMC, respectively. After incubation with curcumin, the DBTRG cells were sonicated and cell lysates were incubated with 2 ml of substrate solution (20 mM HEPES at pH 7.4, 2 mM EDTA, 0.1 % CHAPS, 5 mM DTT and 8.25 μM of caspase substrate) for 1 h at 37 °C as previously described (Uğuz et al., 2009). Substrate cleavage was measured with a fluorescence multiplate reader (Tecan Infinite 200 Pro, Austria) with an excitation wavelength

of 360 nm and an emission wavelength of 460 nm. Former experiments confirmed that caspase 3 or 9 substrate cleavage were not detected in the presence of the caspase 3 or 9 inhibitors, DEVD-CMK or z-LEHDFMK, respectively. The data were calculated as fluorescence units per mg of protein.

Apoptosis assay

Apoptosis assay was performed according to the manufacturer's instruction. When the membrane asymmetry of apoptotic cells lost, the APOPercentage dye is actively linked phosphatidyl serines and transported and accumulated into cells, stains apoptotic but not necrotic cells to red color, and so allows the detection of apoptosis by a multiplate reader (Infinite 200) as previously described, elsewhere (Uğuz and Nazıroğlu, 2012). Data were calculated as fold increase experimental to control.

Statistical analyses

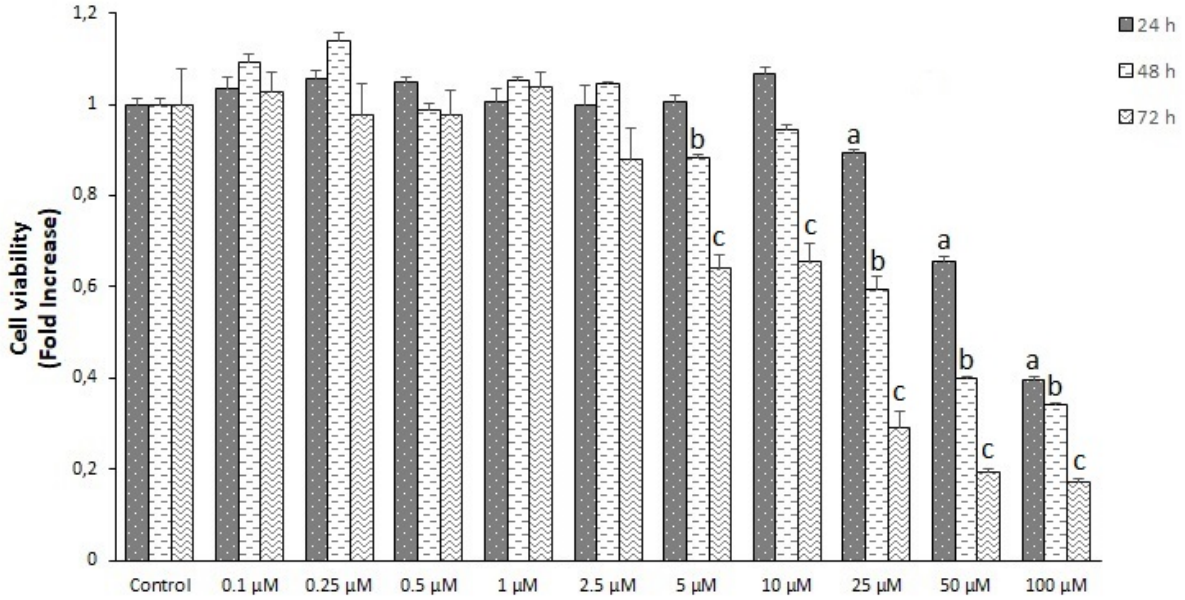
All results were expressed as mean ± standard deviation (SD). The significance of differences among groups were assessed with one way analysis of variance (ANOVA) least significant difference (LSD) test. Data were analyzed using the SPSS, version 9.05 (SPSS, Inc., Chicago, Illinois). $P<0.05$ was considered significant.

Results

Identification of therapeutic doses of curcumin in DBTRG cells

The cell viability (MTT) test results showed that curcumin treatment gradually reduced MTT concentration through dose dependent manner in the DBTRG cells (Figure 1). Cells were incubated with increasing concentrations of curcumin (0.1 µM-100 µM) for different 3 time periods (24, 48 and 72 hours). MTT levels were decreased from 5 µM to 100 µM concentrations. In lower doses (0.1-2.5 µM), it appears that curcumin has not significantly changed cell viability compare to control group. Moreover, curcumin incubation 5 µM and more concentrate significantly ($p<0.001$) inhibits cell viability depend on time. It is shown that inhibitor effect of curcumin elevates as time progress from 24 to 72 h. Hence, the 5, 10, 25, 50 and 100 µM curcumin doses were determined as study groups and the 24 h was elected as suitable time duration of curcumin treatment.

Figure 1. Effect of different doses of curcumin administration on cell viability levels. Curcumin inhibits cellular viability in DBTRG cells dose and time dependent. Curcumin more effective at higher doses (>2.5 µM) compare to lower doses (<2.5 µM) through 24 h and more incubation time. It was observed that in each three different time, dose dependent curcumin administration has significantly inhibitor effect on cell viability in DBTRG cells. (a; $p<0.001$ vs. 0.1-10 µM, b; $p<0.001$ vs. 0.1-5 µM, c; $p<0.001$ vs. 2.5 µM only, n=8 for each group).



Curcumin changes morphological features of DBTRG cells

The morphological change of DBTRG cells was controlled after treatment of curcumin with different concentrations. Cells shrank from a flat and elongated shape to a small and unevenly round shape after treatment with curcumin following dose elevation. Adhered viable cells decreased as the treatment dose increased (Figure 2). Apoptotic feature of DBTRG cells after curcumin treatments were shown by wright-giemsa modified staining kit. Apoptosis of cells developed either after 24 h treatment depend on dose elevation, but the morphological change became apparent when dosage went beyond the 10 µM and more concentrates (Figure 3).

Figure 2. Methylene blue staining shows morphological changes in DBTRG glioblastoma cells after 24 h and different doses of curcumin administrations. Cells were examined and photographed under the light microscope at 200x magnification.

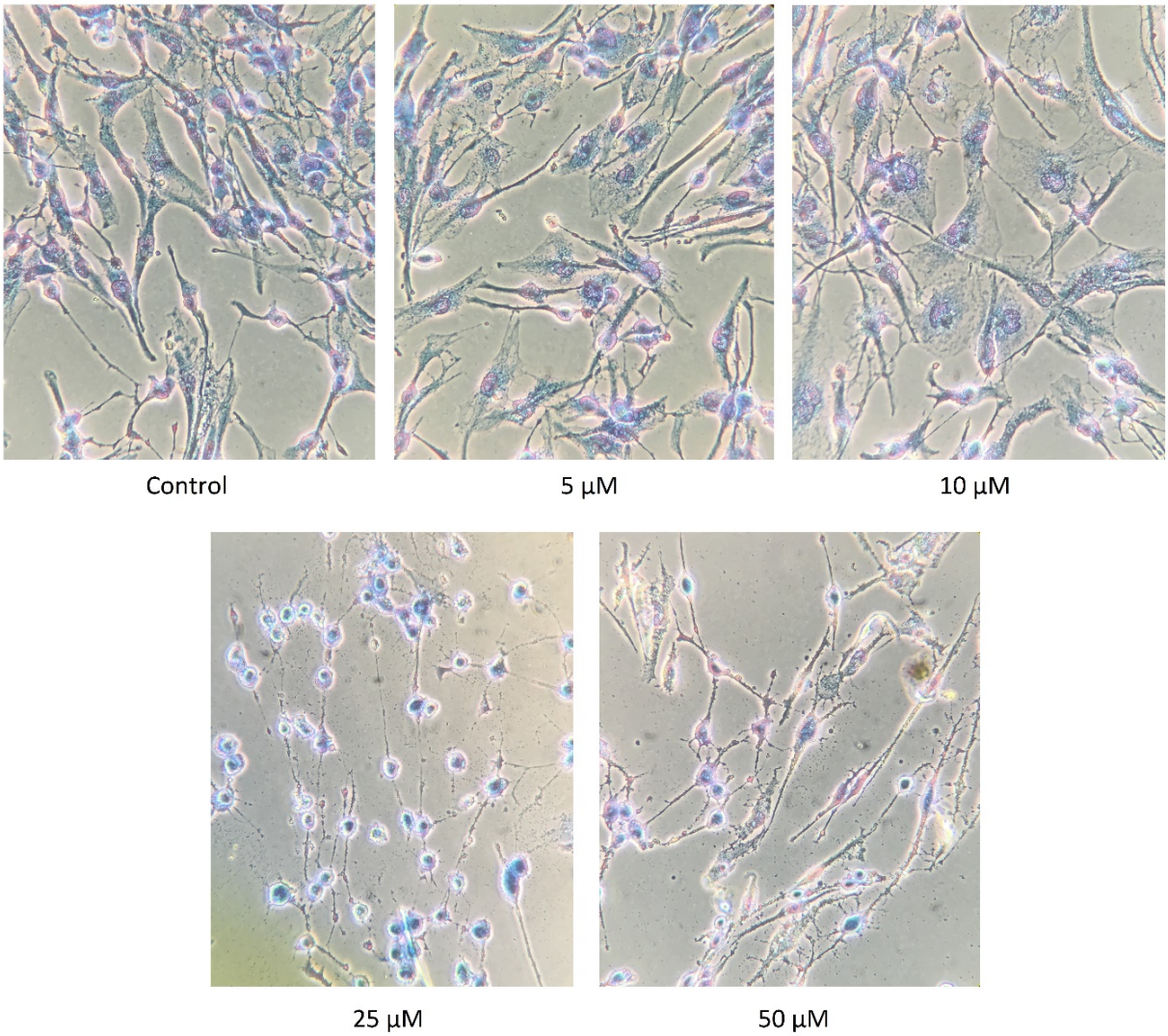
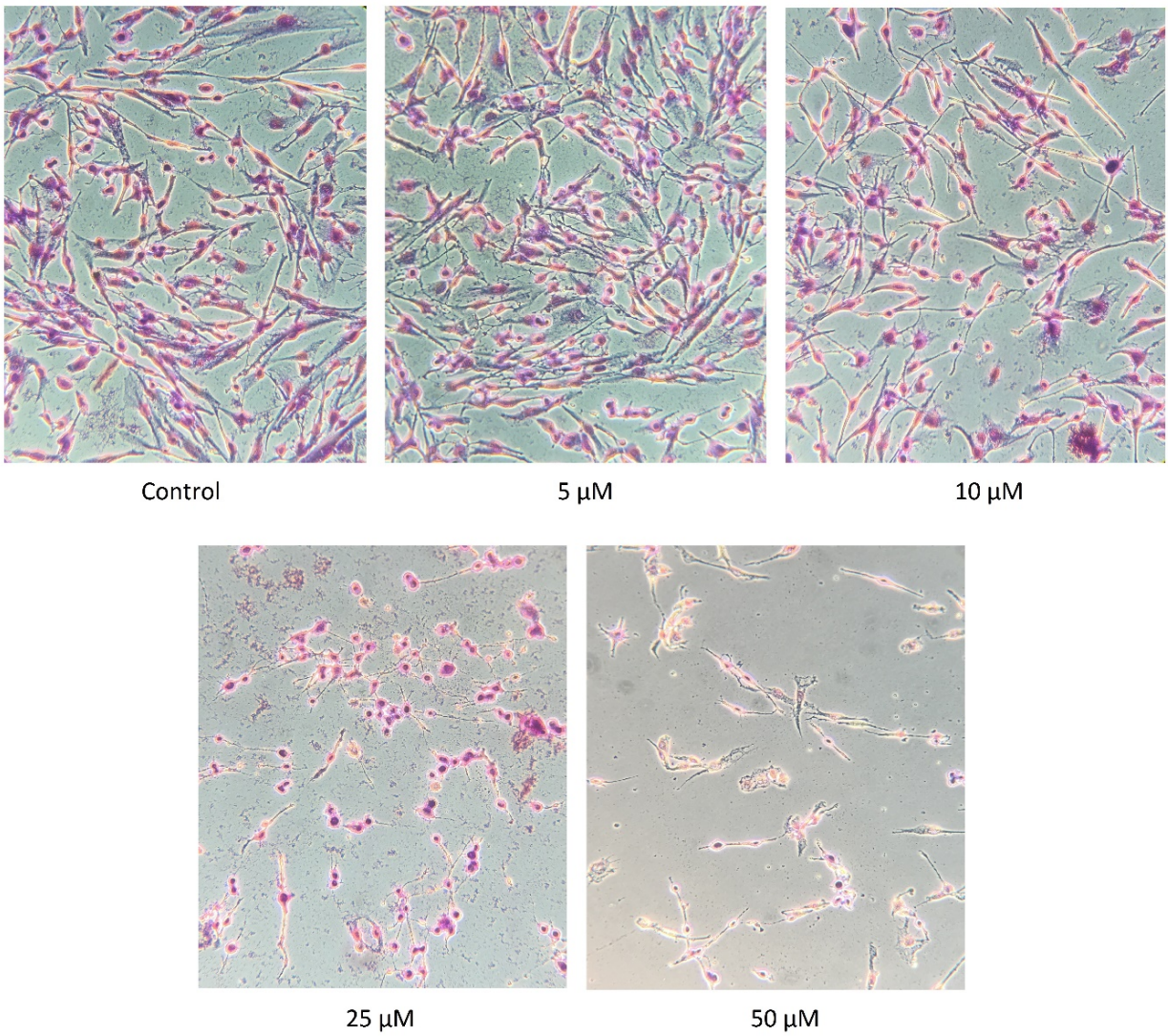


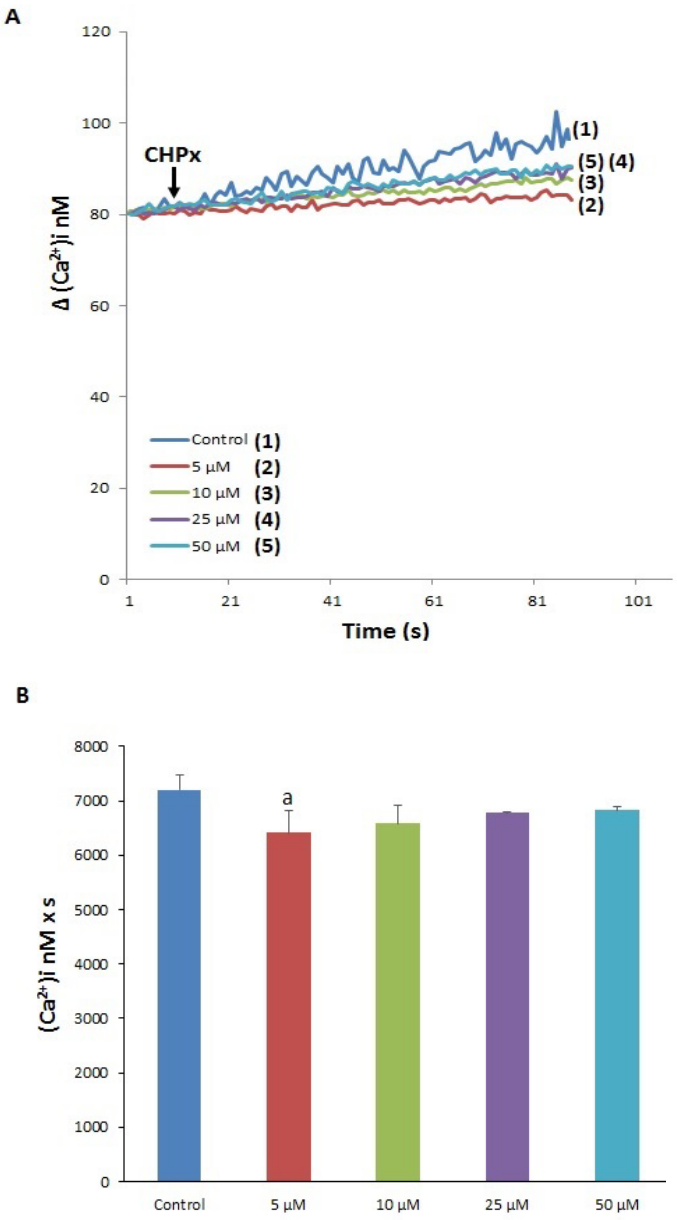
Figure 3. Wright-giemsa staining of DBTRG glioblastoma cells after 24 h and different doses of curcumin administrations. Cells were examined under the light microscope at 100x magnification.



Effects of curcumin on TRPM2 mediated calcium signaling

The $[Ca^{2+}]_i$ concentration results shown as line plots and columns are presented in Figure 4-A and B, respectively. In the analyses, TRPM2 channel activation was stimulated by an experimental oxidizing agent and ROS analogue, cumene hydroperoxide (CHPx and 0.1 mM). The $[Ca^{2+}]_i$ concentration were significantly ($p<0.05$) lower in the 5 µM curcumin incubated group compare to the control group. However, it was not found direct relationship between dose dependent manner of curcumin and TRPM2 channel mediated calcium influx.

Figure 4. (A) Effects of curcumin administrations on calcium signaling through activation TRPM2 channels in DBTRG cells following stimulation with CHPx (0.1 mM). Original time course chart recordings show $[Ca^{2+}]$i transients in DBTRG cells. **(B)** Bar charts show mean ± SD data for $[Ca^{2+}]$i from CHPx stimulated groups (n=6 for each). Note that the significant elevation in $[Ca^{2+}]$i for DBTRG cells compared to control group (a; $p<0.05$).



Curcumin inhibits ROS production

The ROS production levels were determined by DHR-123 non-fluorescent dye. The ROS levels obtained from the study groups are shown in Figure 5. The results indicated that ROS levels were significantly ($p<0.001$) lower in curcumin group compare to the control group. Moreover, curcumin treatment decreased markedly ($p<0.001$) the ROS level in the cells dose dependently.

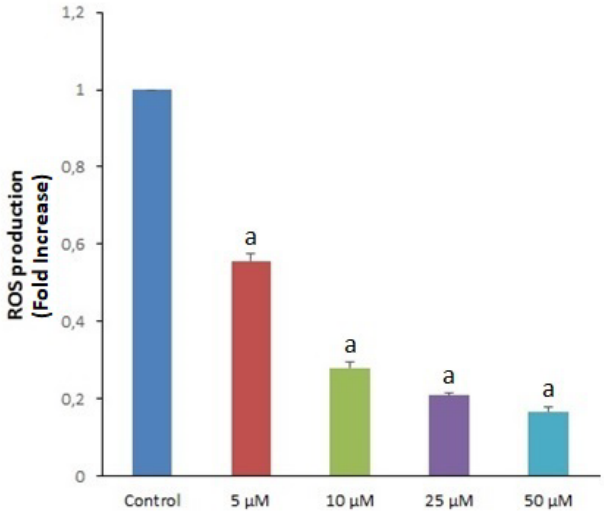


Figure 5. Effects of curcumin administrations on ROS production levels in DBTRG cells (mean ± SD; n=8). The values were expressed as fold increase over the control level. There were statistically difference with curcumin concentrations and control group (a; $p<0.001$ vs. control group).

Effects of curcumin on glutathione (GSH) and glutathione peroxidase (GSH-Px) and lipid peroxidation (LP) values

GSH-Px, GSH and LP values are shown in Table 1. The results indicated that GSH-Px activity was significantly ($p<0.05$) lower in all doses of curcumin treated groups compare to control. GSH levels were markedly ($p<0.001$) increased in the 10 µM and 25 µM curcumin incubated groups compare to control group. However, effect of curcumin on LP level was observed in only 50 µM incubated group compare to control and 5 µM groups ($p<0.05$).

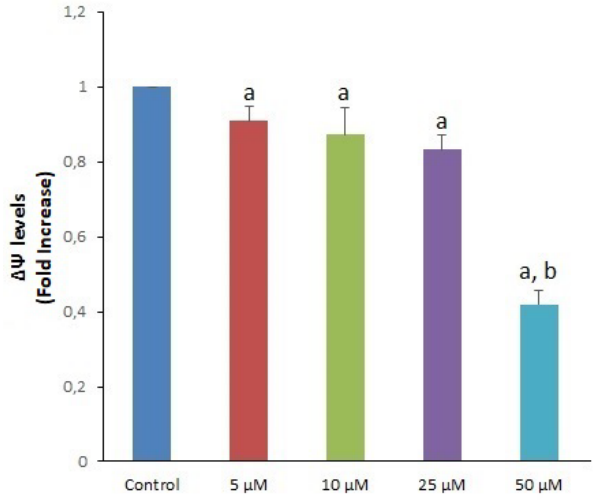
Table 1. Effects of different curcumin doses on GSH, GSH and LP values in the DBTRG cells (mean ± SD; n = 6 for all groups). There were statistically difference among curcumin groups and control group (a; $p<0.05$ vs. control group, b; $p<0.001$ vs. control group, c; $p<0.05$ vs. control and 5 µM groups).

Parameters	Control	5 µM	10 µM	25 µM	50 µM
GSH-Px (IU/g protein)	10.55 ± 0.76	9.20 ± 0.30 [a]	9.21 ± 0.55 [a]	8.42 ± 0.11 [a]	8.65 ± 0.23 [a]
GSH (µmol/g protein)	29.52 ± 0.96	30.70 ± 2.24	76.04 ± 6.13 [b]	54.32 ± 5.59 [b]	28.29 ± 0.66
LP (µmol/g protein)	47.83 ± 3.26	47.83 ± 4.98	44.20 ± 3.32	44.57 ± 4.35	40.94 ± 1.66 [c]

Curcumin decreases mitochondrial membrane potential (ΔΨm) levels

Effects of curcumin on the ΔΨm levels were given in the Figure 6. Curcumin incubations were decreased ΔΨm levels. Concentration dependent reducer effects of curcumin on ΔΨm levels significantly ($p<0.001$) observed from 5 µM to 25 µM groups compare to control group. Moreover, in 50 µM group, the level of ΔΨm were markedly ($p<0.001$) decreased compare to other curcumin treated groups.

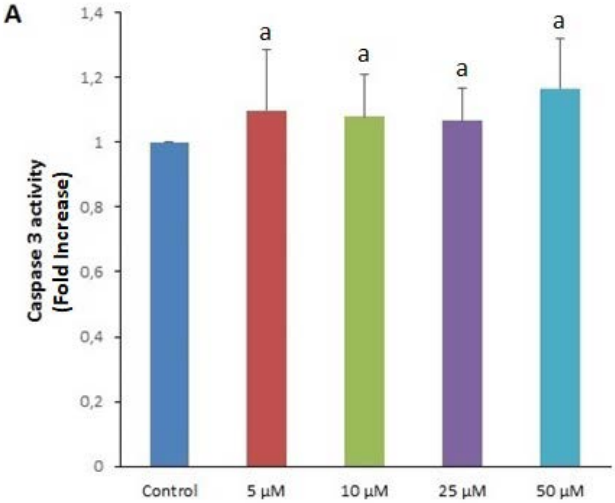
Figure 6. Effects of different curcumin doses on the mitochondrial membrane depolarization (ΔΨ) levels in the DBTRG cells (mean ± SD; n=8). (ap<0.001 vs. control group, bp<0.001 vs. 5, 10 and 25 µM groups).

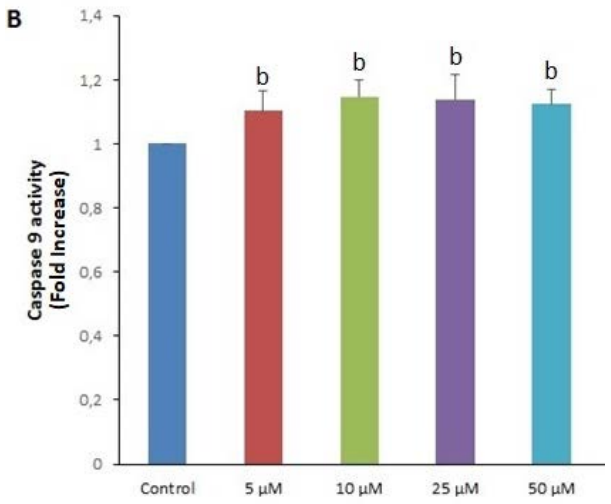


Curcumin elevates caspase 3 and 9 enzyme activities

Effects of curcumin on caspase 3 and 9 enzyme activities are presented in the Figure 7A and 7B. The caspase 3 enzyme activity in curcumin treated all groups were significantly higher than in control group ($p<0.05$). Similarly, the caspase 9 enzyme activity were also markedly ($p<0.001$) higher in curcumin incubated groups compare to control group.

Figure 7. Effects of different curcumin doses on caspase 3 (A) and 9 (B) activity in the DBTRG cells cells (mean ± SD; n=8). ([a]p<0.05 vs. control group, [b]p<0.001 vs. control group).

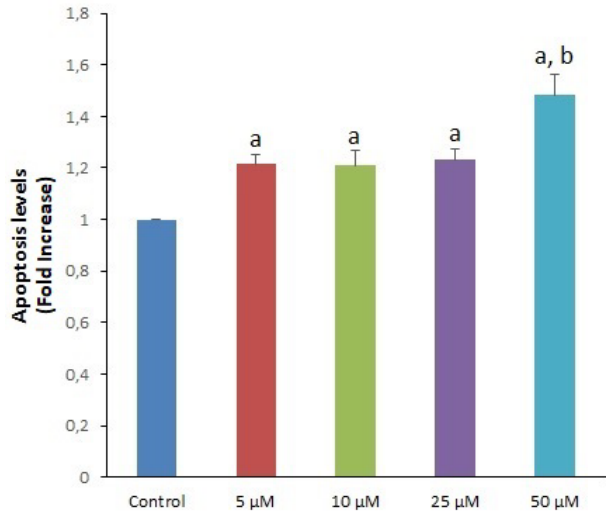




Curcumin induces apoptosis

Effects of curcumin on apoptosis levels in DBTRG cells are shown in Figure 8. The apoptosis levels were relatively higher in curcumin groups as compared to control group ($p<0.001$). Furthermore, apoptosis level were markedly ($p<0.001$) increased in 50 µM group compare to other curcumin treated groups.

Figure 8. Effects of different curcumin doses on apoptosis levels in the DBTRG cells (mean ± SD; n=6). (a; $p<0.001$ vs. control group, b; $p<0.001$ vs. 5, 10 and 25 µM groups).



Discussion

The current results implied that curcumin treatments might increase the levels of apoptosis and caspase 3, 9 activities in DBTRG cells and decrease mitochondrial membrane potential. However, because of curcumin has potent antioxidant capacity, curcumin treatments with increasing concentrations decrease intracellular ROS production. In contrast to higher doses, 5 µM curcumin treatment can also decrease $[Ca^{2+}]_i$. To our knowledge, this is the first evidence for a function of curcumin in a cellular GM model.

GM is known as the most frequent and aggressive brain tumor and has two types. Primary GM is characterized by epidermal growth factor receptor overexpression, p16 tumor suppressor gene deletion and phosphatase / tensin homolog tumor suppressor gene mutation. In secondary GM, common mutations are found in TP53 tumor suppressor and a cell surface protein, platelet derived growth factor receptor-alpha genes (Zhang et al., 2008; Luthra and Lal, 2016). After diagnosis, relative survival estimate for GM patients is very short and less than 5% can survive five years (Kohler et al., 2011). In a study, GM patients have highly expressed some TRP cation channels in brain tissues. Moreover, researchers also firstly resulted that there was a correlation between TRP channel overexpression and surviving rate more than one year in GM patients (Alptekin et al., 2015). TRPM2 channels are mostly expressed in brain and neurological tissues including dorsal root ganglia (DRG) and hippocampus (Fonfria et al., 2006). Perraud et al (2001) showed that TRPM2 channels can be activated by intracellular ADPR and it regulates Ca^{+2} and cationic influx in U937 monocyte cells. TRPM2 also gating by ROS indirectly, and it is highly unique because of both channel and enzymatic structure (chanzyme) of the C-terminal nudix homology region which named ADPR pyrophosphatase domain (Perraud et al., 2001). Previous studies remarked that the C-terminal region of the TRPM2 channels are also independently activated by ROS (Hara et al, 2002; Naziroglu and Lückhoff, 2008a). Although many blockers have been identified, none of them completely and efficiently block these channels. Because of these channels play an oxidant sensor role in cellular physiology of neuronal cells, more than a decade, studies have been focused on indirectly inhibitory effects of antioxidants and trace elements on TRPM2 mediated cationic currents by scavenging the ROS generation. Although vitamin E, C and GSH have not inhibitor effects (Naziroğlu and Lückhoff, 2008b), it has been showed melatonin and selenium act as

inhibitor on TRPM2 channel currents in transfected Chinese hamster ovary (CHO) cells (Celik and Nazıroğlu, 2012; Nazıroğlu et al., 2013). Curcumin has nearly eight fold more potent antioxidant properties those of vitamin E (Ak and Gülçin, 2008) and its effect on TRPM2 currents has not yet been studied in cellular model of GM. Latest findings showed that non-selective TRPM2 cationic influxes can be inhibited by dose dependent extracellular curcumin administration in cultured rat hepatocytes (Kheradpezhouh et al., 2016). The importance of the current study, being one of the novel findings explaining the interaction between curcumin and its effect on TRPM2 channel related Ca^{2+} signaling and apoptosis processes in DBTRG cells. In our work, Ca^{2+} signaling results demonstrated that 5 µM curcumin incubation significantly ($p<0.05$) inhibits Ca^{2+} release in DBTRG cells. However, more than 5 µM of curcumin treatment seems not to be inhibitor capacity on TRPM2 channels in DBTRG cells. Our results confirmed to a novel TRPM2 study. Similarly, Kheradpezhouh et al., (2016) determined that 5 µM curcumin incubation has inhibited paracetamol and H_2O_2 induced $[Ca^{2+}]_i$ elevation in rat hepatocytes (Kheradpezhouh et al., 2016). In a recent study, 5 µM curcumin incubation was also inhibited $[Ca^{2+}]_i$ transients in SH-SY5Y neuroblastoma cells (Uğuz et al., 2016). Furthermore, it was firstly demonstrated that increased concentrations of curcumin has not same inhibitor effects compare to 5 µM.

Intracellular ROS production levels have been demonstrated that curcumin has strong ability on ROS scavenging. However, when the GSH-Px activity, GSH and LP values assessed together intracellular ROS production result, curcumin has not provide antioxidant support on these parameters. In the coupled mitochondria, ATP production is at normal levels and mitochondrial membrane potential (ΔΨm, MMP) is high, and mitochondrial membrane depolarization (MMD) is low but some structural changes of mitochondria, namely, cristal unraveling and matrix condensation, are a consequence of the decline in MMP or resulted in increase of MMD (Gottlieb et al., 2003; Perl et al., 2004). Decreasing of the MMP levels can induce different apoptotic stimuli (Susin et al., 1997). In the current study, we demonstrated that curcumin incubations significantly reduce the MMP levels by concentration dependently. Increased caspase 3 and caspase 9 enzyme activities and apoptosis assay results also were corroborated that curcumin stimulates DBTRG cells to undergo apoptotic pathways like similar studies (Karmakar et al., 2007, Huang et al., 2010). MTT results demonstrated that curcumin indicated cytotoxic effects in the DBTRG cells dose and time dependently. Hence, this *in vitro* GM model study, we concluded that the possible inhibitor effect of curcumin on cell viability and inductor effect on apoptosis are seem to directly related with mitochondrial membrane integrity and caspase 3 and 9 related apoptotic pathways, but not TRPM2 mediated Ca^{2+} signaling.

In conclusion, curcumin shows protective effect against to oxidative stress, and protects antioxidant redox system in the DBTRG cells. In addition, low dose curcumin modulated Ca^{2+} release in the cell line system. Higher doses of curcumin decreases cell viability, increases apoptosis and shows concentration dependent ROS scavenging ability. Our current study on DBTRG cells taken together with other GM studies suggest a therefore unappreciated therapeutic potential of curcumin in psychiatric diseases such as schizophrenia and depression diseases that are characterized by oxidant stress.

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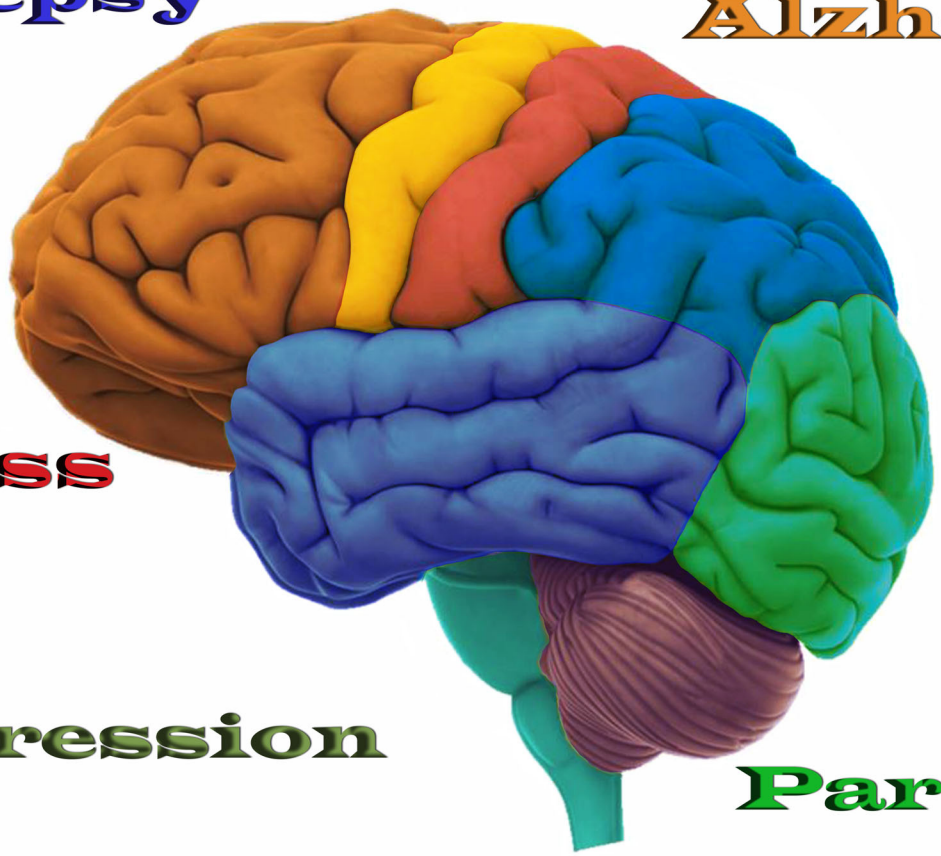

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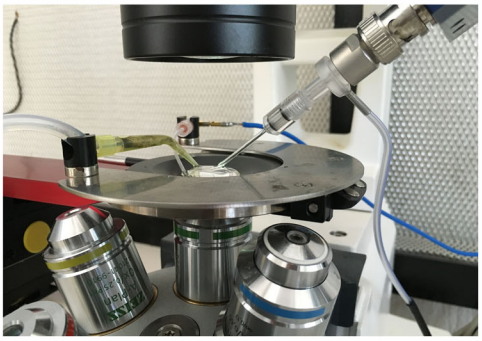

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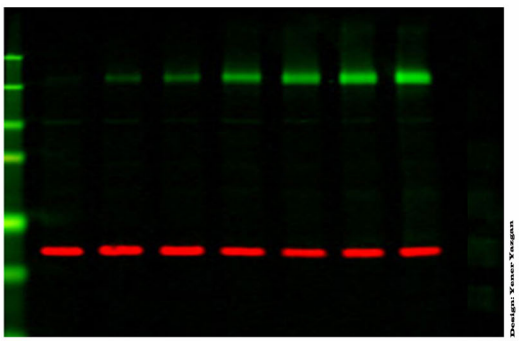

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