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Thymoquinone Protects Dopaminergic Neurons against MPP+ and Rotenone

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Thymoquinone is the main active constituent of *Nigella sativa* seeds with antioxidant and antiinflammatory properties. In the present study, primary dopaminergic cultures from mouse mesencephala were used to investigate the neuroprotective effects of thymoquinone against MPP+ and rotenone toxicities. MPP+ (10 μm on day 10 *in vitro* (i.v.) for 48 h) significantly decreased the number of THir by 40% compared with untreated control cultures. Rotenone at both short (20 nM on day 10 i.v. for 48 h) and long-term (1 nM on day 6 i.v. for 6 consecutive days) toxicities reduced the number of THir neurons by 33% and 24%, respectively. Treatment of cultures with thymoquinone (0.01, 0.1, 1, 10 μM on day 8 i.v. for 4 days) rescued about 25% of THir neurons at concentrations of 0.1 μM and 1 μM against MPP+-induced cell death. Against rotenone, thymoquinone afforded significant protection in both short- and long-term models. In short-term rotenone toxicity, thymoquinone (from days 8–12 i.v.) saved about 65%, 74% and 79% of THir neurons at concentrations of 0.01, 0.1 and 1 μM, respectively, compared with cell loss induced by rotenone. In long-term rotenone toxicity, concomitant treatment of cultures with thymoquinone significantly rescued about 83–100% of THir neurons compared with rotenone-treated cultures. In conclusion, the current study presents for the first time the potential of thymoquinone to protect primary dopaminergic neurons against MPP+ and rotenone relevant to Parkinson's disease.

Keywords: thymoquinone; MPP+; rotenone; dopaminergic; Parkinson's disease.

INTRODUCTION

Parkinson's disease (PD) is a slowly progressive neurodegenerative disorder characterized clinically by bradykinesia, resting tremor, rigidity and disturbance in posture and gait, and pathologically by a loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of Lewy bodies in degenerated neurons (Paulus and Jellinger, 1991). Symptomatic treatment of PD relies on neurotransmitter replacement therapy by L-DOPA or dopamine agonists which compensate for the loss of dopamine in the striatum as the result of dopaminergic cell death (Olanow *et al*., 2004). At present, there are no proven neuroprotective drugs against the demise of dopaminergic neurons in PD (Litvan *et al*., 2007; Sakai *et al*., 2007).

Phytodrugs are complex mixtures of many compounds extracted from a single or various plants. They have been used largely in the past decade as a substantial component of complementary and alternative medicine (Calapai and Caputi, 2007). According to their anti-inflammatory and antioxidative properties, various herbs are used to treat inflammatory disorders as well as those caused by reactive oxygen species (Kaplan *et al*., 2007). For example, Lin *et al*. (2007) reported that ginsenoside Rd protected dopaminergic neurons against lipopolysaccharides in primary mesencephalic culture through antiinflammatory processes. Chen *et al*. (2003) observed that ginsenoside Rg1 rescued PC12 cells against dopamine-induced apoptotic cell death through suppression of intracellular oxidative stress.

Thymoquinone (TQ) (2-isopropyl-5-methyl-1,2-benzoquinone) is a pharmacologically active quinone that constitutes 18–24% of the volatile oil of *Nigella sativa* seed (El-Tahir *et al*., 1993; Al-Majed *et al*., 2006). Thymoquinone possesses several properties including antioxidant (Houghton *et al*., 1993) and antiinflammatory actions (El-Gazzar *et al*., 2007). For instance, Kanter *et al*. (2005) found that the antioxidant effect of TQ is attributed to a decrease of lipid peroxidation and an increase of antioxidant activity in carbon tetrachloride-treated rats. Houghton *et al*. (1993) returned the antiinflammatory effects of TQ to the inhibition of eicosanoid generation as thromboxane B2 and leukotriene B4. Moreover, TQ has also chemopreventive, anticarcinogenic and antimutagenic activity (Nair *et al*., 1991; El Daly, 1998).

Searching for new compounds that slow down or stop the progression of PD is an important target of research in CNS drug development. Accordingly, this study is designed to investigate the potential of TQ to protect against MPP+- and rotenone-induced cell death in primary dopaminergic cultures.

MATERIALS AND METHODS

Preparation of primary dopaminergic cell culture. Pregnant mice (OF1/SPF, Himberg, Austria) were cared for and handled in accordance with the guidelines of the

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European Union Council (86/609/EU) for the use of laboratory animals. At gestation day 14, uterine horns were dissected after abdominal laparotomy and transferred to a petri dish containing sterile Dulbecco's phosphate buffered saline (DPBS, Invitrogen, Germany). Embryos were removed carefully under aseptic conditions and collected in DPBS at room temperature. Under a stereoscope (Nikon SMZ-1B, 100× magnification), the brains were dissected, ventral mesencephala excised and primary cultures were prepared according to Radad *et al*. (2008). Briefly, after careful removal of the meninges, tissues were mechanically cut into small pieces in DPBS and transferred into a sterile test tube containing 2 mL of 0.1% trypsin (Invitrogen, Germany) and 2 mL 0.02% DNase I (Roche, Germany) in DPBS. The tube was incubated in a water bath at 37 °C for 7 min. Then, 2 mL of trypsin inhibitor (0.125 mg/mL in DPBS) (Invitrogen, Germany) was added, the tissue was centrifuged (Hettich, ROTIXA/AP) at 100 × **g** for 4 min and the supernatant was aspirated. The tissue pellet was triturated with a fire-polished Pasteur pipette in Dulbecco's modified Eagle's medium (DMEM, Sigma, Germany) containing 0.02% DNase I. Dissociated cells were collected in DMEM supplemented with HEPES buffer (25 mM), glucose (30 mM), glutamine (2 mM), penicillin–streptomycin (10 U/mL and 0.1 mg/mL, respectively) and heat inactivated fetal calf serum (FCS, 10%) (all from Sigma, Germany). The cell suspension was plated into 4-well multidishes (Nunclon, Germany) precoated with poly-D-lysine (50 μg/mL) (Sigma, Germany). Cultures were grown at 37 °C in an atmosphere of 5% CO_2/95% air and 100% relative humidity. The medium was exchanged on day 1 *in vitro* (i.v.) and on day 3 i.v. On day 5 i.v. half of the medium was replaced with serum-free DMEM containing 0.02 mL B-27/mL (Invitrogen, Germany). Serum-free supplemented DMEM was used for feeding from day 6 i.v. and subsequently replaced every 2 days.

Treatment of cultures with TQ. A stock solution of TQ (10 mM) was prepared by dissolving 1.642 mg in 1 mL of dimethyl sulphoxide (DMSO). Final concentrations of TQ were prepared in DMEM. The DMSO concentration in the culture medium did not exceed 0.01%. To investigate the effect of TQ on the survival rate of dopaminergic neurons, cultures were treated only with TQ (0.01, 0.1, 1 and 10 μM) on day 6 i.v. for 6 consecutive days. During the treatment period, the culture medium was changed every 2 days with the same concentrations of freshly prepared TQ.

Treatment of cultures with TQ and MPP^+. To investigate the neuroprotective potential of TQ against MPP^+ toxicity, TQ-treated cultures from days 8–12 i.v. were additionally exposed to 10 μM of MPP^+ on day 10 i.v. for 48 h.

Treatment of cultures with TQ and rotenone. In this treatment protocol, the neuroprotective potential of TQ against both short- and long-term rotenone exposures was investigated. Against short-term rotenone exposure, cultures were treated with TQ on day 8 i.v. for 4 days and then 20 nM of rotenone, dissolved in DMSO, was added to the cultures on day 10 i.v. for 48 h. For long-term rotenone exposure, both TQ and rotenone (1 nM) were concomitantly added from days 6–12 i.v.

Identification of THir neurons. On day 12 i.v. the cultures were rinsed carefully with PBS (pH 7.2) and fixed in 4% paraformaldehyde for 45 min at 4 °C. Fixed cells were permeabilized with 0.4% Triton X-100 for 30 min at room temperature. Cultures were washed three times with PBS and incubated with 5% horse serum (Vectastain ABC Kit) for 90 min to block non-specific binding sites. Cells were sequentially incubated with anti-TH antibody overnight at 4 °C, biotinylated secondary antibody (Vectastain) and avidin–biotin–horseradish peroxidase complex (Vectastain) for 90 min at room temperature and washed with PBS between incubations. The reaction product was developed in a solution of diaminobenzidine (1.4 mM) in PBS containing 3.3 mM hydrogen peroxide (H_2O_2). THir neurons were counted in ten randomly selected fields at 100× magnification with a Nikon inverted microscope. On day 12 i.v., the average number of THir cells were between 21–75 cells/field in the various control experiments, 12–26 cells/field when treated with MPP^+ for 48 h, 12–28 cells/field when treated with 20 nM rotenone for 48 h and 15–49 cells/field when treated with 1 nM rotenone for 6 consecutive days.

Statistics. THir neurons were counted in 12 wells (from three repeats) for each treatment condition. Data were expressed as mean ± standard error of mean (SEM). Comparisons were made using ANOVA and post-hoc Duncan's test using statistical program SAS 1998. $p < 0.05$ was considered as statistically significant.

RESULTS

Effect of TQ on the survival of dopaminergic neurons

Treatment of cultures with TQ (0.01, 0.1, 1 and 10 μM) on day 6 i.v. for 6 consecutive days produced no significant effects on either the survival rate or the morphology of THir neurons (data not shown).

Effect of TQ on MPP^+-treated dopaminergic neurons

Treatment of primary dopaminergic cultures with MPP^+ (10 μM on day 10 i.v. for 48 h) decreased THir neurons by about 40% compared with untreated cultures (Fig. 1A and B). Moreover, it resulted in mis-shaped neuronal perikarya and shortened and fewer neurites of THir neurons (Fig. 1B). Thymoquinone rescued about 25% of THir neurons at concentrations of 0.1 μM and 1 μM against MPP^+-induced cell death (Fig. 1A).

Effect of TQ on rotenone-treated dopaminergic neurons

Short-term exposure of primary dopaminergic cultures to rotenone (20 nM on day 10 i.v. for 48 h) resulted in a loss of THir neurons by 33% (Fig. 2A) and decreased the number and length of the processes of the surviving neurons (Fig. 2B). Treatment with TQ (0.01, 0.1, 1 and 10 μM on day 8 i.v. for 4 days) significantly protected THir by 65%, 74% and 79% at concentrations of 0.01,

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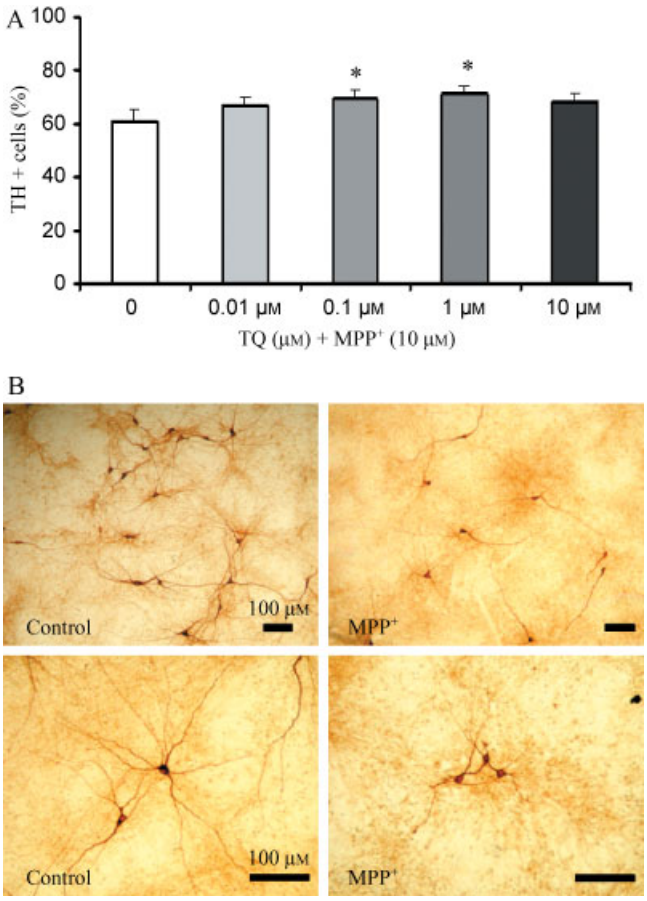

Figure 1. (A) Effect of TQ on the survival of MPP^+-treated THir neurons. 100% corresponds to the total number of THir neurons after day 12 i.v. in untreated controls. Values represent the mean ± SEM of three independent experiments with four wells in each treatment. In each well ten randomly selected fields were counted for TH immunocyochemistry ($p = 0.0001$). (B) Representative micrographs of THir neurons after day 12 i.v. Untreated control cultures had many THir neurons with long and branched processes and in contrast, MPP^+-treated cultures showed fewer THir neurons and lost and shortened neurites.

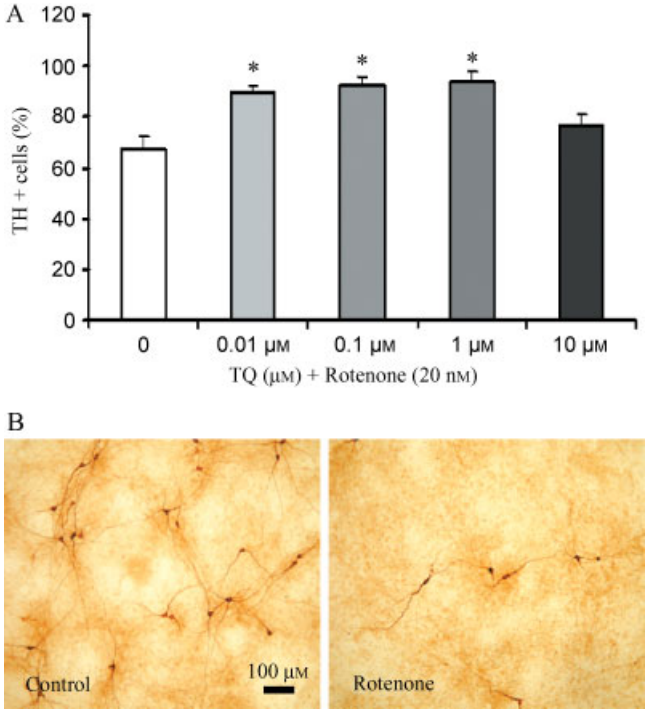

Figure 2. Effect of TQ on the survival of THir neurons against short-term rotenone toxicity. 100% corresponds to the total number of THir neurons after day 12 i.v. in untreated controls. Values represent the mean ± SEM of three independent experiments with four wells in each treatment. In each well ten randomly selected fields were counted for TH immunocyochemistry ($p = 0.0001$). (B) Representative micrographs of THir neurons after day 12 i.v. THir neurons appeared many in number and with intact processes in untreated cultures. Treatment with rotenone (20 nM on day 10 i.v. for 48 h) resulted in loss of many THir neurons.

0.1 and 1 μM, respectively, compared with the cell loss induced by rotenone (Fig. 2A).

Long-term rotenone toxicity (1 nM on days 6–12 i.v.) only decreased the number of THir by 24% compared with untreated cultures (Fig. 3A and B). Concomitant treatment of cultures with thymoquinone significantly rescued about 83–100% of THir neurons compared with rotenone-treated cultures (Fig. 3A).

DISCUSSION

The use of herbs for the overall maintenance of health and protection from diseases has a very long history and today many pharmaceutical companies work to develop their active ingredients as alternatives for prescription medications. For instance, ginseng, the famous Chinese herb, has served as a general tonic and adaptogen to help the body to resist the adverse influences of a wide range of physical, chemical and biological factors and to restore homeostasis for thousands of years (Nocerino *et al.*, 2000; Brekman and Dardymov, 1969). Herbs are also recently used in both *in vitro* and *in vivo* studies to halt or slow down neuronal death relevant to neurodegenerative diseases. In this context, it was reported previously that ginsenoside Rb1 rescued dopaminergic neurons against age-induced cell death and increased lengths and numbers of their neurites following MPP^+ treatment in primary mesencephalic cell culture (Radad *et al.*, 2004). Other than that, Hou *et al.* (2008) found that green tea extracts attenuated the apoptosis of PC12 cells induced by paraquat and Ishikawa *et al.* (2000) showed the effectiveness of kampo medication which consists of ten Chinese medicinal herbs against the tremors of antipsychotic-induced parkinsonism.

Nigella sativa oil and its active constituent, thymoquinone, were reported to have a variety of actions against many symptoms. For example, Daba and Abdel-Rahman (1998) and Mansour *et al.* (2001) found that TQ prevented oxidative injury in hepatocytes induced by carbon tetrachloride or tert-butyl hydroperoxide in different *in vitro* and *in vivo* hepatotoxicity models, respectively. Tekeoglu *et al.* (2007) found that TQ suppressed adjuvant-induced arthritis in rats. However, there is a little evidence in the literature concerning the effects of *Nigella sativa* oil and TQ on neuronal cells. Therefore, this study was designed to investigate the effects of TQ against dopaminergic insults in primary mesencephalic cell culture relevant to PD. It

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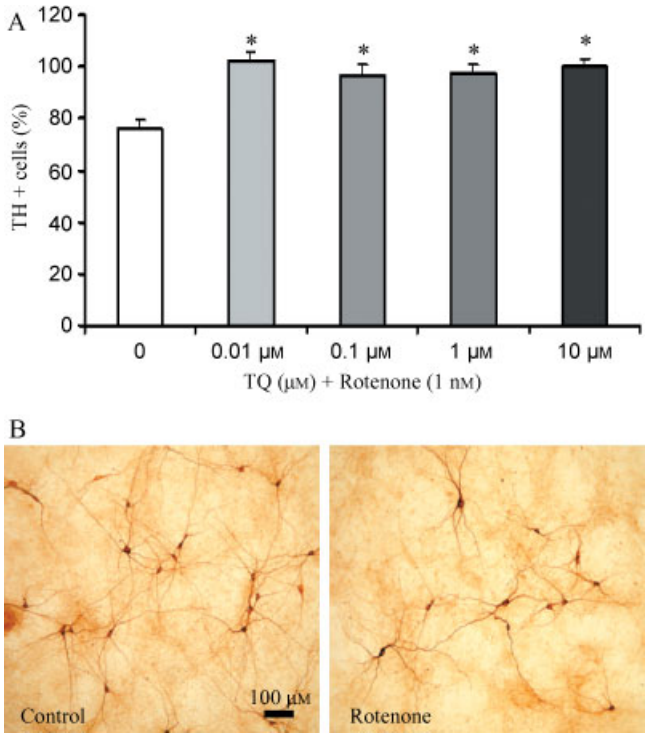

Figure 3. Effect of TQ on the survival of THir neurons against long-term rotenone toxicity. 100% corresponds to the total number of THir neurons after day 12 i.v. in untreated controls. Values represent the mean ± SEM of three independent experiments with four wells in each treatment. In each well ten randomly selected fields were counted for TH immunocyochemistry ($p = 0.0001$). (B) Representative micrographs of THir neurons after day i.v. Treatment with 1 nM rotenone on day 6 i.v. for 6 consecutive days decreased the number of THir compared with control cultures. However, the cell morphology was not severely deteriorated.

was found that TQ, at concentrations of 0.1 and 1 µM, significantly preserved the total number of THir neurons compared with MPP^+-treated cultures (Fig. 1A). Moreover, TQ significantly rescued THir neurons against both short- and long-term rotenone toxicity (Figs 2A and 3A). Similar neuroprotection by TQ was described in only a few investigations using other neurodegeneration models. For instance, Martin *et al.* (2006) found that TQ protected SH-SYSY cells against L-DOPA toxicity. Al-Majed *et al.* (2006) reported that TQ significantly reduced neuronal cell death in the hippocampal CA1 region from ischemia-induced brain injury. Also, Kanter (2008) reported significant protection of hippocampal neurons by TQ against toluene toxicity in rats. The antioxidant properties of TQ appeared to play an important role in rescuing THir neurons from either MPP^+ or rotenone-induced cell death in primary mesencephalic cultures. This might be due to the fact that oxidative stress mediates the neurotoxic effects of MPP^+ or rotenone to a large extent (Amazzal *et al.*, 2007; Nehru *et al.*, 2008). This is consistent with the report that TQ protected hippocampal CA1 neurons from ischemia-induced injury through an antioxidative process. Thymoquinone decreased the elevated levels of malondialdehyde and reversed the decreased levels of GSH, catalase and SOD activities to normal levels (Al-Majed *et al.*, 2006). Also, Hosseinzadeh *et al.* (2007) found that TQ provided an overall protection against lipid peroxidation during cerebral ischemia reperfusion injury. Neuroinflammatory processes were also reported to underlie dopaminergic cell death in MPP^+ (Miwa *et al.*, 2004) and rotenone (Zhou *et al.*, 2007) models as well as in parkinsonian patients (Mosley *et al.*, 2006). The well-known antiinflammatory action of TQ reported by El-Gazzar *et al.* (2007) may therefore be responsible, to some extent, for the protection of THir neurons against MPP^+ or rotenone in the present study.

In conclusion, the study presented for the first time the neuroprotective potential of TQ against MPP^+ and rotenone-induced cell death in primary dopaminergic cell cultures relevant to PD. Thymoquinone was found to protect significantly the total number of THir neurons from either MPP^+ or rotenone exposure.

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