Free Radical Research, January 2006; 40(1): 95–102

Taylor & Francis
Taylor & Francis Group

Quercetin protects C6 glial cells from oxidative stress induced by tertiary-butylhydroperoxide

B. GITIKA, M. SAI RAM, S. K. SHARMA, G. ILAVAZHAGAN, & P. K. BANERJEE

Defence Institute of Physiology & Allied Sciences, Timarpur, Lucknow Road, Delhi 110054, India

Accepted by Professor A. Collins

(Received 28 March 2005; in revised form 1 September 2005)

Abstract
The anti-oxidant and cyto-protective activity of quercetin against tertiary-butylhydroperoxide (*t*-BOOH) induced oxidative stress on C6 glial cells is reported. Exposure of the cells to *t*-BOOH resulted in a significant increase in cytotoxicity, reactive oxygen species (ROS) generation and lipid peroxidation. There was a significant increase in DNA strand breaks and fall in reduced GSH levels in cells exposed to *t*-BOOH. A significant increase in calcium ion influx was noticed in cells exposed to *t*-BOOH. Pre-treatment of cells with quercetin, vitamin C (vit C), Trolox, and deferoxamine (DFO) significantly inhibited *t*-BOOH induced cytotoxicity and ROS generation. Pretreatment of cells with quercetin, Trolox and DFO inhibited the DNA damage, maintained higher GSH levels and prevented calcium influx significantly. Although vit C protected the cells from cytotoxicity induced by *t*-BOOH, the intracellular Ca^{2+} levels were significantly higher than the control cells. However, anti-oxidants like butylated hydroxy toluene (BHT), vitamin E (vit E), *N*-acetyl cysteine (NAC) did not have significant cytoprotection against *t*-BOOH induced oxidative injury in C6 glial cells.

Keywords: *C6 glial cells, t*-BOOH, oxidative stress, quercetin

Introduction

Brain is highly susceptible to oxidative insults owing to the fact that it has high iron and lipid content. It utilizes 20% of the total oxygen consumed by body though it comprises only 2% of the body weight [1]. Oxidative stress leads to enhanced production of reactive oxygen species (ROS), which can modify DNA, proteins, lipids and carbohydrates in cells [2,3] resulting in various neurological disorders [4,5]. In addition, the brain has much lower levels of super-oxide dismutase, glutathione peroxidase and catalase relative to kidney and liver [6]. It has been reported that the adult brain consists of about 10^{11}–10^{12} neurons and at least twice the number of glial cells [7]. The astrocytes are largely responsible for protecting neurons against oxidative injuries [8,9]. Apart from providing protection to neurons against oxidative insults, astrocytes have recently been reported to perform a variety of important functions in brain. These studies indicate that astrocytes play an important role in synaptogenesis and proper neuronal synaptic functions, maintaining the ionic environment and pH of the extracellular spaces, secreting growth factors, insulating the neurons and forming the blood brain barrier (BBB) [10,11]. All of these functions are vital for sustaining neuronal functions and structure. Any injury to astrocytes will thus be indirectly responsible for degeneration of structural and functional integrity of neurons, which in turn may lead to neurodegenerative disorders. Astrocytes as they age become more vulnerable to oxidative stress even though their antioxidant defense system seems to be maintained. The increase in vulnerability is related to increase in iron content [12] and altered iron deposition and metabolism has been implicated to

Correspondence: Dr M. Sairam, Defence Institute of Physiology & Allied Sciences, Timarpur,Lucknow Road, Delhi 110054, India.
E-mail: sairam64@rediffmail.com

ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760500335447

play a significant role in neurodegenerative diseases including Parkinsonism and Alzheimer's disease [13].

Flavonoids comprise a large group of low molecular weight polyphenolic substances reported to have a number of biological activities [14,15]. They are one of the bioactive dietary components responsible for antioxidant effects of fruits and vegetables. Currently, there is no accurate information available on dietary intake of flavonoids. Hertog et al. [16] reported that the daily intake of two flavonoids, i.e. flavones and flavonols, was found to be 23 mg in Dutch diet. However, Kuhnau [17] estimated the intake of the three same flavonoids to be at 115 mg/day. The differences in these studies were attributed to the differences in the analytical techniques employed. The health promoting properties of flavonoids against a number of degenerative diseases like cardiovascular, cerebro-vascular, certain forms of cancer, Alzheimer's disease, Parkinson's diseases, etc. have received much attention in recent years [18–21]. These compounds are known to possess a range of pharmacological effects of which many are linked to their ability to scavenge free radicals, to chelate metal ions and to synergistic effects with other anti-oxidants [22–24]. Most studies of the neuroprotective activities of flavonoids against oxidative injury have been carried out on neurons [18,25,26]. Recently, van Meeteren et al. [27] reported that the flavonoids protect oligodendrocytes from ROS induced cytotoxicity. However, to the best of our knowledge, not many studies have been carried out to evaluate the cyto-protective activity of flavonoids against the oxidative injury on the astrocytes.

Thus, present study was designed to determine the cyto-protective efficacy of quercetin against tertiary-butylhydroperoxide (*t*-BOOH) induced oxidative stress using C6 glioma cells as model system. C6 cells exhibit many properties of astrocytes including the expression of astrocyte specific markers glial fibrillary acidic protein (GFAP) and S-100 protein [28,29]. An attempt has also been made to compare its activity with a number of anti-oxidants such as iron chelator deferoxamine mesylate (DFO), antioxidants such as butylated hydroxy toluene (BHT), vitamin C (vit C), Trolox and vitamin E (vit E) and glutathione precursors such as *N*-acetyl cysteine (NAC) in providing cytoprotection against *t*-BOOH induced oxidative stress.

Materials and methods

Cell culture

C6 glioma cells were obtained from National Center for Cell Science (NCCS), Pune, India. The cells were propagated in MEM supplemented with 10% fetal calf serum, 100 μg/ml ampicillin and 100 μg/ml streptomycin at 37°C in a humidified CO_2 incubator. The cells were grown to density of 1×10^4/well in 96 well plates (Greinner, Germany) for determination of cytotoxicity and ROS levels. The cells were grown in 24 well plates (Falcon make) to a density of about 1×10^5/well for determination of anti-oxidant levels, DNA strand breaks and intracellular Ca^{2+} levels.

Experimental setup

In pilot experiments, the optimal incubation time and concentration of *t*-BOOH required to produce cytotoxicity on C6 glial cells were determined (data were not shown). The range of doses used for various antioxidants used in the study was based on preliminary *in vitro* experiments. The lowest concentration of the antioxidants that provided maximum cytoprotection against *t*-BOOH induced cytotoxicity was used in the present study. C6 glial cells were incubated with 100 μM of *t*-BOOH for 3 h in the absence or presence of different antioxidants for determination of cytotoxicity and ROS. The cells were exposed to 100 μM *t*-BOOH for 1 h (optimum time) for determination of DNA strand breaks and intracellular calcium levels. For measuring lipid peroxidation, the cells were exposed to 2 mM *t*-BOOH for 30 min. For evaluating GSH levels, the cells were exposed to *t*-BOOH for 1, 2 and 3 h. To determine the efficacy of various antioxidants, the cells were supplemented with respective antioxidants 30 min before *t*-BOOH addition. Quercetin and Trolox were dissolved in ethanol (the final ethanol concentration was less than 0.5% in the medium) while vit C, NAC and DFO were dissolved in PBS. Vit E and BHT were emulsified in PBS containing 0.1% Tween 80. The final concentration of antioxidants in the culture medium was: quercetin 75 μM, vit C 60 μM, Trolox 500 μM, BHT 200 μM, DFO 1.5 mM, NAC 1 mM and vit E 500 μM.

Determination of cytotoxicity

Cytotoxicity was studied by using neutral red, a supra-vital dye that is selectively taken up by the live cells [30]. Briefly 10 μl of neutral red dye (0.1%) was added to the cells and incubated at 37°C in CO_2 incubator for 3 h. Later the cells were washed three times with saline followed by the addition of 200 μl of ethanol–acetic acid (50:1) solution. The O.D. was then measured at 570 nm using (molecular devices) ELISA reader.

Measurement of reactive oxygen species (ROS)

It was determined by using a fluorescent probe 2,7-dichloro fluorescein-diacetate (DCFH-DA) as described previously [31]. Briefly, after treatment, 10 μl of DCFH-DA stock solution (200 μM in DMSO) was added to the 190 μl of medium in 96

well plates to get a final concentration of 10 μM. The cells were incubated at 37°C for 30 min in CO_2 incubator. The cells were then washed three times with PBS and the fluorescence was measured by multiwell spectrofluorimeter (Varian, USA) with an excitation at 485 nm and emission at 530 nm.

Single cell gel electrophoresis or Comet assay

It was carried out as described by Tice [32]. Microscope slides were pre-coated with normal melting point agarose (0.1%). After incubation, the cells were washed twice in PBS and 100 μl of cell suspension (3.5×10^4 cells) was mixed in warm low gelling agarose (0.75%), pipetted onto the slides and allowed the agarose to solidify. The slides were placed in cold lysis solution (SDS 2.5%, sodium sarcosinate 1% and EDTA 25 mM, pH 9.5) for 15 min and electrophoresed for 5 min at 2 V/cm. The slides were washed in distilled water, stained with propidium iodide (25 μg/ml) and the cells were then visualized using Hunds fluorescence microscope. One hundred nuclei on each slide were scored and given a score from 0 (undamaged nuclei) to 4 (severely damaged nucleus) as per the method described earlier [33]. Samples were scored in random order and results were expressed as percentage DNA damage taking control as zero.

Determination of lipid peroxidation

The extent of lipid peroxidation was measured using a fluorescent probe, *cis*-parinaric acid (9,11,13,15-octadecatetraenoic acid) as described by Van den Berg et al. [34]. *cis*-Parinaric acid has four conjugated double bonds that render it fluorescent and which are readily attacked in lipid peroxidation reaction. After incubation with antioxidants, 10 μl of *cis*-parinaric acid (2 μg/ml) was added to the cells and incubated for 25 min. Later, *t*-BOOH was added to a final concentration of 2 mM and the fall in fluorescence was measured with excitation at 313 nm and emission at 413 nm at different time intervals (0, 15, 30 min).

Determination of reduced glutathione levels

After incubation, the cells were lysed by adding 200 μl of lysis buffer (10 mM Tris, 20 mM EDTA, 0.25% Triton X-100 pH 8). The glutathione (GSH) levels in cell lysates were determined fluorimetrically as described earlier [35].

Calcium influx assay

Calcium influx was determined flow cytometrically using fluo3-AM as described earlier [36]. Since increase in calcium levels is an early event in cellular injury, therefore, calcium levels were measured after 1 h of exposure to *t*-BOOH. Briefly, after treatment, the cells were incubated with 8 μM of fluo3-AM for 45 min at 37°C in dark. The cells were washed twice with PBS and incubated for another 20 min at 37°C in HEPES buffered Ringer's Medium (in mM—120 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1.0 $CaCl_2$, 11 glucose, 20 μM HEPES and 0.2% BSA at pH 7.4). After 20 min incubation, the cells were washed with same buffer devoid of calcium, trypsinized and suspended in 500 μl of PBS for flow-cytometric analysis (FACS Calibure, Becton Dickinson, USA).

Results

Cytotoxicity

The cyto-protective activity of quercetin and various other anti-oxidants was evaluated by neutral red assay. There is a significant increase in cytotoxicity (76%) when the cells were exposed to 100 μM of *t*-BOOH for 3 h (Figure 1). Pre-treatment of cells with quercetin significantly attenuated the cytotoxicity induced by *t*-BOOH. Similarly, water-soluble free radical scavengers (vit C and Trolox) and metal ion chelator DFO gave significant cytoprotection ($p < 0.001$). However, lipophilic free radical scavengers (vit E and BHT) and glutathione precursor (NAC) had no appreciable effect on the cytotoxicity induced by *t*-BOOH in C6 glial cells.

ROS generation and DNA damage

There is a significant increase in ROS generation by about 2.8 times in cells exposed to *t*-BOOH relative to control cells (Figure 2). Pre-treatment of cells with quercetin, vit C, Trolox and DFO appreciably

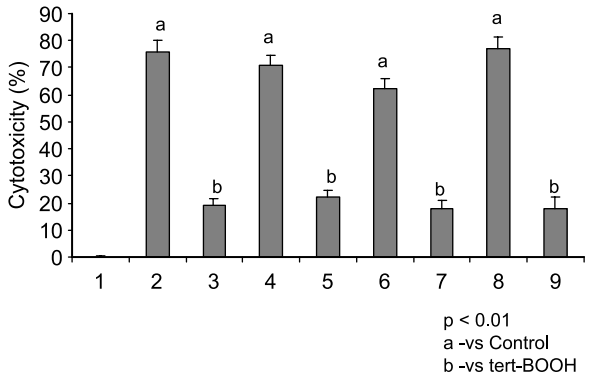

Figure 1. Cytoprotective activity of quercetin against *t*-BOOH induced cytotoxicity in C6 glial cells. Cells were grown to a density of 1×10^4/well in 96 microtitre plate and incubated with quercetin or other antioxidants for 30 min before exposing the cells to 100 μM of *t*-BOOH. After 3 h of treatment, cells were assessed for cytotoxicity by neutral red assay. (1) Control, (2) *t*-BOOH (100 μM), (3) vit C (60 μM) + *t*-BOOH, (4) NAC (1 mM) + *t*-BOOH, (5) quercetin (75 μM) + *t*-BOOH, (6) BHT (200 μM) + *t*-BOOH, (7) DFO (1.5 mM) + *t*-BOOH, (8) vit E (500 μM) + *t*-BOOH, (9) Trolox (500 μM) + *t*-BOOH.

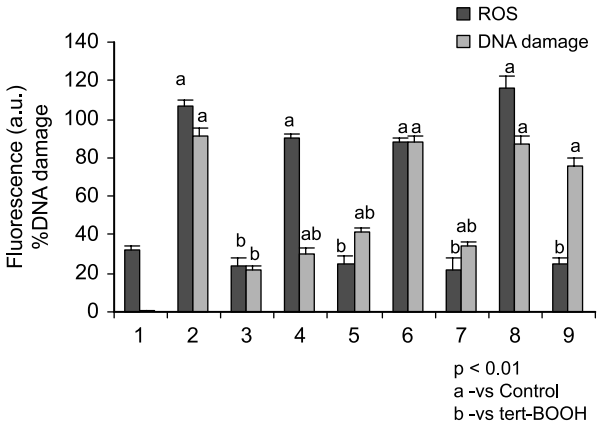

Figure 2. Effect of quercetin on *t*-BOOH induced ROS generation and DNA damage. ROS generation was measured by incubating cells with DCFH-DA (20 μM) for 30 min at 37°C after 3 h exposure of cells to 100 μM of *t*-BOOH in the presence or absence of quercetin or various antioxidants. DNA strand breaks were determined at the end of 1 h of incubation with 100 μM *t*-BOOH in the presence or absence of quercetin or other antioxidants using comet assay. (1) Control, (2) *t*-BOOH (100 μM), (3) vit C (60 μM) + *t*-BOOH, (4) NAC (1 mM) + *t*-BOOH, (5) quercetin (75 μM) + *t*-BOOH, (6) BHT (200 μM) + *t*-BOOH, (7) DFO (1.5 mM) + *t*-BOOH, (8) vit E (500 μM) + *t*-BOOH, (9) Trolox (500 μM) + *t*-BOOH.

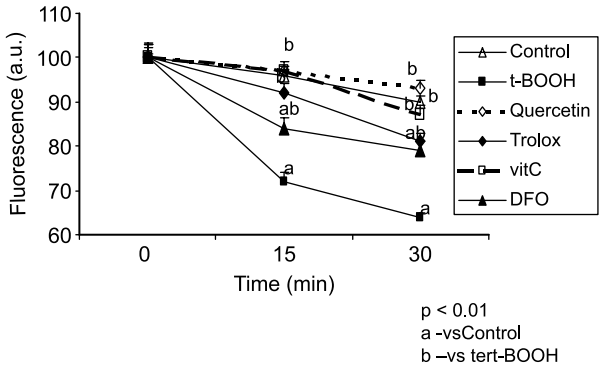

Figure 3. Effect of quercetin on *t*-BOOH induced lipid peroxidation using *cis*-parinaric acid (2 μg/ml). The cells (1×10^6 cells/ml) were incubated with quercetin or other antioxidants for 30 min before labeling with *cis*-parinaric acid for 25 min. The cells were then exposed to 2 mM of *t*-BOOH for 30 min and the fall in fluorescence was monitored spectrofluorometrically every 15 min.

inhibited the ROS generation and maintained their values similar to that of control cells. However, supplementation of NAC, BHT marginally inhibited the ROS generation induced by *t*-BOOH while vit E had no significant effect (Figure 2).

Exposure of cells to *t*-BOOH resulted in a significant increase in DNA strand breaks by about 90% relative to that of control cells. Pre-treatment of cells with quercetin, vit C, Trolox, NAC and DFO prevented DNA damage considerably. However, supplementation of vit E or BHT had little effect on *t*-BOOH induced DNA damage. (Figure 2).

Lipid peroxidation

Since quercetin, vit C, Trolox and DFO had significant protection against cytotoxicity and genotoxicity, we determined the relative abilities of these antioxidants to inhibit the lipid peroxidation induced by *t*-BOOH using *cis*-parinaric acid as a fluorescent probe. There was an appreciable increase in lipid peroxidation when the cells were exposed to *t*-BOOH (Figure 3). Pre-treatment of cells with quercetin, vit C, Trolox and DFO inhibited the lipid peroxidation significantly, the order of activity is quercetin > vit C > Trolox > DFO (Figure 3).

GSH levels

The GSH levels were determined both during 1, 2 and 3 h of *t*-BOOH treatment to understand the dynamics of GSH metabolism in C6 glial cells. Since, no significant difference was observed between the GSH levels at 1 and 2 h, the data at 2 h are not shown. Exposure of cells to *t*-BOOH for 1 h resulted in a considerable fall in intracellular GSH levels by about 30% when compared to control cells; however, GSH levels dropped further to 40% of control cells after 3 h of incubation in the presence of *t*-BOOH (Figure 4). Supplementation of quercetin or DFO maintained higher GSH levels in C6 glial cells both at 1 and 3 h of *t*-BOOH treatment. In the presence of vit C, Trolox and BHT, the cells maintained GSH levels similar to that of control cells throughout the period of incubation.

Supplementation of NAC or vit E maintained higher GSH levels in C6 glial cells by about 2.5 and

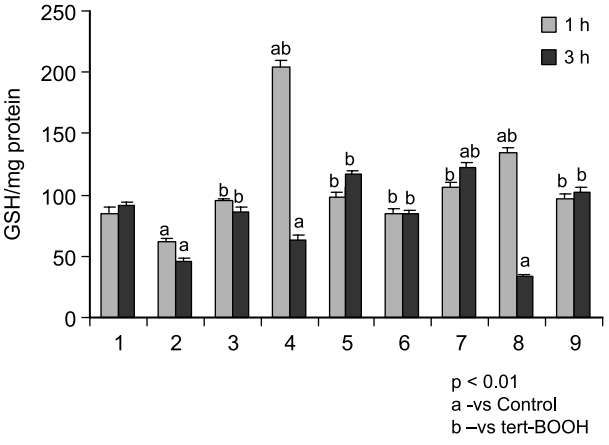

Figure 4. Effect of quercetin on intracellular GSH levels in C6 glial cells. Cells were grown in 24 well plate to a density of 1×10^5/well and exposed to 100 μM of *t*-BOOH for 1 or 3 h in the presence or absence of quercetin or other antioxidants. The GSH levels were determined fluorometrically in cell lysates. (1) Control, (2) *t*-BOOH (100 μM), (3) vit C (60 μM) + *t*-BOOH, (4) NAC (1 mM) + *t*-BOOH, (5) quercetin (75 μM) + *t*-BOOH, (6) BHT (200 μM) + *t*-BOOH, (7) DFO (1.5 mM) + *t*-BOOH, (8) vit E (500 μM) + *t*-BOOH, (9) Trolox (500 μM) + *t*-BOOH.

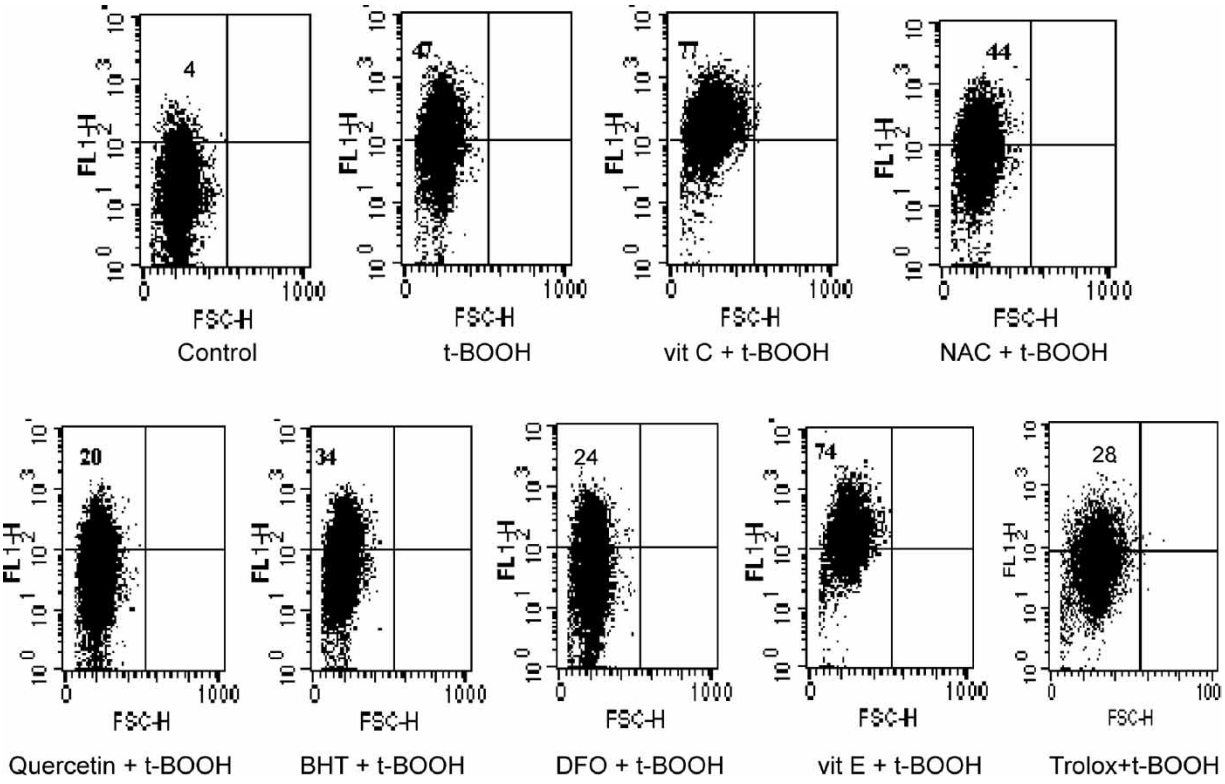

Figure 5. Effect of quercetin on intracellular calcium levels in cells exposed to *t*-BOOH. Cells were treated with 100 μM *t*-BOOH for 1 h in the presence or absence of quercetin or other antioxidants. After treatment, the cells were incubated with Fluo-3AM (8 μM) for 45 min and intracellular calcium levels were determined flow cytometrically.

1.8 times, respectively, to that of control cells during 1 h of *t*-BOOH treatment. However, their levels dropped sharply at 3 h following pre-treatment with NAC or vit E in the presence of *t*-BOOH (Figure 4).

Intracellular calcium levels

There is a considerable increase in the intracellular calcium level by about 10 times in cells exposed to *t*-BOOH relative to control cells. Supplementation of cells with quercetin or Trolox or DFO significantly inhibited intracellular calcium levels as compared to *t*-BOOH treated cells. However, supplementation of NAC or BHT had no significant effect on the influx of calcium ions induced by *t*-BOOH (Figure 5). Interestingly, in the presence of vitamins C and E, the calcium levels were much higher than *t*-BOOH treated cells.

Discussion

The above results show that the flavonoid quercetin protects the C6 glial cells from oxidative injury induced by *t*-BOOH. In the present study, we determined the ability of quercetin in preventing cytotoxicity, ROS generation, lipid peroxidation and DNA damage induced by *t*-BOOH. We also compared the relative efficacy of quercetin with many conventional anti-oxidants in providing the cyto-protection against *t*-BOOH induced oxidative injury.

Flavonoids are found ubiquitously in fruits and vegetables and are widely regarded to possess a number of biological activities, some of which are believed to be due to their potent anti-oxidant and cyto-protective activities [37,38]. Flavonoids have been reported to exert beneficial effects in a multitude of disease states such as cardiovascular diseases [39], atherosclerosis [40] and cancer [41]. Flavonoids have been reported to possess their antioxidant activity by three mechanisms: (1) increasing intra-cellular GSH levels, (2) scavenging ROS, (3) blocking Ca^{2+} influx [18]. The anti-oxidant activity of the flavonoids forms a strong basis for neuro-protective activity in the brain. In spite of its anti-oxidant potency *in vitro* and cyto-protective activity in cell cultures, it has been reported that quercetin did not protect against 6-hydroxydopamine (6-OHDA) induced death of dopaminergic neurons in substantia nigra [42].

In the present study, we report the cyto-protective and anti-oxidant activity of quercetin against *t*-BOOH induced oxidative injury in C6 glial cells. *t*-BOOH is an organic hydroperoxide and is useful compound for inducing oxidative injury. Its toxicity is attributed to its decomposition to alkoxyl or peroxyl radicals that accelerate lipid peroxidation chain reaction [43,44]. Addition of *t*-BOOH to C6 glial cells resulted in

a significant increase in cytotoxicity. This was attributed to the increased production of ROS as revealed by increased fluorescence of DCFH-DA in the presence of *t*-BOOH as compared to the control cells. Pre-treatment of cells with quercetin, vit C, Trolox and DFO significantly inhibited the cytotoxicity induced by *t*-BOOH. They also inhibited ROS generation induced by *t*-BOOH significantly. Earlier studies revealed the efficacy of flavonoids in scavenging various free radicals such as superoxide, hydroxyl and peroxyl radicals and chelating metal ions [44,45]. It is interesting to note that quercetin increased cell survival in an oxidative injury model where free radical scavengers like BHT and vit E failed to protect the glial cells from oxidative injury. This suggests that quercetin, by virtue of its hydrophilic nature has an access to alkoxyl and peroxyl radicals generated by *t*-BOOH. This was confirmed by the fact that all the hydrophilic antioxidants used in the study, i.e. vit C, Trolox (which is a water-soluble form of vit E) and DFO inhibited the cytotoxicity induced by *t*-BOOH. In this regard our results fall in confirmation with earlier study [45]. However, both BHT and vit E owing to their lipophilic nature were ineffective in attenuating the ROS generation induced by *t*-BOOH, which in turn was responsible for their inability to provide appreciable cytoprotection against *t*-BOOH induced toxicity.

Exposure of C6 glial cells to 100 μM *t*-BOOH resulted in the formation of DNA strand breaks significantly. The DNA damage induced by *t*-BOOH may be triggered by two mechanisms: (1) iron mediated butoxyl radical formation via Fenton reaction and (2) oxidation of thiol groups in proteins followed by activation of Ca^{2+} dependent endonucleases which can lead to DNA strand breaks [44]. In the present study, we found that pre-treatment of cells with quercetin protected the cells significantly from DNA damage induced by *t*-BOOH. Further, iron-specific chelator DFO also inhibited the DNA damage induced by *t*-BOOH significantly. In this regard our results fall in confirmation with earlier studies by Ishige et al. [18] who reported that flavonoids quercetin and rutin protect against DNA damage induced by H_2O_2 in Caco2 cells. As expected, lipophilic antioxidants BHT and vit E had no significant protection against DNA damage induced by *t*-BOOH. Since, Trolox was also unable to prevent genotoxicity induced by *t*-BOOH, it is clear that quercetin prevented the DNA damage by its metal chelating action. Earlier, Sestili et al. [46] reported that quercetin but not vit E, Trolox and BHT prevented *t*-BOOH induced genotoxicity in U-937 macrophages by its metal chelating action.

In the present study, we observed that quercetin inhibited *t*-BOOH induced lipid peroxidation in C6 glial cells significantly compared to metal chelator DFO as revealed by fall in the fluorescence of *cis*-parinaric acid. Since, the cells were suspended in PBS before labeling with *cis*-parinaric acid, it is unlikely that iron chelation property of quercetin contributes to the antioxidant activity observed here. Recently, Wen-Peng [47] reported that flavonoids prevent lipid peroxidation induced by H_2O_2 and $FeSO_4$ by their ability to quench ROS. The fact that free radical scavengers vit C and to some extent Trolox attenuated lipid peroxidation better than DFO supports the idea that quercetin inhibited the lipid peroxidation induced by *t*-BOOH by its ability to quench alkoxyl and peroxyl radicals. Flavonoids have been shown to interact with the polar zone of the phospholipids and lipid solubility is not critical for preventing lipid peroxidation [48].

GSH is an important anti-oxidant in cells of the brain and is a potential target for therapeutic manipulations [49]. GSH plays an important role in the protection of cells against oxidative injury and is reported to be present in high concentrations in astrocytes [50]. The cells can be recovered from ROS induced cellular injury by mechanisms either dependent or independent of GSH metabolism. Therefore, it is prudent to determine the effect of quercetin and other antioxidants on GSH levels during *t*-BOOH induced oxidative stress in C6 glial cells. There was a significant decrease in GSH levels both at 1 and 3 h of *t*-BOOH treatment in C6 glial cells. In the present study, we found no significant increase in intracellular GSH levels in the presence of quercetin, vit C, vit E, Trolox, BHT and DFO in control cells (data not shown). However, supplementation of quercetin, vit C, Trolox and DFO attenuated *t*-BOOH induced fall in GSH levels. However, pre-treatment of cells with-NAC increased GSH levels at 1 h, its levels dropped sharply by 3 h in the presence of *t*-BOOH. Earlier, Ishige et al. [18] reported that some cyto-protective flavonoids such as flavonol, galangin did not increase GSH levels but still found to protect the HT-22 neurons from glutamate induced cytotoxicity. While flavonoids like quercetin, fisetin increased GSH levels by 40–50% relative to untreated cells. It has been shown that cells survive glutamate toxicity if the cellular GSH levels are $>20\%$ of the control level [51]. In the present study, we found that quercetin maintained higher GSH levels during *t*-BOOH induced oxidative stress. Interestingly, all other antioxidant compounds which provided cytoprotection against *t*-BOOH, i.e. vit C, Trolox and DFO also maintained higher GSH levels. It is speculated that by attenuating ROS production, these antioxidants spared GSH which in turn was responsible for its higher levels. Earlier Kim et al. [52] also reported that flavonoids prevent diminution of GSH induced by glutamate but did not influence synthesis of GSH.

In *t*-BOOH induced oxidative stress, there was a 10 times increase in intracellular Ca^{2+} levels. There is increasing evidence that Ca^{2+} ions play a critical role

in cell death. In a variety of experimental systems increased Ca^{2+} has been found to be an early event in cellular injury [53,54]. Increased Ca^{2+} ions are known to activate various proteases, phospholipases and endonucleases resulting in the disruption of cellular cytoskeletal organization [55]. Since quercetin protects the cells from *t*-BOOH induced cytotoxicity, it was asked whether pretreatment of cells with quercetin blocks intracellular Ca^{2+} levels in C6 glial cells. In the present study, we found that quercetin significantly attenuated Ca^{2+} influx induced by *t*-BOOH. Dobrydneva et al. [56] reported that flavonoid *trans*-resveratrol inhibited thrombin induced Ca^{2+} influx in human platelets by blocking Ca^{2+} channels. Flavonoids have also been shown to prevent calcium entry induced by H_2O_2 in PC12 cells [57]. More recently, Kempuraj et al. [58] reported that flavonoids inhibit IgE mediated Ca^{2+} influx in mast cells.

The application of dietary compounds like flavonoids for preventing neurodegenerative diseases merits further study with special reference to systemic bioavailability of these compounds. Unlike other tissues, the BBB appears to be an added obstacle to flavonoids reaching the brain. Recent studies by Youdim et al. [59] reported measurable permeability of quercetin and naringenin both in *in vitro* and *in situ* (rat) models paving the way for possible application of flavonoids for treating neurodegenerative diseases.

In conclusion, the flavonoid quercetin protected the C6 glial cells from *t*-BOOH induced oxidative stress by attenuating ROS generation, DNA damage and Ca^{2+} influx and maintaining higher GSH levels. At present, the cellular and molecular mechanisms that underlie the actions of quercetin are not fully understood. However, our results reveal that quercetin inhibited lipid peroxidation by its ROS scavenging activity while its genoprotective activity was due to its metal chelating property. Natural compounds that inhibit ROS induced neurotoxicity offer a useful therapeutic choice in the treatment of neurodegenerative diseases caused by oxidative injury.

References

[1] Clarke DD, Sokoloff L. Circulation and energy metabolism of brain. In: Siegel GJ, Agranoff BW, Albers RW, Fisher SK, Uheer MD, editors. Basic neurochemistry: Molecular, cellular and medical aspects. Philadelphia PA: Lippincott-Raven; 1999. p 637–669.

[2] Halliwell B, Gutteridge JMC, Cross CE. Free radicals, antioxidants and human diseases: Where are we now? J Lab Clin Med 1997;119:598–620.

[3] Diplock AT, Chanleux JL, Crozier WG, Kok FJ, Rice-Evans C, Roberfroid M, Stahl W, Vina-Ribes J. Functional food science and defense against reactive oxygen species. Br J Nutr 1998;80:s77–s112.

[4] Halliwell B. Antioxidants and human diseases: A general introduction. Nutr Rev 1997;55:544–552.

[5] Coyle JT, Puttfarcken P. Oxidative stress, glutamate and neurodegenerative disorders. Science 1993;262:689–700.

[6] Cooper AJL. Glutathione in the brain: Disorders of glutathione metabolism. In: Rosenberg RN, Prusiner SB, DiMauro S, Barchi RL, Kunk LM, editors. The molecular and genetic basis of neurological diseases. Boston: Butterworth-Heinemann; 1997. p 1195–1230.

[7] Emerit J, Edeas M, Bricaire F. Neurodegenerative diseases and oxidative stress. Biomed Pharmacother 2004;58:39–46.

[8] Desagher S, Glowinski J, Premont J. Astrocytes protect neurons from hydrogen peroxide toxicity. J Neurosci 1996;16:2553–2562.

[9] Mena MA, Casarejos MJ, Carazo A, Paino CL, Yebens JG. Glia protect fetal midbrain dopamine neurons in culture from L-DOPA toxicity through multiple mechanisms. J Neural Transm 1997;104:317–328.

[10] Akerud P, Alberch J, Eketjall S, Wanger J, Arenas E. Differential effects of glial cell line-derived neurotrophic factor and neurturin on developing and adult substantia nigra dopaminergic neurons. J Neurochem 1999;73:70–78.

[11] Abbott NJ. Astrocyte–endothelial interactions and blood–brain barrier permeability. J Anat 2002;200:629–638.

[12] Papadopoulos MC, Loumenis IL, Yuan TY, Giffard RG. Increasing vulnerability of astrocytes to oxidative injury with age despite constant antioxidant defenses. Neuroscience 1998;82:915–925.

[13] Gerlack M, Benshachar D, Riederer P, Youdim MBH. Altered brain metabolism of iron as a cause of neurodegenerative diseases? J Neurochem 1994;63:7095–7104.

[14] Afanas'ev IB, Dorozhko AI, Brodskii AV, Kostyuk VA, Potapovitch AI. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. Biochem Pharmacol 1989; 38:1763–1769.

[15] Formica JV, Regelson W. Review of the biology of quercetin and related bio-flavonoids. Food Chem Toxicol 1995;33:1061–1080.

[16] Hertog MGL, Hollman PCH, Katan MB, Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. Nutr Cancer 1993;20:21–29.

[17] Kuhnau J. The flavonoids: A class of semi-essential food components: Their role in human nutrition. Rev Nutr Diet 1976;24:117–191.

[18] Ishige K, Schubertm D, Sagaram Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. Free Radic Biol Med 2001;30:443–446.

[19] Hertog MGL, Feskens EJM, Hollman PCH, Katan MB, Kromhoutatan MGL. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. Lancet 1993;342:1007–1011.

[20] Scalbert A, Johnson IT, Saltmarsh M. Polyphenols: Antioxidants and beyond. Am J Clin Nutr 2005;81:215S–217S.

[21] Zhao B. Natural antioxidants for neurodegenerative diseases. Mol Neurobiol 2005;31:283–294.

[22] Vinson JA. Flavonoids in foods as *in vitro* and *in vivo* antioxidants. Adv Exp Med Biol 1998;439:151–163.

[23] Nijveldt RJ, van Nood E, van Hoorn EC, Boelens PG, va Norren K, vam Leeuwen PAM. Flavonoids: A review of probable mechanisms of action and potential applications. Am J Clin Nutr 2001;74:418–425.

[24] Filipe P, Lanca V, Silva JN, Morliere P, Santus R, Fernandes A. Flavonoids and urate antioxidant interplay in plasma oxidative stress. Mol Cell Biochem 2001;221:79–87.

[25] Dajas F, Rivera-Megret F, Blasina F, Arredondo F, Abin-Carriquiry JA, Costa G, Echeverry C, Lafon L, Heizen H, Ferreira M, Morquio A. Neuroprotection by flavonoids. Braz J Med Biol Res 2003;36:1613–1620.

[26] Bastianetto SB, Zheng W, Quirion R. Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide related to toxicity in cultured hippocampal neurons. Br J Pharmacol 2000;131:711–720.
[27] van Meeteren ME, Hendriks JJA, Dijkstra CD, van Tol AF. Dietary compounds prevent oxidative damage and nitric oxide production by cells involved in demyelinating disease. Biochem Pharmacol 2004;67:967–975.
[28] Pfeiffer SE, Herschman HR, Lightbody J, Sato G. Synthesis by a clonal line of rat glial cells of a protein unique to the nervous system. J cell Physiol 1970;75:329–339.
[29] Bissel MG, Rubinstein LJ, Bugnami A, Herman MM. Characterisation of the rat C6 glioma maintained on organ culture system. Production of glial fibrillary acidic protein in the absence of gliofibrillogenesis. Brain Res 1974;82:77.
[30] Babich H, Borefreund E. Applications of the neutral red cytotoxicity assay to *in vitro* toxicology (Review). Altern Lab Anim 1990;18:129–144.
[31] Geetha S, SaiRam M, Singh V, Ilavazhagan G, Sawhney RC. Antioxidant and immunomodulatory properties of Seabuckthorn (*Hippophae rhamnoides*) — an *in vitro* study. J Ethnopharmacol 2002;79:373–378.
[32] Tice RR, Andrews PW, Hira IO, Singh NP. The single cell gel (SCG) assay: An electrophoretic technique for the detection of DNA damage in individual cells. In: Witmer CM, editor. Biological and reactive intermediates IV. New York: Plenum Press; 1990. p 157–164.
[33] Collins AR, Ai-guo M, Duthie SJ. The kinetics of oxidative DNA damage (strand breaks and oxidized pyrimidines) in human cells. Mutat Res 1995;336:69–77.
[34] Van den Berg J, Kuypers F, Qju J, Chiu D, Lubin B, Roelofsen B, Op den Kamp J. The use of *cis*-parinaric acid to determine lipid peroxidation in human erythrocyte membranes. Comparison of normal and sickle erythrocytes membranes. Biochim Biophys Acta 1988;944:29–39.
[35] Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976;76:214–226.
[36] Xiaopeng B, Francis MH, Huang JY, John AC, James WP. Roles of cytoplasmic Ca^{++} and intracellular Ca^{++} stores in induction and suppression of apoptosis in S49 cells. Am J Physiol (Cell Physiol 41) 1997;272:C1241–C1249.
[37] Torel J, Cillard J, Cillard P. Antioxidant activity of flavonoid and reactivity with peroxyl radical. Phytochemistry 1986;25:383–385.
[38] Robak J, Gryglewski RL. Flavonoids are scavengers of superoxide anions. Biochem Pharmacol 1988;37:837–841.
[39] Manach A, Mazur A, Scalbert A. Polyphenols and prevention of cardiovascular diseases. Curr Opin Lipidol 2005;16:77–84.
[40] Cherbuni A, Vigna GB, Zuliani G, Ruggiero C, Senin U, Fellin R. Role of antioxidants in atherosclerosis: Epidemiological and clinical update. Curr Pharm Des 2005;11:2017–2032.
[41] Galati G, O'Brien PJ. Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. Free Radic Biol Med 2004;37:287–303.
[42] Dajas F, Costa G, Abin-Carriguiry JA, Echeverry C, Martinez-Borges A, Dajas-Bailder F. Antioxidants and cholinergic neuroprotective mechanisms in experimentative Parkinsonism. Funct Neurol 2001;17:37–44.
[43] Sandstrom B. Induction and rejoining of DNA single strand breaks in relation to cellular growth in human cells exposed to three hydroperoxide at 0°C and 37°C. Free Radic Res Commun 1991;15:79–89.
[44] Aherne SA, O'Brien N. Mechanism of protection by flavonoids, quercetin and rutin against *tert*-butylhydroperoxide and menadione induced DNA single strand breaks in Caco-2 cells. Free Radic Biol Med 2000;29:507514.
[45] Bastianetto SB, Zheng W, Quirion R. Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide related to toxicity in cultured hippocampal neurons. Br J Pharmacol 2000;131:711–720.
[46] Sestili P, Guidarelli A, Dacha M, Cantoni O. Quercetin prevents DNA single strand breakage and cytotoxicity caused by *tert*-butyl hydroperoxide free radical scavenging versus iron chelating mechanism. Free Radic Biol Med 1998;25:196–200.
[47] Wen-Peng I, Kuo SM. Flavonoid structure affects the inhibition of lipid peroxidation in Caco-2 intestinal cells at physiological concentrations. J Nutr 2003;133:2184–2187.
[48] Ratty AK, Sunamoto J, Das NP. Interaction of flavonoids with 1,1-diphenyl-2-picrylhydrazyl free radical, liposomal membranes and soybean lipoxygenase-1. Biochem Pharmacol 1988;37:989–995.
[49] Makar TK, Nodergaard M, Preuss A, Gelbard AS, Perumal AS, Cooper JL. Vitamin E, ascorbate, glutathione, glutathione disulfide and enzymes of glutathione metabolism in cultures of chick astrocytes and neurons: Evidence that astrocytes play an important role in antioxidative processes in the brain. J Neurochem 1994;62:45–53.
[50] Saito Y, Matuyama K, Saido TC, Kawashima S. N23K, a gene transiently up-regulated during neuronal differentiation, encides a precursor protein for a newly identified neuropeptide niciceptin. Biochem Biophys Res Commun 1995;217:539–545.
[51] Sagara Y, Schubert D. The activation of metabotropic glutamate receptors protects neuronal cells from oxidative stress. J Neurosci 1998;18:6662–6671.
[52] Kim MS, Cheong YP, So HS, Lee KM, Kim TY, Oh J, Chung YT, Son Y, Kim BR, Park R. Protective effects of morphine in peroxynitrite-induced apoptosis of primary neonatal astrocytes: Potential involvement of G protein and phosphatidylinositol 3-kinase (PI3 kinase). Biochem Pharmacol 2001;61:779–786.
[53] Di Monte D, Bellomo G, Thor H, Nicotera O, Orrenius S. Menadione induced cytotoxicity is associated with protein thiol oxidation and alteration in intracellular Ca^{2+} homeostasis. Arch Biochem Biophys 1984;235:343–350.
[54] Nicotera P, McConkey D, Svemsson SA, Bellomo G, Orrenius S. Correlation between cytosolic Ca^{2+} concentration and cytotoxicity in hepatocytes exposed to oxidative stress. Toxicology 1988;52:55–63.
[55] Orrenius S, McCabe MJ, Nicotera P. $Ca^{(2+)}$ dependent mechanisms of cytotoxicity and programmed cell death. Toxicol Lett 1992;64-65:357–364.
[56] Dobrydneva Y, Williams RL, Blackmore PF. *trans*-Resveratol inhibits calcium influx in thrombin-stimulated human platelets. Br J Pharmacol 1999;128:149–157.
[57] Wang H, Joseph JA. Structure relationships of quercetin in antagonizing hydrogen peroxide-induced calcium dysregulation in PC12 cells. Free Radic Biol Med 1999;27:683–694.
[58] Kempuraj D, Madhappan B, Christodoulou S, Boucher W, Cao J, Papadopoulou N, Cetrulo CL, Theoharides TC. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. Br J Pharmacol 2005; 145: 934–44.
[59] Youdim KA, Qaiser MZ, Begley DJ, Rice-Evans CA, Abbott NJ. Flavonoid permeability across an *in situ* model of the blood–brain barrier. Free Radic Biol Med 2004;36:592–604.