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Quebrachitol (2-*O*-methyl-L-inositol) attenuates 6-hydroxydopamine-induced cytotoxicity in rat fetal mesencephalic cell cultures

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Abstract

Naturally occurring plant substances have the potential to prevent oxidative damage in various pathophysiological conditions including neurodegenerative disorders. Recent findings indicate that impaired energy metabolism plays a prominent role in neurodegeneration. The present study investigated whether quebrachitol (2-*O*-methyl-L-inositol) (QCT), a sugar like natural compound that was suggested to have both antioxidant and membrane stabilization activity prevents the cytotoxic effect of 6-hydroxydopamine (6-OHDA, 200 μM) on cultured rat fetal mesencephalic cells. While QCT (0.1–100 μg/ml) produced no effect per se on cell viability as measured in the 3[4,5-dimethylthiazole-2il]-2,5-diphenyltetrazolium bromide (MTT) test, it offered concentration-related protection against cell death induced by 6-OHDA. In addition, QCT demonstrated an antioxidant activity against 6-OHDA-induced oxidative stress as evidenced by reduced formation of nitrite–nitrate and thiobarbituric acid-related substances. Fluorescence microscopy using acridine orange/ethidium bromide double staining further affirmed the absence of 6-OHDA (200 μM)-induced morphological changes characteristic of apoptosis/necrosis in cultures pretreated with QCT (100 μg/ml). Also, results of tyrosine hydroxylase immunoreactivity indicated that 6-OHDA induces cell death in mesencephalic cultures affecting both TH^+ positive and TH^- negative (TH^+ and TH^-, respectively) and QCT pretreatment protects them from cell death, in a non-specific manner. Our data indicate that QCT has a cytoprotective role due, at least in part, to an antioxidant and free radical scavenging mechanism. Furthermore, the study suggests that inositol compounds might serve as leads in developing drugs for the treatment of various neurodegenerative disorders.

Keywords: Quebrachitol; 2-*O*-Methyl-L-inositol; 6-Hydroxydopamine; Cytotoxicity; Mesencephalic cell cultures; Fetal rat

1. Introduction

Although the etiology of neurodegeneration in Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington disease (HD) is not yet clear, oxidative stress has been strongly implicated (Anderson, 2004; Cui et al., 2004). Oxidative stress reflects a state of imbalance between generation of reactive oxygen species and activity of the antioxidant defences. 6-hydroxydopamine (6-OHDA), an endogenously produced toxic metabolite of dopamine, has been the toxin widely used in vitro and in vivo as a model to investigate the drugs which may be effective in Parkinson's disease and to study their mechanisms of action (Bahat-Stroomza et al., 2005; Jakel et al., 2005). 6-OHDA may be a likely candidate in creating oxidative stress through the production of hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS), capable of destroying cellular structural and functional apparatus (Foley and Riederer, 2000; Ferger et al.,

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2001; Cui et al., 2004). The toxic effects of 6-OHDA are largely attributed to increased lipid peroxidation, exhaustion of endogenous glutathione, loss of mitochondrial respiration and neuronal apoptosis (Dragunow et al., 1997; Ogawa et al., 2004), and some plant-derived antioxidants are neuroprotective (Kim et al., 2004a; Im et al., 2005). 6-OHDA has been the widely used in vitro, and in vivo model to investigate the drugs effective in Parkinson's disease and to study the action mechanisms (Bahat-Stroomza et al., 2005; Jakel et al., 2005).

In the recent past, a large number of antioxidants such as Vitamin E, ebselen, carotenoids, curcumin, flavonoids, lipoic acid and lazaroid have been investigated as potential therapeutic agents to control oxidative stress in neurodegenerative disorders (Sen, 1998; Gilgun-Sherki et al., 2001, 2002; Nobre Junior et al., 2003; Kim et al., 2004a; Leal et al., 2005; Mercer et al., 2005; Wang et al., 2005). Although such compounds have shown neuroprotection when used in animal models or in small clinical studies, their use is still relatively limited mainly because of poor bioavailability and/or inadequate antioxidant activity under physiological conditions (Gilgun-Sherki et al., 2002). Consequently, the search for novel free radical scavengers with improved clinical efficacy and safety margins has continued as a potential therapeutic strategy for neurodegenerative disorders (Lapchak and Araujo, 2002). It has been suggested that antioxidant constituents in tropical fruits, medicinal and food plant extracts protect against free radical mediated damage and offer neuroprotection (Aruoma et al., 2003; Hassimotto et al., 2005). Quebrachitol (2-*O*-methyl-L-inositol) (QCT) is a bioactive plant constituent and has similar physiological effects as inositol. Inositol is a polyalcohol required for the proper formulation of cell membranes. In mammals, it exists in phosphoinositides and in free form and participates in transmembrane signaling mechanism (Almeida et al., 2003). It has a membrane stabilization property and acts as a cryoprotectant (Orthen and Popp, 2000; Hincha and Hagemann, 2004). Although QCT closely resembles glucose in structure, when taken by mouth it does not relieve hypoglycemia, raise the blood sugar or lead to the deposition of glycogen in the liver (McCance and Lawrence, 1933). Peroxynitrite ($ONOO^-$) scavenging and laxative effects of QCT have been described in literature (Aurousseau et al., 1964; Kim et al., 2004b). QCT also showed free radical scavenging ability in DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (Machado et al., 2006). High concentration of 6-OHDA (200 μM) may induce cytotoxicity to mesencephalic cell cultures in vitro affecting both neuronal and non-neuronal cells (Mazzio et al., 2004). In cultured fetal rat ventral mesencephalon cells, mixed neuronal populations are present and 6-OHDA at low concentration (5 μM) selectively affects the dopaminergic neurons (Parkinson's disease model) whereas at high levels, it may cause non-specific neuronal damage creating severe oxidative stress (Michel and Hefti, 1990). Since QCT possesses membrane stabilization and free radical scavenging properties, the present study aims to verify whether it can manifest cytoprotection against cell death induced by 6-OHDA (200 μM) in rat mesencephalic cell cultures in vitro.

2. Materials and methods

2.1. Plant material and isolation of QCT (2-O-methyl-L-inositol)

Magonia glabrata St. Hill (Sapindaceae) fruits were collected in Iraucuba, Ceará, Brazil, in January 2003, and identified by Dr. Afrânio G. Fernandes, botanist of the Federal University of Ceara. A voucher specimen (#15198) has been deposited at Herbarium Prisco Bezerra. QCT (Fig. 1) was extracted and isolated from the pericarp of *M. glabrata* dried fruits as per procedures described earlier (Araújo et al., 1994), and on dry weight basis, its yield was 7.2%. The chemical identification was done based on spectral analysis, determination of physical properties and by comparison with literature data.

2.2. Chemicals

6-hydroxydopamine hydrobromide, poly-L-lysine, Eagles Minimal Essential Medium (MEM) and sulfanilamide were purchased from Sigma, USA. *N*-(1-naphthyl)-ethylenediamine dihydrochloride was from Merck, USA. All other chemicals and reagents used were of analytical grade.

2.3. Animals

Female Wistar albino rats (200 g) obtained from the Animal House of the Federal University of Ceará were used. The animals were housed in polypropylene cages at 23–25 °C with a 12 h light–12 h dark cycle, and had free access to standard laboratory rat pellet diet (Purina Chow) and tap water. The Institutional Ethics Committee approved the experimental protocol according to the Guidelines of National Institute of Health for the Care and Use of Laboratory Animals.

2.4. Mesencephalic cell cultures

Pregnant rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), killed by cervical dislocation and through an abdominal incision fetuses (days 17–20) were removed. Fetal brains were excised under sterile conditions and the ventral mesencephali were dissected according to standard procedures (Franke et al., 2003). Briefly, the mesencephalic explants were rinsed twice in Eagles Minimal Essential Medium (MEM) and mechanically dissociated in 2 ml of cold MEM. Immediately after dissociation, 10 ml of MEM was added to the suspension. The supernatant containing the individual cells were then suspended in MEM supplemented with 10% horse serum, streptomycin (100 mg/ml), penicillin (1000 UI/ml), actinomycin C (2.5 mg/ml), sodium bicarbonate (24 mM), and glucose (11 mM). In MTT and nitrite–nitrate assays, cell suspensions were plated on poly-L-lysine-coated-96 well plates at a density of 5×10^4 cells/well, whereas for lipid peroxidation assay and microscopy studies the cells

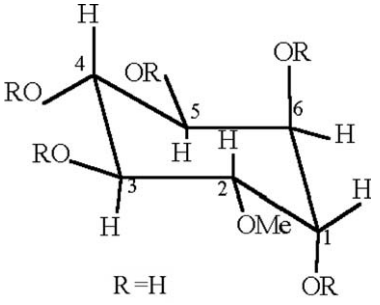

Fig. 1. Quebrachitol (2-*O*-methyl-L-inositol).

were plated on 24 well plates at a density of 5×10^5 cells/well. Besides, for fluorescence microscopy and tyrosine hydroxylase immunoreactivity studies the cells were cultivated on glass coverslips. Cultures are maintained at 37 °C in a humidified 5% CO_2 atmosphere (Choi et al., 1987). Medium was changed to a fresh MEM at 24 h after plating. The cells were used for experiments after four days.

2.5. Culture treatments

After 4 days in culture, wells were randomly exposed to either the treatment groups or to the control group. QCT (0.1–100 μg/ml) was added to the cell culture, 3 h prior to the addition of 6-OHDA (200 μM), and incubated for 24 h at 37 °C in 5% CO_2 atmosphere before being subjected to the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium (MTT) assays. QCT and 6-OHDA were dissolved in cold PBS. Cytotoxicity of 6-OHDA and its protection by QCT was assessed by cell viability, morphological analysis of cell death, and by biochemical assays.

2.6. Cell viability (MTT assay)

The MTT test was performed to study the cell survival as described by Mosmann (1983). This assay is based on the ability of living cells to reduce MTT to insoluble formazan and therefore the measure of the amount of formazan produced reflects the cell viability. Briefly, 24 h after treatment of cultures with the test compounds or vehicle, the medium was replaced and MTT (10 μl of a 5 mg/ml in PBS) was added to each well so as to obtain a concentration of 50 μg in 100 μl. The cell culture was incubated for 3 h at 37 °C under 5% CO_2 atmosphere. The cells were then washed with PBS and 100 μl of DMSO was added. After 5 min stirring, the absorbance was measured at 550 nm, using a microplate reader. The inhibition of MTT reduction indicates the degree of 6-OHDA-induced toxicity to cells. Experiments were performed in triplicate in three independent experiments. Cell viability was expressed as a percentage of cells untreated with 6-hydroxydopamine, which served as the control group and was designated as 100%. The results are expressed as percentage of the control.

2.7. Analysis of cell death by fluorescence microscopy

Acridine orange/ethidium bromide double staining (Branton and Clarke, 1999) was used to study the morphological changes associated with 6-OHDA-induced cell death. Acridine orange penetrates into living and dead cells, emitting green fluorescence as a result of intercalation in double-stranded DNA and red–orange fluorescence after binding with single-stranded RNA and due to its accumulation in lysosomes. Ethidium bromide emits red fluorescence after intercalation in DNA of cells with an altered cell membrane (at a late stage of apoptosis and necrosis). Using this double staining, four cell stages can be identified: normal cells – uniformly stained green; early apoptosis – cell membrane still continuous but chromatin condensation and an irregular green nucleus are visible; late apoptosis so-called 'secondary necrosis' or 'apoptotic necrosis' – ethidium bromide penetrates through altered cell membrane and stains the nuclei orange, while fragmentation or condensation of chromatin is still observed; and necrosis – uniformly orange-stained cell nuclei but with nuclear morphology resembling that of viable cells. Following the addition of fluorochromes (100 μg/ml acridine orange and 100 μg/ml ethidium bromide), 200 cells were analyzed in each of the three independent experiments in duplicate using fluorescence microscopy (Olympus BX 60 F5 from Olympus Optical Co. Ltd., and 480 and 520 nm filters).

2.8. Tyrosine hydroxylase immunoreactivity

After the culture medium had been aspirated, cells were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4. After washing and quenching of endogenous peroxidase (3% hydrogen peroxide), the cells were incubated with primary rabbit antibody, tyrosine hydroxylase (Dako, 1:200), for overnight at 4 °C. After washing and incubation with secondary antibody, the immunoreactivity was visualized using a colorimetric-based detection kit following the manufacturer protocol (Dako LSAB + Kit, peroxidase, DAKO, USA). Stained cells were visualized by optic microscopy, using a Nikon microscope, and counted by a blinded observer. TH^+ neurons were counted all over the entire coverslip, while TH^- cells were determined by counting negative stained cells in four random fields from each well. Experiments were performed in duplicate in three independent experiments.

2.9. Nitrite–nitrate determination

In order to assess the effects of treatments on nitric oxide (NO) production, nitrite–nitrate levels were determined in cell culture supernatants by a previously described method (Green et al., 1981). Briefly, 100 μl of Griess reagent (1% sulfanilamide in 1% H_3PO_4/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/1% H_3PO_4/distilled water, 1:1:1:1) was added to 100 μl of the cell culture supernatant and incubated at room temperature for 10 min. The standard curve was prepared with several concentrations of $NaNO_2$ (ranging from 0.75 to 100 μM) under the same conditions. Blanks were prepared by adding 100 μl of the Griess reagent to 100 μl of the culture medium. The absorbance was measured with a microplate reader at 560 nm. Experiments were performed in triplicate in three independent experiments.

2.10. Lipid peroxidation assay

Lipid peroxidation was measured in cell cultures, as previously described (Draper and Hadely, 1990). This method is based on the oxidation of polyunsaturated fatty acids in biologic membranes, giving rise to a variety of lipid breakdown products such as malondialdehyde (MDA). MDA reacts with thiobarbituric acid, forming thiobarbituric acid reactive substances (TBARS), which is a measure of lipid peroxidation. Briefly, after drug exposure, the supernatant was discarded and mesencephalic cells were lysed with Triton ×100. Then, 250 μl of the lysed cells were introduced into glass tubes and the mixtures were incubated in a water bath at 37 °C, for 1 h, followed by the addition of 400 μl of 35% perchloric acid to stop lipid peroxidation. The mixture was centrifuged at 1400*g* for 10 min and to the supernatant (600 μl), 200 μl of 1.2% sodium 2-thiobarbiturate solution was added. The glass tubes were then placed in a water bath and heated at 95 °C for 30 min. After cooling, absorbance was measured with a microplate reader at 535 nm. Experiments were performed in triplicate in three independent experiments.

2.11. Statistical analysis

All data are expressed as mean ± SEM. For statistical analysis, the results of two or three independent experiments were averaged together due to similarity. The statistical significance of differences among groups was determined by analysis of variance (ANOVA) followed by a Tukey posthoc test using GraphPad Prism Software (San Diego, CA, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Effect on cell viability

Fig. 2 shows the cell viability as measured by the MTT assay in mesencephalic cultures after 24-h exposure to 6-OHDA 200 μM in the presence and absence of QCT. 6-OHDA significantly reduced the cell viability by 65.85% as evidenced by a decrease in the optical density value. Apparently, the cell viability was not influenced by QCT in the absence of 6-OHDA. QCT at 0.1, 1, 10 and 100 μg/ml produced a significant concentration-related

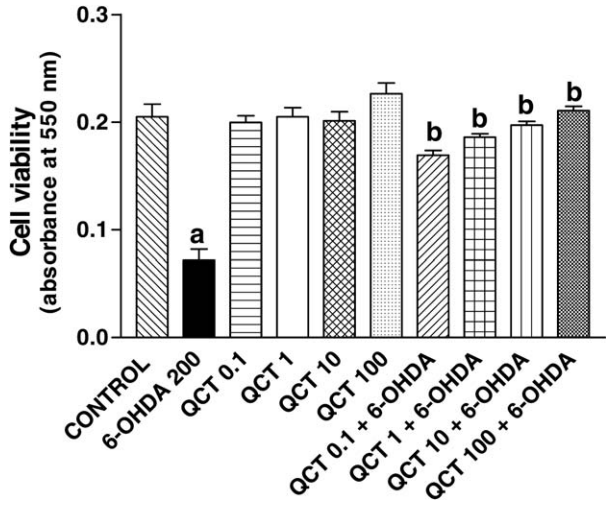

Fig. 2. Protective effect of quebrachitol (QCT) on cell death induced by 6-OHDA. Cultured fetal rat mesencephalic cells were exposed to QCT (0.1; 1; 10 and 100 μg/ml) 3 h before the addition of 6-OHDA (200 μM). After 24 h, the cell viability was evaluated by MTT assay. Each column represents the mean ± SEM ($n = 6$). [a]vs. control, [b]vs. 6-OHDA ($P < 0.05$, ANOVA followed by Tukey's test).

inhibition of 6-OHDA-induced cell-death and at 100 μg/ml, the cell death was completely abolished (% of cell-death: 6-OHDA – 65.85%; QCT 0.1 + 6-OHDA – 17.56%; QCT 1 + 6-OHDA – 9.46%; QCT 10 + 6-OHDA – 3.91%; QCT 100 + 6-OHDA – 0.0%).

3.2. Fluorescence microscopy findings

Acridine orange/ethidium bromide double staining enables the distinction between apoptotic and necrotic cells to be observed. A higher percentage of late apoptotic and necrotic cells accompanied by a small percentage of normal cells was observed after treatment with 200 μM 6-OHDA (normal – 0.25%, early apoptosis (EA) – 1.75%, late apoptosis (LA) – 28.38%, necrosis – 69.75%) and this apoptotic pattern was almost completely blocked by QCT (100 μg/ml) treatment (normal – 76.83%, EA – 2.00%, LA – 2.50%, necrosis – 19.67%) (Fig. 3).

3.3. Tyrosine hydroxylase immunoreactivity

Tyrosine hydroxylase immunoreactivity in cultures exposed to 6-OHDA (200 μM) for 24 h in the presence or absence of QCT (100 μg/ml) is shown in Fig. 4. The neurotoxin 6-OHDA greatly decreased the percentage cell survival affecting both TH^+ and TH^- cells compared to control (6-OHDA – $TH^+ = 6.25\%$, $TH^- = 36.56\%$). Addition of QCT significantly prevented the neuronal cell death induced by 6-OHDA, also in a non-selective manner (QCT + 6-OHDA – $TH^+ = 59.70\%$, $TH^- = 83.73\%$).

3.4. Effect on the release of NO

Mesencephalic cells exposed to 6-OHDA (200 μM), showed an increased generation of NO, as evidenced by a 7.3-fold increase in nitrite–nitrate levels as compared to control value (control – 8.39 ± 0.33; 6-OHDA – 59.38 ± 2.24 μM) (Fig. 5). QCT treatment significantly reduced the 6-OHDA-enhanced levels of NO metabolites in a concentration-related way (QCT 0.1 + 6-OHDA = 49.5; QCT 1 + 6-OHDA = 37.9; QCT 10 + 6-OHDA = 32.3; QCT 100 + 6-OHDA = 21.10 μM).

3.5. Effect on lipid peroxidation

In cells exposed to 6-OHDA (200 μM), a 2.7-fold increase in TBARS formation was observed as compared

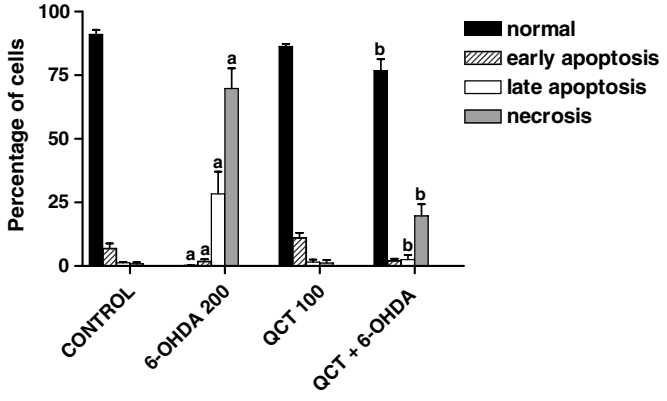

Fig. 3. Effect of quebrachitol (QCT) on 6-OHDA-induced morphological changes in cultured fetal rat mesencephalic cells. Cells were exposed to QCT (0.1; 1; 10 and 100 μg/ml) 3 h before the addition of 6-OHDA (200 μM). After 24 h, the cells were double stained with acridine orange/ethidium bromide and the pattern of apoptosis/necrosis was analyzed by fluorescence microscopy. Each column represents the mean ± SEM ($n = 6$). [a]vs. control, [b]vs. 6-OHDA ($P < 0.05$, ANOVA followed by Tukey's test).

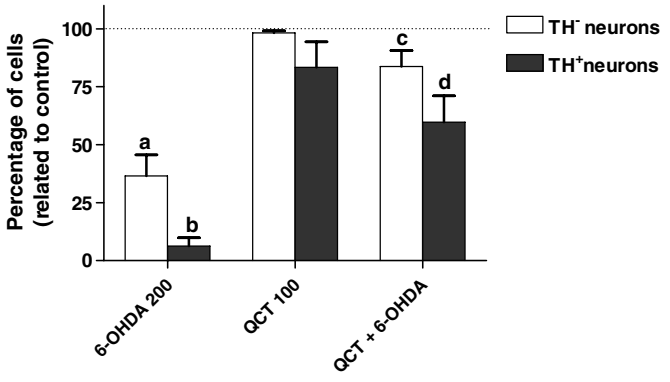

Fig. 4. Effect of quebrachitol (QCT) and 6-OHDA on tyrosine hydroxylase immunoreactivity in rat fetal mesencephalic cell cultures. Cells were incubated with quebrachitol (100 μg/ml), 3 h before 6-OHDA (200 μM). After 24 h exposure the cells were analysed for tyrosine hydroxylase-positive (TH^+) and -negative (TH^-) immunoreactivity. Data represent mean ± SEM ($n = 6$). [a]vs. TH^-control, [b]vs. TH^+ control, [c]vs. TH^- 6-OHDA, [d]vs TH^+ 6-OHDA at their corresponding concentrations ($P < 0.05$, ANOVA followed by Tukey's test).

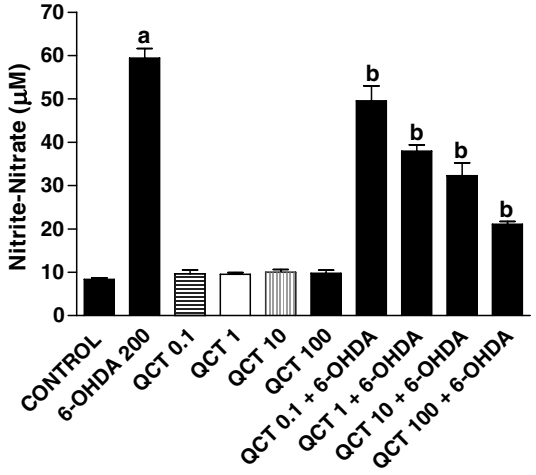

Fig. 5. Effect of quebrachitol (QCT) on 6-OHDA-induced nitrite–nitrate formation in fetal rat mesencephalic cell cultures. Cells were exposed to QCT (0.1; 1; 10 and 100 μg/ml) 3 h before the addition of 6-OHDA (200 μM). After 24 h, nitrite–nitrate levels in culture supernatants were measured by the Griess reaction. Each column represents the mean ± SEM ($n = 6$). [a]vs. control, [b]vs. 6-OHDA ($P < 0.05$, ANOVA followed by Tukey's test).

to controls (TBARS absorbance: control – 0.187 ± 0.047; 6-OHDA – 0.499 ± 0.06). When cells were pretreated with QCT (1–100 μg/ml), 6-OHDA-stimulated TBARS formation was significantly less and was concentration-dependent (Fig. 6). The pretreatment of cell cultures with QCT at concentrations of 1, 10 and 100 μg/ml attenuated this increase in TBARS levels by 1.9-, 2.3- and 3.3-fold, respectively.

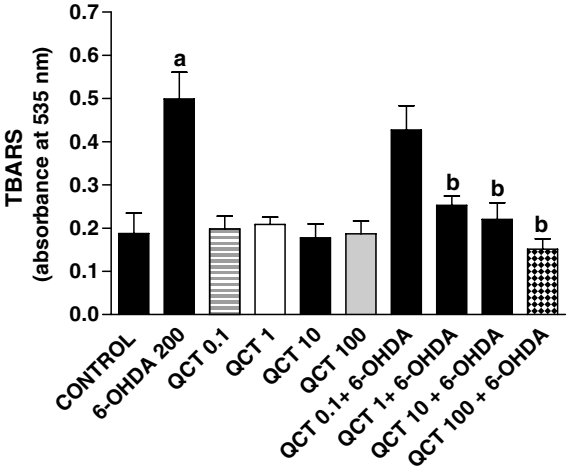

Fig. 6. Effect of quebrachitol (QCT) on 6-OHDA-induced TBARS formation in fetal rat mesencephalic cell cultures. Cells were exposed to QCT (0.1; 1; 10 and 100 μg/ml) 3 h before the addition of 6-OHDA (200 μM). After 24 h, the levels of TBARS in culture supernatants were measured as an index of lipid peroxidation. Each column represents the mean ± SEM ($n = 6$). [a]vs. control, [b]vs. 6-OHDA ($P < 0.05$, ANOVA followed by Tukey's test).

4. Discussion

Oxidative stress is a ubiquitously observed hallmark of neurodegenerative disorders. Toxic free radicals, including those arising from dopamine-, lipid- and nitrergic-related mechanisms play a prominent role in neurodegeneration (Jenner, 1991). In the recent past, there has been much interest in dietary strategies to combat oxidant stress-related damage in various pathophysiological conditions (Youdim and Joseph, 2001; Aruoma et al., 2003). QCT is a natural inositol endowed with antioxidant and membrane stabilizing properties. The present study verified a possible neuro/cytoprotection by QCT against cell death in rat fetal mesencephalic cultures exposed to a high concentration of 6-OHDA (200 μM). In both cultured and intact foetal ventral mesencephalon, mixed neuronal populations are present. These populations are likely to be affected during oxidative stress. It has been reported that concentrations of 6-OHDA as low as 5 μM can ensure selective toxicity for dopaminergic neurons in vitro (Carrasco and Werner, 2002), but higher concentrations loose this selectivity causing cell damage to both neuronal and non-neuronal cells (Michel and Hefti, 1990). The oxidative stress induced by 6-OHDA occurs via the generation of H_2O_2 and related hydroxyl radicals through auto-oxidation (Kumar et al., 1995). Antioxidants, which have the capacity to prevent lipid peroxidation and modulate nitrergic-involvement, provide protection against the oxidative damage occurring in Parkinson's disease and other neurodegenerative conditions, which have the capacity to prevent lipid peroxidation and modulate nitrergic-involvement. In the current study, consistent with earlier findings (Im et al., 2005; Leal et al., 2005), we observed marked increases in nitric oxide-related metabolites (nitrite–nitrate) and lipid peroxidation-derived TBARS formation in cultured mesencephalic cells exposed to 6-OHDA. QCT pretreatment not only prevented the 6-OHDA associated cell death but also showed an apparent antioxidant effect reducing effectively the levels of nitrite–nitrate and TBARS formation.

As reported earlier, challenge of mesencephalic culture with 6-OHDA resulted in cell death characterized by focal degeneration and necrosis with complete loss of cell integrity and architecture (Ding et al., 2004). In order to analyse the effects of 6-OHDA and QCT on neurotoxicity, we quantified the number of TH^+ and TH^- cells in mesencephalic culture. The results show that at the concentration used (200 μM), 6-OHDA caused cell death in both neuronal and non-neuronal cells as indicated by tyrosine hydroxylase immunoreactivity. QCT pretreatment markedly reduced the 6-OHDA associated cell death of both TH^+ (neuronal) and TH^- cells (neuronal and non-neuronal cells). The model system designed in this study is not ideal to evaluate the cell death mechanism in in vitro model of PD. In fact, we used a low dose 6-OHDA (40 μM) also in our experiments. The proportion of DA neurons in this mixed cell system is very low. The total cell death appeared to be low with small dose compared to a high dose

6-OHDA (200 μM). However, the extent of cytoprotection by QCT was almost similar whether we used a small dose or a high dose 6-OHDA, thus showing no selective protection for dopaminergic cells (data not shown). Therefore, the main focus in our study was to verify the antioxidant capacity of QCT in a mixed cell system (mesencephalic cell culture) exposed to high dose 6-OHDA. The results obtained show a clear cytoprotection by QCT, which may have some clinical significance in combating oxidative stress in neurodegenerative conditions.

The mechanism of QCT neuro/cytoprotection is unclear from the current study. Amongst the potential mechanisms, an antioxidant effect of QCT may be important in cell survival as it inhibited the nitric oxide generation and lipoperoxidation process in mesencephalic cell cultures exposed to neurotoxin 6-OHDA. QCT possesses multiple hydroxyl groups in its chemical structure, which may largely contribute to the antioxidant efficiency as was observed in DPPH assay (Machado et al., 2006). Acting as an antioxidant, it might control the reactive oxygen species-nitric oxide (ROS-NO) pathway involved in 6-OHDA-induced cell death, described by Guo et al. (2005). Peroxynitrite ($ONOO^-$) is one of the cytotoxic species produced by the reaction between superoxide and nitric oxide that can cause irreversible damage to proteins, lipids, and DNA. A recent study reported that QCT scavenges $ONOO^-$ (Kim et al., 2004b) and in the current study we showed that it inhibits 6-OHDA-induced NO generation in cultured mesencephalic cells. These effects of QCT may be beneficial in overcoming the cellular oxidative and nitrosative stress. Besides the scavenging action on free radicals, being a sugar derivative, QCT possibly might improve the mitochondrial energy metabolism preventing the decreases in high-energy phosphate stores (ATP) induced by 6-OHDA. Furthermore, QCT as an inositol can serve as a building block for the synthesis of biologically active chiral inositol phosphates required for the proper formation of cell membranes (Almeida et al., 2003) and it may also attenuate endoplasmic reticulum stress response, which may be a consequence of oxidative stress. Although the molecular mechanisms associated with QCT neuroprotection remain to be elucidated in more detail, our results clearly demonstrate an antioxidant mechanism in its effect.

Our study is the first to show neuroprotective effect of QCT on neurotoxin (6-OHDA)-initiated cell death in mesencephalic cultures. Parkinson's disease (PD) is a neurodegenerative disease marked by severe loss of dopamine (DA) neurons in the nigrostriatal system, which results in depletion of striatal DA. Transplantation of embryonic ventral mesencephalic DA neurons into the striatum is a currently explored experimental treatment aimed at replacing lost DA in the nigrostriatal system, but is plagued with poor survival (5–20%) of implanted neurons (Kanaan et al., 2006). In this context, the results obtained in this study are interesting and suggest the possibility of enhancing the survival of embryonic mesencephalic DA neurons when QCT is administered with grafted cells in the above experimental model of PD.

This study demonstrated only a preventive effect of QCT on 6-OHDA-induced cell death. Since the action of 6-OHDA neurotoxin is very rapid in its onset (within 2 h in mesencephalic cell cultures), we did not test QCT after the neurotoxin insult. Many polyphenolic compounds like flavonoids have been shown to be neuroprotective in vitro (Lee et al., 2005; Mercer et al., 2005), but their efficacy and mechanisms of neuroprotective effects of antioxidants in vivo have not been clearly elucidated. It was postulated that age-associated neurodegeneration is caused by an imbalance between pro-oxidants and antioxidants resulting in oxidative stress. Oxidative damage of biomolecules increases with age and is postulated to be a major causal factor of various neurodegenerative disorders. A recent study had shown that antioxidants could attenuate cognitive deficits in OXYS rats, providing evidence for therapeutic role of antioxidants (Kolosova et al., 2005). We consider that QCT might be beneficial as a neuroprotective, especially in combating neurodegeneration associated with senescence onset or subjects who are at high risk of developing degenerative diseases of brain. Nevertheless, the therapeutic potential of QCT and the mechanism/s of neuroprotection need to be further investigated.

In conclusion, our study demonstrated a clear cytoprotective role of QCT in a rat fetal mesencephalic culture system affording protection against toxic insults on both neuronal and non-neuronal cells possibly involving multiple mechanisms amongst which an antioxidant mechanism at least, in part, is important. Further studies are warranted to determine the exact mechanism/s through which QCT provides neuro/cytoprotection, particularly in relation to its effect on glia and on mitochondrial function. This study further suggests that inositol compounds might serve as leads in developing drugs for the treatment of various neurodegenerative disorders.

Acknowledgements

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References

Almeida, M.V., Silva, A.D., Souza, M.V.N., Benicio, A.A.A., 2003. A cascata dos fosfoinositideos. Química Nova 26, 105–111.

Anderson, J.K., 2004. Oxidative stress in neurodegeneration: cause or consequence? Neurodegeneration 434, S18–S25.

Araújo, F.W.L., Lemos, T.L.G., Militão, J.S.L., Braz Filho, R., 1994. 2-O-metilinositol e proantocianidina de *Magonia glabrata* St. Hill. Química Nova 17, 128–136.

Aruoma, O.L., Bahorun, T., Jen, L.-S., 2003. Neuroprotection by bioactive components in medicinal and food plant extracts. Mutation Research 544, 203–215.

Aurousseau, M., Lagrange, E., Roquet, F., 1964. Study of some pharmacodynamic properties of a laxative substance of plant origin: Quebrachitol. Therapie 19, 1071–1083.

Bahat-Stroomza, M., Gilgun-Sherki, Y., Offen, D., Panet, H., Saada, A., Krool-Galron, N.A., Attas, D., Metamed, F., 2005. A novel thiol antioxidant that crosses the blood brain barrier protects dopaminergic neurons in experimental models of Parkinson's disease. European Journal of Neuroscience 21, 637–646.

Branton, R.L., Clarke, D.J., 1999. Apoptosis in primary cultures of E14 rat ventral mesencephala: time course of dopaminergic cell death and implications for neural transplantation. Experimental Neurology 160, 88–98.

Carrasco, E., Werner, P., 2002. Selective destruction of dopaminergic neurons by low concentrations of 6-OHDA and MPP^+: protection by acetylsalicylic acid (aspirin). Parkinsonism and Related Disorders 8, 407–411.

Choi, D.W., Maulucci-Gedde, M.A., Kreigstein, A.R., 1987. Glutamate neurotoxicity in cortical cell culture. Journal of Neuroscience 7, 357–368.

Cui, K., Luo, X., Xu, K., Vem Murthy, M.R., 2004. Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and neutraceutical antioxidants. Progress in Neuro-Psychopharmacology and Biological Psychiatry 28, 771–799.

Ding, Y.M., Jaumotte, J.D., Signore, A.P., Zigmond, M.J., 2004. Effects of 6-hydroxydopamine on primary cultures of substancia nigra: specific damage to dopamine neurons and the impact of glial cell line-derived neurotropic factor. Journal of Neurochemistry 89, 776–787.

Dragunow, M., MacGibbon, G.A., Lawlor, P., Butterworth, N., Connor, B., Henderson, C., Walton, M., Woodgate, A., Hughes, P., Faull, R.L., 1997. Apoptosis, neurotrophic factors and neurodegeneration. Reviews in the Neurosciences 8, 223–265.

Draper, H.H., Hadely, M., 1990. Malondialdehyde determination as an index of lipid peroxidation. Methods in Enzymology 186, 421–431.

Ferger, B., Rose, S., Jenner, A., Halliwell, B., Jenner, P., 2001. 6-hydroxydopamine increases hydroxyl free radical production and DNA damage in rat striatum. Neuroreport 12, 1155–1159.

Foley, P., Riederer, P., 2000. Influence of neurotoxins and oxidative stress on the onset and progression of Parkinson's disease. Journal of Neurology 247 (Suppl. 2), II82–II94.

Franke, H., Schelhorn, N., Illes, P., 2003. Dopaminergic neurons develop axonal projections to their target areas in organotypic co-cultures of the ventral mesencephalon and the striatum/prefrontal cortex. Neurochemistry International 42, 431–439.

Gilgun-Sherki, Y., Melamed, E., Offen, D., 2001. Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood–brain barrier. Neuropharmacology 40, 959–975.

Gilgun-Sherki, Y., Rosenbaum, Z., Melamed, E., Offen, D., 2002. Antioxidant therapy in acute central nervous system injury: current state. Pharmacological Reviews 54, 271–284.

Green, L.C., Tannenbaum, S.R., Goldman, P., 1981. Nitrate synthesis in the germfree and conventional rat. Science 212, 56–58.

Guo, S., Bezard, E., Zhao, B., 2005. Protective effect of green tea polyphenols on the SH-SY5Y cells against 6-OHDA induced apoptosis through ROS-NO pathway. Free Radical Biology and Medicine 39, 682–695.

Hassimotto, N.M., Genovese, M.I., Lajolo, F.M., 2005. Antioxidant activity of dietary fruits, vegetables, and commercial frozen fruit pulps. Journal of Agriculture and Food Chemistry 53, 2928–2935.

Hincha, D.K., Hagemann, M., 2004. Stabilization of model membranes during drying by compatible solutes involved in the stress tolerance of plants and microorganisms. Biochemistry Journal 383, 277–283.

Im, H.-I., Joo, W.S., Nam, E., Lee, E., Hwang, Y., Kim, Y.S., 2005. Baicalein prevents 6-hydroxydopamine-induced dopaminergic dysfunction and lipid peroxidation in mice. Journal of Pharmacological Sciences 98, 185–189.

Jakel, R.J., Kern, J.T., Johnson, D.A., Johnson, J.A., 2005. Induction of the protective antioxidant response element pathway by 6-hydroxydopamine in vivo and in vitro. Toxicological Science 87, 176–186.

Jenner, P., 1991. Oxidative stress as a cause of Parkinson's disease. Acta Neurologica Scandinavica 136, 6–15.

Kanaan, N.M., Collier, T.J., Marchionini, D.M., McGuire, S.O., Fleming, M.F., Sertwell, C.E., 2006. Exogenous erythropoietin provides neuroprotection of grafted dopamine neurons in a rodent model of Parkinson's disease. Brain Research 1068, 221–229.

Kim, M.S., Lee, H., Lee, W.Y., Kim, S.E., 2004a. Neuroprotective effect of *Ginkgo biloba* L. extract in a rat model of Parkinson's disease. Phytotherapy Research 18, 663–666.

Kim, A.R., Zou, Y.N., Park, T.H., Shim, K.H., Kim, M.S., Kim, N.D., Kim, J.D., Bae, S.J., Choi, J.S., Chung, H.Y., 2004b. Active components from *Aremisia iwayomogi* displaying $ONOO^-$ scavenging activity. Phytotherapy Research 18, 1–7.

Kolosova, N.G., Scheglova, T.V., Sergeeva, S.V., Loskutova, L.V., 2005. Long-term antioxidant supplementation attenuates oxidative stress markers and cognitive deficits in senescent-accelerated OXYS rats. Neurobiology of Ageing, in press, doi:10.1016/j.Neurobiolaging.2005.07.022.

Kumar, R., Agarwal, R.K., Seth, P.K., 1995. Free radical-generated neurotoxicity of 6-hydroxydopamine. Journal of Neurochemistry 64, 1703–1707.

Lapchak, P.A., Araujo, D.M., 2002. Spin trap agents: a new approach to stroke therapy. Drug News Perspectives 15, 220–225.

Leal, L.K.A.M., Nóbre Júnior, H.V., Cunha, G.M.A., Moraes, M.O., Pessoa, C., Oliveira, R.A., Silveira, E.R., Canuto, K.M., Viana, G.S.B., 2005. Amburoside A, a glucoside from *Amburana cearensis*, protects mesencephalic cells against 6-hydroxydopamine-induced neurotoxicity. Neuroscience Letters 388, 86–90.

Lee, H.J., Noh, Y.H., Lee do, Y., Kim, Y.S., Kim, K.Y., Chung, Y.H., Lee, W.B., Kim, S.S., 2005. Baicalein attenuates 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y cells. European Journal of Cell Biology 84, 897–905.

Machado, L.L., Souza, J.S.N., Fonseca, A.M., Maia, J.L., Pessoa, D.L.P., Lemos, T.L.G., 2006. Antioxidant, icthyotoxicity and brine shrimp lethality tests of *Magonia glabrata* (Sapindaceae). Fitoterapia, in press.

Mazzio, E.A., Reams, R.R., Soliman, K.F.A., 2004. The role of oxidative stress, impaired glycolysis and mitochondrial respiratory redox failure in the cytotoxic effects of 6-hydroxydopamine *in vitro*. Brain Research 1004, 29–44.

McCance, R.A., Lawrence, R.D., 1933. CXXV111. An investigation of quebrachitol as a sweetening agent for diabetics. Biochemical Journal 27, 986–989.

Mercer, L.D., Kelly, B.L., Horne, M.K., Beart, P.M., 2005. Dietary polyphenols protect dopamine neurons from oxidative insults and apoptosis: investigations in primary rat mesencephalic cultures. Biochemical Pharmacology 69, 399–445.

Michel, P.P., Hefti, F., 1990. Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture. Journal of Neuroscience Research 26, 428–435.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunology Methods 65, 55–63.

Nobre Junior, H.V., Cunha, G.M., Maia, F.D., Oliveira, R.A., Moraes, M.O., Rao, V.S., 2003. Catechin attenuates 6-hydroxydopamine (6-OHDA)-induced cell death in primary cultures of mesencephalic cells. Comparative Biochemistry and Physiology C Toxicology and Pharmacology 136, 175–180.

Ogawa, N., Asanuma, M., Miyoshi, K., 2004. Mechanism of specific dopaminergic neuronal death in Parkinson's disease. Nippon Rinsho 62, 629–634.

Orthen, B., Popp, M., 2000. Cyclitols as cryoprotectants for spinach and chickpea thylakoids. Environmental Experimental Toxicology 44, 125–132.

Sen, C.K., 1998. Redox signaling and the emerging therapeutic potential of thiol antioxidants. Biochemical Pharmacology 55, 1747–1758.

Wang, Q., Sun, A.Y., Simonyi, A., Jensen, M.D., Shelat, P.B., Rottinghaus, G.E., Macdonald, R.S., Miller, D.K., Lubahn, D.E., Weisman, G.A., Sun, G.Y., 2005. Neuroprotective mechanisms of curcumin against cerebral ischemia-induced neuronal apoptosis and behavioral deficits. Journal of Neuroscience Research 82, 138–148.

Youdim, K., Joseph, J., 2001. A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: a multiplicity of effects. Free Radical Biology and Medicine 30, 583–594.