

Cell death and neuronal arborization upon quercetin treatment in rat neurons

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Quercetin, one of the major flavonoids, exhibits many beneficial effects on human organism as antihistamine, antioxidant, anti-inflammatory, anticancer and antiviral drug. It is recommended as supplement of healthy diet but still the knowledge of its beneficial effect on normal cells is not satisfactory. We decided to examine the effect of flavonoid on neurons morphology and their susceptibility to cell death. Fractal analysis of rat neurons revealed that 24 hours long incubation with quercetin diminished neuronal arborisation in cortical neurons. Neurons also appeared to be very sensitive to cell death after flavonoid treatment in concentration dependent manner. Over 50% of cells died after incubation with 15 µg/ml of flavonoid while 1 µg/ml of quercetin induced cell death only in 5%. Staining with Hoechst 33342 and propidium iodide revealed the two types of cell death: apoptosis and necrosis. The number of apoptotic cells was comparable with necrotic ones. These results suggest toxic effect of quercetin on neurons what should be taken into consideration in further studies on using quercetin as therapeutic agent.

Key words: neurons, quercetin, apoptosis, necrosis, fractal analysis

INTRODUCTION

Quercetin (3,3',4',5,7-pentahydroksyflavone) is a flavonoid present in many fruits, vegetables and beverages like green tea, red wine. Average daily consumption of quercetin is about 30 mg/day. Only 30–50% of ingested quercetin is absorbed, the rest passes through gastro-intestinal tract. Manufacturers recommend daily dose of quercetin supplements ranges from 400–1 200 mg/day. It is marketed as diet supplement with antihistamine, anti-inflammatory, antiviral, immunomodulatory and antioxidant properties (Ross and Kasum 2002). At the molecular level quercetin activates mitogen-activated protein kinase (MAPK) pathway (including extracellular regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and

p38) leading to the expression of survival genes (*c-fos* and *c-jun*) and activates survival and protective mechanisms (Kong et al. 2000, Spencer et al. 2003b). It acts as anticancer agent by down-regulating the expression of oncogenes (*H-ras*, *c-myc* and *K-ras*) and anti-oncogenes (*p53*) or up-regulating cell cycle control protein (p21WAF1 and p27KIP1) (Ranelletti et al. 2000, Casagrande and Darbon 2001). Activation of caspase-3, caspase-7 and caspase-9, cleavage of poly ADP-ribose polymerase (PARP) and release of cytochrome c by quercetin was observed in HL-60 leukemic cells and A549 lung cancer cells (Wang et al. 1999, Nguyen et al. 2004). The flavonoid down-regulates the antiapoptotic protein Bcl-x1 and up-regulates the proapoptotic Bax protein (Vijayababu et al. 2005).

The central nervous system has high rate of oxidative metabolism and exhibits increased vulnerability for effects of oxidative stress (Esiri 2007). The antioxidant profile of quercetin and the ability to scavenge free radicals would be a strong advantage for neuron-

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protective activity in the brain, including neuroprotection in age-related dementias and Alzheimer's disease (Esposito et al. 2002, Luo et al. 2002, Zimmermann et al. 2002). Mercer and others (2005) showed that the ability of flavonoid to protect mesencephalic dopamine (DA) neurons from injury and to reduce apoptosis caused by oxidative stressors *in vitro* could provide benefits in the therapy of Parkinson's disease. However, although there is growing evidence in favor of protective effect of flavonoid, there is still uncertainty about potential side effect at higher drug concentration, especially after information about its occasional pro-oxidative (Metodiewa et al. 1999) and cytotoxic (Spencer et al. 2003a) behavior. Surprisingly, quercetin did not protect DA neurons of substantia nigra against apoptosis caused by oxidative stress *in vivo* (Costa et al. 2001, Zbarsky et al. 2005).

The knowledge of flavonoid effect on neurons morphology and viability is not satisfactory. Thus in this study we have investigated the effect of quercetin on neurons arborisation and their susceptibility to cell death.

METHODS

Cells and culture conditions

Neuronal cell culture was prepared from cortices of 18-day-old Wistar rat fetuses as previously described (Rzeski et al. 2004). Tissue was pooled into ice-cold glucose (33 mM) Hank's Balanced Salt Solution (HBSS, Sigma, St. Louis, MO), cut into small pieces and incubated for 30 min at 37°C with 0.25% trypsin-EDTA solution (Sigma, St. Louis, MO). Single cell suspension was obtained by gentle pipeting of the cortex fragments in presence of 10% FBS (Life Technologies, Karlsruhe, Germany) and 0.01% DNase I (Sigma, St. Louis, MI). Cells were then sieved through a 70 µm cell strainer (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ), centrifuged at 800 rpm for 10 min and plated at a density of 5×10^5 cells/ml on poly-L-lysine (MW 70 000–150 000) coated 96-multiwell plates or Lab-Tek Chamber Slide (NUNC, Roskilde, Denmark). The culture medium consisted of B-27 supplemented Neurobasal Medium (Life Technologies, Karlsruhe, Germany), 0.5 mM L-glutamine and 1% of antibiotic-antimycotic solution (Life Technologies, Karlsruhe, Germany). For the initial plating, the culture medium

was supplemented with 25 µM of glutamate. Incubation was carried out at 37°C in a humidified 95% air and 5% CO₂ atmosphere. The culture medium was changed every three days, until the culture reached 8 days *in vitro*. Neuronal identity was confirmed by positive staining with mouse anti-neuron specific enolase γγ monoclonal antibody (Chemicon International, Temecula, CA). The presence of functional NMDA receptors was verified by NMDA receptor subunit NR1 immunoreactivity using a mouse anti-NR1 monoclonal antibody (Chemicon International, Temecula, CA).

Green monkey kidney cell line (GMK) was cultured in RPMI 1640 medium while culture of human skin fibroblasts (HSF) in MEM+DMEM medium, both supplemented with 10% FBS (Life Technologies, Karlsruhe, Germany), penicillin (100 u/ml) (Sigma) and streptomycin (100 mg/ml) (Sigma). Cultures were kept at 37°C in humidified atmosphere of 95% air and 5% CO₂. Cells were seeded on Lab-Tech Chamber Slides (Nunc) at the concentration 1×10^5 cell/ml.

Drug treatment

Quercetin (3,3',4',5,7-pentahydroksyflavone) (Sigma) at a final concentrations 1, 5, 10, 15 mg/ml were used in the experiments. The drug was dissolved in dimethyl sulfoxide (DMSO, Sigma). The final concentration of DMSO in the culture medium did not exceed 0.1%, which as indicated in preliminary experiments did not influence cell viability and the expression of studied proteins. Neurons were incubated with different quercetin concentrations for 24 hours.

As control, cells were incubated with 0.1% of DMSO.

Apoptosis and necrosis detection

For apoptosis and necrosis identification, the neurons were stained with fluorescent dye Hoechst 33342 (Sigma) and propidium iodide (Sigma) (Jankowska et al. 1997). Morphological analysis was performed under fluorescence microscope (NIKON E – 800). The cells exhibiting blue fluorescent nuclei (intact or fragmented) were interpreted as apoptotic. The cells exhibiting pink fluorescent nuclei were interpreted as necrotic. At least 1000 cells in randomly selected microscopic fields were counted under microscope. The obtained results

were analysed for significance by one-way ANOVA test and were presented as mean \pm SD.

MTT assay

Neurons growing on 96-well microplates were exposed to quercetin (0.5–50 $\mu\text{g/ml}$) for 24 h. Cell viability was assessed by means of MTT assay (Cell proliferation kit I, Roche Diagnostics, Germany) in which the yellow tetrazolium salt (MTT) is metabolized by viable cells to purple formazan crystals. Neurons were incubated for 3 h with MTT solution (5 mg/ml). Formazan crystals were solubilized overnight in SDS buffer (10% SDS in 0.01N HCl) and the product was quantified spectrophotometrically by measuring absorbance at 570 nm wavelength using E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA). The obtained results were analyzed for significance by Student *t*-test.

Cell staining

Control cells and cells treated with quercetin were stained with fluorescent dye 3,3'-dihexyloxacarbocyanine iodide ($\text{DiO}_{6(3)}$) (Sigma). Cells were washed with PBS, permeabilized with 3.7% paraformaldehyde, washed three times with PBS and stained with $\text{DiO}_{6(3)}$ (final concentration 0.5 $\mu\text{g/ml}$) for 10 min at room temperature. After washing with PBS cells were analysed under microscope Nikon E-800. The fluorescent channel was $\lambda = 525$ nm.

Fractal Analysis

The images of neurons after $\text{DiO}_{6(3)}$ staining were analysed applying box-counting method of fractal analysis. The box counting method is based on the concept of covering the image border with sets of squares. Each set is characterized by the square side r . The corresponding number of squares $N(r)$ necessary to cover the border is presented as function of r . The fractal dimension of an image is determined from slope S of log–log relationship between $N(r)$ and r as $D=1-S$. Fractal analysis of at least 1 000 cells was carried out using the ImageJ 1.38u programme (Smith et al. 1996, Fernández and Jelinek 2001, Milošević et al. 2005). Additionally, using the same programme, the percent of area of spreading neurons was examined.

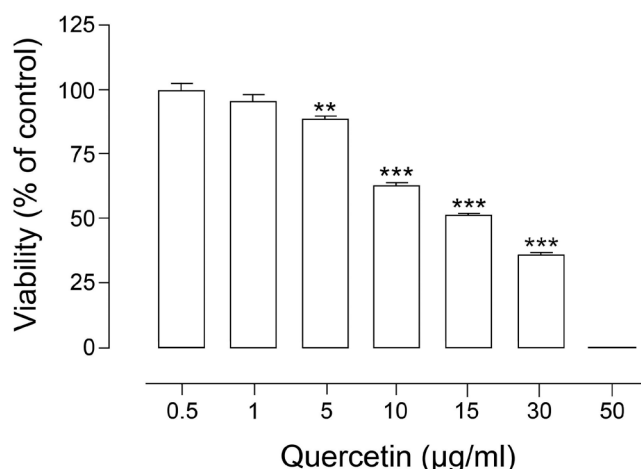


Fig. The effect of 24 h long incubation of neurons with quercetin (1–50 $\mu\text{g/ml}$) on cellular viability as assessed by MTT assay. ** $P < 0.01$; *** $P < 0.001$.

The obtained results were analysed for significance by Mann-Whitney *U* test.

RESULTS

The effect of quercetin on neurons viability

MTT viability test revealed that quercetin caused neurons death in concentration dependent manner (Fig. 1). Gradual decrease of survival cells was correlated with parallel increase in the flavonoid concentration. Quercetin in concentration 1 $\mu\text{g/ml}$ caused neurons death in about 5% of cells while about 50% of dead cells was observed after incubation with 15 $\mu\text{g/ml}$ of quercetin. It may suggest toxic effect of quercetin to neurons. Staining with Hoechst 33342 and propidium iodide showed that neurons died in apoptotic and necrotic way (Table I). Similarly to MTT proliferation test correlation between drug concentration and cell death was observed. Quercetin in concentration 1 $\mu\text{g/ml}$ increased the number of apoptotic cells by about 4.35% and necrotic by about 3.4% in comparison to control. After treatment with quercetin in concentration 15 $\mu\text{g/ml}$, the number of apoptotic cells increased by 32.2% and in the case of necrosis by 32% in comparison to control. Quercetin induced apoptosis and necrosis at the similar level.

In contrast to neurons, GMK and HSF cells appeared to be very resistant to pro-apoptotic and pro-necrotic effect of quercetin (Table I).

Table I

	Neurons		HSF		GMK	
Quercetin ($\mu\text{g/ml}$)	Apoptosis (%)	Necrosis (%)	Apoptosis (%)	Necrosis (%)	Apoptosis (%)	Necrosis (%)
Control	0.95 ± 0.21	3.0 ± 0.14	0.8 ± 0.07	0.0	0.35 ± 0.07	0.15 ± 0.07
1	$5.3^{**} \pm 0.14$	$6.4^{**} \pm 0.28$	0.75 ± 0.07	0.55 ± 0.21	0.65 ± 0.07	0.5 ± 0.0
5	$12.45^* \pm 2.19$	12.35 ± 3.46	0.85 ± 0.35	0.65 ± 0.21	0.8 ± 0.21	0.25 ± 0.0
10	$19.05^{**} \pm 1.2$	$16.95^{**} \pm 0.64$	1.5 ± 0.14	0.4 ± 0.0	$0.85^* \pm 0.07$	0.35 ± 0.07
15	$33.15^{***} \pm 0.64$	$35^{**} \pm 4.24$	$1.8^* \pm 0.14$	0.7 ± 0.14	$1.05^* \pm 0.07$	0.5 ± 0.07

The effect of quercetin on neurons morphology

The morphology of neurons after quercetin treatment was analysed using two parameters: fractal analysis (D) and the percent of area of spreading neurons. Nerve cells do not cover the area completely and the values of fractal dimension vary between 1 and 2. The less neuronal branches the smaller D value.

Quercetin significantly diminished neuronal arborization in neurons (Figs 3 and 4). Flavonoid in concentration 1 $\mu\text{g/ml}$ significantly diminished D

value from 1.21 (control) to 1.11. Concentration 5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ of quercetin were more effective. The strongest effect on diminishing neuronal arborization was observed after incubation with 15 $\mu\text{g/ml}$ of quercetin when D value was about 1.03. Flavonoid at all studied concentrations significantly diminished the percent of area of neurons (Fig. 2). Control cells covered about 28% of area. The percent of area covered by neurons treated with quercetin at all studied concentrations was similar and the mean value ranged from 9% to 13%.

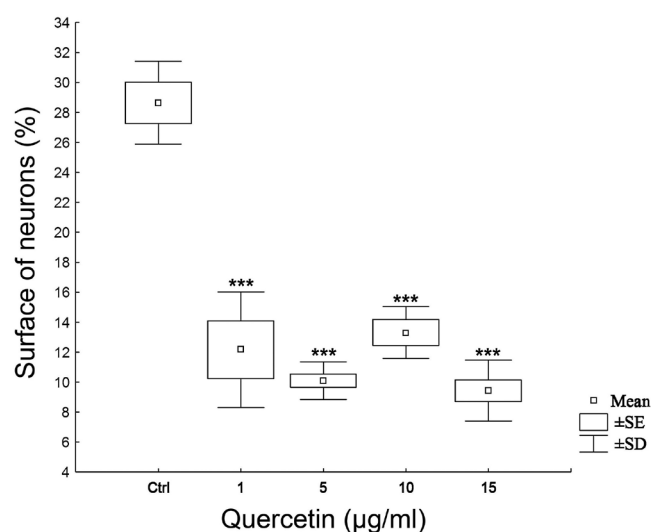


Fig. 2 The effect of 1–15 $\mu\text{g/ml}$ of quercetin on the percent of surface of neurons (%). (Ctrl) control; *** $P < 0.0001$.

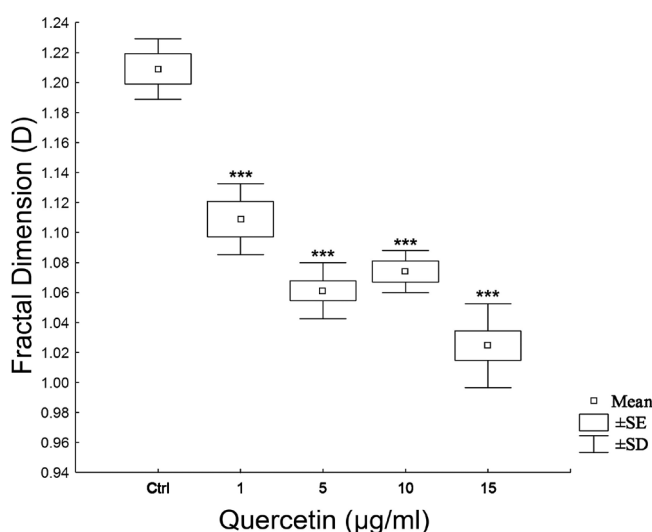


Fig. 3. Fractal dimension of neuronal arborization after 24 h long incubation with 1–15 $\mu\text{g/ml}$ of quercetin. (Ctrl) control; *** $P < 0.0001$

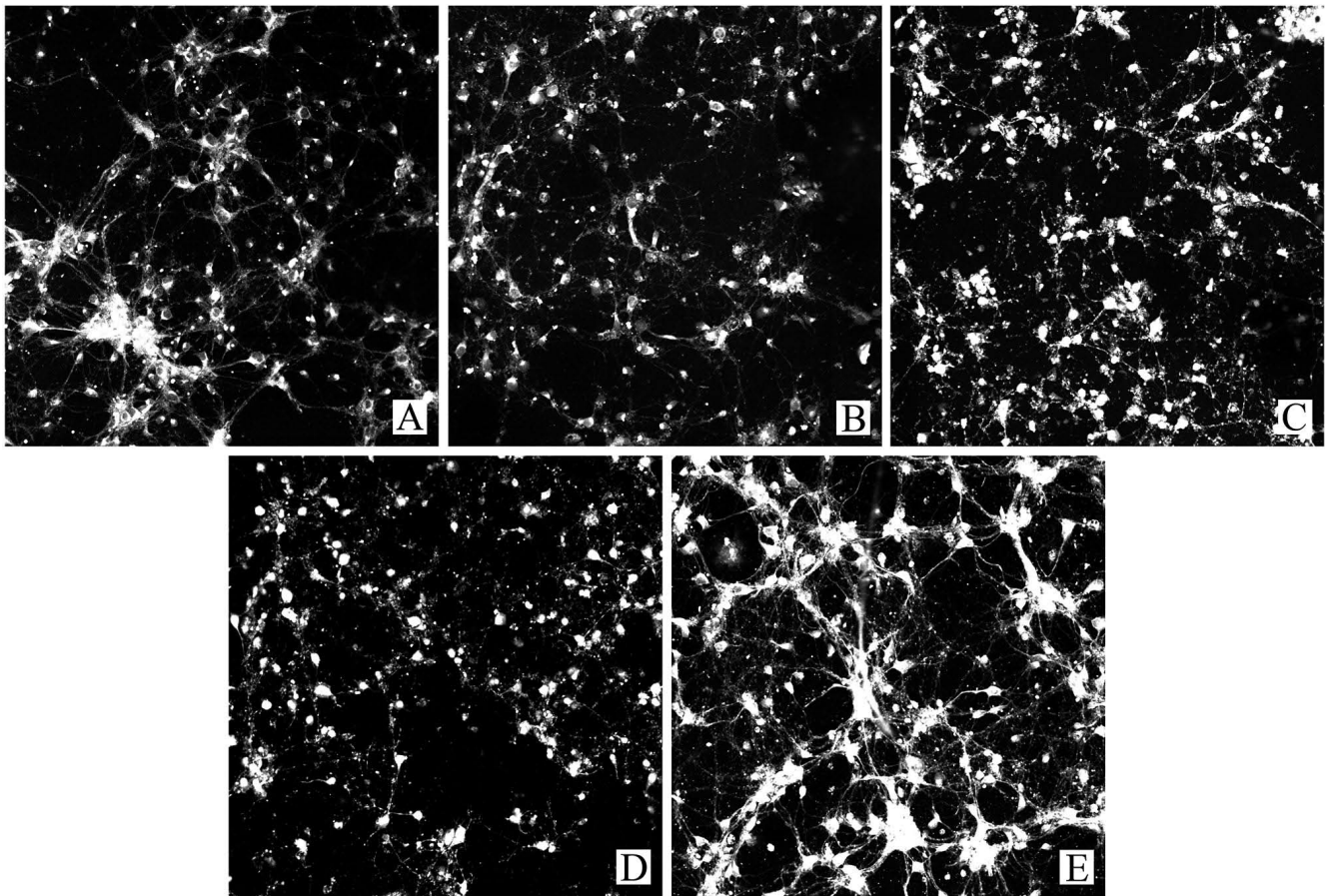


Fig. 4. Changes in the morphology of neurons after treatment with 1 $\mu\text{g/ml}$ (A), 5 $\mu\text{g/ml}$ (B), 10 $\mu\text{g/ml}$ (C), 15 $\mu\text{g/ml}$ (D) of quercetin in comparison to control (E).

Discussion

Neuronal development and differentiation is associated with specific biochemical, morphological and physiological features. Development of specific neuronal morphology is critical to the function of neurons and to establish the appropriate connections (Kaczmarek 2000, Womelsdorf et al. 2007). Epidemiological studies suggested that quercetin may exhibit beneficial role in neuroprotection (Dajas et al. 2003, Heo and Lee 2004, Cho et al. 2006, Lu et al. 2006) but many concerns about its side effects still exist and the effect of flavonoid on neurons require further precise studies.

Neuronal structure is among the most complex cellular morphologies and fractal analysis has been considered as useful tool for measuring this complexity (Smith et al. 1996, Fernández and Jelinek 2001). This is an appropriate method for quantifying neuronal arborization (Caserta et al. 1995) and for describing how neurons fill its dendritic fields or to study the straightness

of individual dendrits (Montague and Friedlander 1991). Up to date several fractal analysis methods were applied to analyze biological structures. The mass–radius method, cumulative intersection method and the vectorized intersection method are generally described by the relationship between the surface of the image found within the circle defined by radius covering the image (Sholl 1953, Caserta et al. 1995, Shierwagen 1990, Cornforth et al. 2002). These methods cannot produce satisfactory results because the radius of gyration does not cover the entire object and the periphery of neurons is also ignored. The box counting method is more precise than fractal methods mentioned above. It is based on idea of covering the whole image with sets of squares covering the entire object. This method is so sensitive that enables distinguishing between populations of neurons from different laminae of human spinal cord (Smith et al. 1996, Milošević et al. 2005). In our experiments, the box counting method revealed that quercetin diminished neurons arborisation in cortical neurons. The area

covered by neurons was also smaller after quercetin treatment. Such morphological changes may affect proper contact and signal transmission between neurons leading to cell death and brain disfunction in consequence.

We observed that quercetin, significantly increase the number of apoptotic and necrotic cells in the culture of neurons. Our observations are in agreement with results presented by Spencer and colleagues (2003b). They found that quercetin may be neurotoxic at higher concentrations. The flavonoid and its O-methylated metabolites inhibited pro-survival protein kinase cascades in cortical neurons. High concentrations of quercetin produce a sustained deactivation of Akt/PKB which led to extensive caspase-3 activation. Lower concentrations of quercetin (less than 10 μM) induced reversible inhibition of Akt/PKB phosphorylation and activation (Spencer et al. 2003b). What is worth to note, quercetin had no significant impact on cell death in GMK and HSF cells suggesting selective sensitivity of neurons to the flavonoid treatment. However it is difficult to relate these observations to what occurs *in vivo*. Cells in culture accumulate high amounts of quercetin (Spencer et al. 2003a). *In vivo*, quercetin is extensively metabolized to O-methylated and glucuronide metabolites during absorption in the small intestine and in the liver (Boulton et al. 1999, Spencer et al. 1999). In consequence, low amounts of quercetin and higher levels of its metabolites are found in the circulation (Azuma et al. 2002, Oliveira et al. 2002). On the other hand, the elimination of quercetin and its metabolites from organism is quite slow, what could favor its accumulation in plasma with repeated intakes. Baseline quercetin concentrations generally vary between 50 and 80 nmol/L. After 28 days of supplementation with over 1 g/d of quercetin the baseline concentration increased to 1.5 $\mu\text{mol/L}$ (Conquer et al. 1998). *In vivo*, quercetin toxicity toward neurons may be limited by blood–brain barrier – an obstacle to reach the brain. Few papers reported that quercetin and some of its metabolites (especially O-methylated metabolites) are able to enter central nervous system (CNS) by crossing the BBB but its flux into different brain regions was lower than of other flavonoids like naringenin. Additionally, quercetin was found to be a substrate for BBB efflux transports what may limit its brain access (Youdim et al. 2004). Mixing quercetin with lecithin increased the possibility of flavonoid to cross BBB (Dajas et al. 2003).

CONCLUSION

Our results indicate that quercetin induce apoptosis and necrosis in neurons rather than protect them against cell death. The toxic effect of quercetin on neurons, especially at higher concentrations should be taken into consideration in further studies on using quercetin as therapeutic agent.

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