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The antioxidant drink ''effective microorganism-X (EM-X)'' pre-treatment attenuates the loss of nigrostriatal dopaminergic neurons in 6-hydroxydopamine-lesion rat model of Parkinson's disease

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Abstract

There is continued interest in the assessment and potential use of antioxidants as neuroprotective agents in diseases associated with increased oxidative stress, such as Parkinson's disease. The neuroprotective effect of a natural antioxidant drink, EM-X (a ferment derivative of unpolished rice, papaya and seaweeds with effective microorganisms), was investigated using the 6-hydroxydopamine (6-OHDA)-lesion rat model of Parkinson's disease. The nigrostriatal dopaminergic neurons were unilaterally lesioned with 6-OHDA (8 μg) in rats that were treated with a 10-times diluted EM-X drink (dilEM-X), standard EM-X drink (stdEM-X) or tap water for 4 days. Seven days post lesion, the integrity (no. of tyrosine hydroxylase positive cells (TH+ cells) in the substantia nigra pars compacta (SNpc)) and functionality (dopamine and its metabolites DOPAC and HVA content in the striata) of nigrostriatal dopaminergic neurons were assessed. In the vehicle-treated rats, infusion of 8 μg of 6-OHDA significantly reduced the number of TH+ cells in the SNpc as well as the levels of dopamine, DOPAC and HVA in the striata on the lesion side. The loss of TH+ cells, dopamine and HVA, but not the DOPAC levels, was significantly attenuated by stdEM-X pretreatment, but not by the dilEM-X pretreatment. There were no significant changes in the TH+ cells, or in the monoamine levels with the EM-X pretreatment per se, except for a small but significant fall in the levels of dopamine with the stdEM-X. The evidence presented supports the potential neuroprotective effects of stdEM-X drink, although its effect on dopamine levels needs further investigation.

Introduction

Parkinson's disease is the second most common neurodegenerative disorder affecting predominantly the aging population. It is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, resulting in the reduction of dopamine content in the striata (Calne & Langston 1983). Although the precise mechanisms of neuronal loss are not fully characterized, post-mortem studies on the brains of Parkinson's disease patients suggest an active involvement of oxidative stress mechanisms (Jenner et al 1992; Finkel & Holbrook 2000), as indicated by increased iron deposition (Dexter et al 1987), lipid peroxidation (Dexter et al 1989), impaired mitochondrial complex-I activity (Orth & Schapira 2002), decreased cellular antioxidant reduced glutathione (GSH) levels (Sian et al 1994), increased glutathione transferase activity (Smythies et al 2002) and increased proliferation of reactive microglia resulting in increased cytokines (Mogi et al 1994). This has led to the suggestion that agents that reduce oxidative stress, such as antioxidants, might reduce neuronal loss in Parkinson's disease (Aruoma 1996; Drukarch & van Muiswinkel 2000). Indeed, treatment of newly diagnosed Parkinson's disease patients with high-dose vitamin E and C has been shown to delay the requirement to start L-DOPA therapy by up to 2.5 years (Fahn 1992), while vitamin E alone in another study had no effect on disease progression (Shoulson 1998),

suggesting a finite window for the efficacy of dietary antioxidants to be established by use of in-vivo models.

Polyphenolic compounds, including flavonoids, are widely distributed in fruits, vegetables, nuts, seeds, grain, tea and wine and have been shown to have antioxidant, anti-inflammatory and anti-apoptotic properties both in-vitro and in-vivo (Bravo 1998; Di Carlo et al 1999; Pietta 2000; Luximon-Ramma et al 2002). These polyphenolics seem ideal candidates for reducing oxidative stress as they have been shown to possess free-radical scavenging and metal-ion chelating properties (van Acker et al 1996, 1998; Pietta 2000). The cocktail drink, EM-X, has been shown to be rich in minerals, lycopenes, ubiquinone and various saponins, as well as antioxidant polyphenolics compounds, such as quercetin, kaempferol, ascorbic acid and alpha-tocopherol (Higa & Ke 2001). EM-X drink is derived from the ferment of unpolished rice, papaya and seaweeds with effective microorganisms (selected from actobacillaceae, saccharomycetes, funguses, actinomyces and photosynthetic bacteria (Higa & Ke 2001)), and is widely available in South East Asia where it has been accepted as a clinical supplement in the treatment of cancer, hypertension, various allergies, diabetes and tuberculosis (Higa & Ke 2001).

Infusion of the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) selectively destroys dopaminergic neurons in rats by increasing oxidative stress, and is an established and widely used animal model of Parkinson's disease (Jeon et al 1995). Datla et al (2001b) showed that pretreatment of rats with the citrus flavonoid tangeretin was neuroprotective in this model. Similarly, treatment with tea-extract, which is enriched with antioxidants, reduced the 6-OHDA toxicity in rat pheochromocytoma (PC12) and human neuroblastoma (NB) SH-SY5Y cells in-vitro (Levites et al 2002). As antioxidants with a similar structure to tangeretin, and those found in tea extracts, were also known to be present in stdEM-X drink (Figure 1), our aim was to assess the neuroprotective effects of EM-X drink using the 6-OHDA lesion rat model of Parkinson's disease, following the same 4-day pretreatment schedule as in our tangeretin studies. The neuroprotective effects were established by assessing both the integrity and functionality of nigrostriatal dopaminergic neuronal pathways by quantifying the number of tyrosine hydroxylase (rate-limiting enzyme for the synthesis of dopamine) positive cells (TH+ cells) in the substantia nigra pars compacta (SNpc) and assaying dopamine and its metabolites DOPAC and HVA in the striata by HPLC with electrochemical detection.

Materials and Methods

Materials

EM-X drink was supplied by EM Research Organization Inc. (Okinawa, Japan). 6-Hydroxydopamine and all the chemicals used were purchased from Sigma-Aldrich (Dorset, UK). Polyclonal TH antibodies were purchased from Chemicon (Harrow, UK), while ABC immunostaining kits were purchased from Vector Laboratories (UK).

Drug treatment

Male Sprague-Dawley rats (Charles River, UK), 250 ± 25 g (starting weight), were housed in groups of 3 with free access to food, under controlled temperature ($21 \pm 1\,^{\circ}C$) and a 12-h light–dark cycle (light on 0700 h). Groups of rats ($n = 6$ per group) received either dilEM-X drink (10-times diluted standard EM-X drink), stdEM-X (standard EM-X drink) or tap water for 4 days. The EM-X drink was the only source of fluid for the EM-X-treated group. The consumption of fluids was monitored daily in all groups and the amount of fluid consumed was comparable

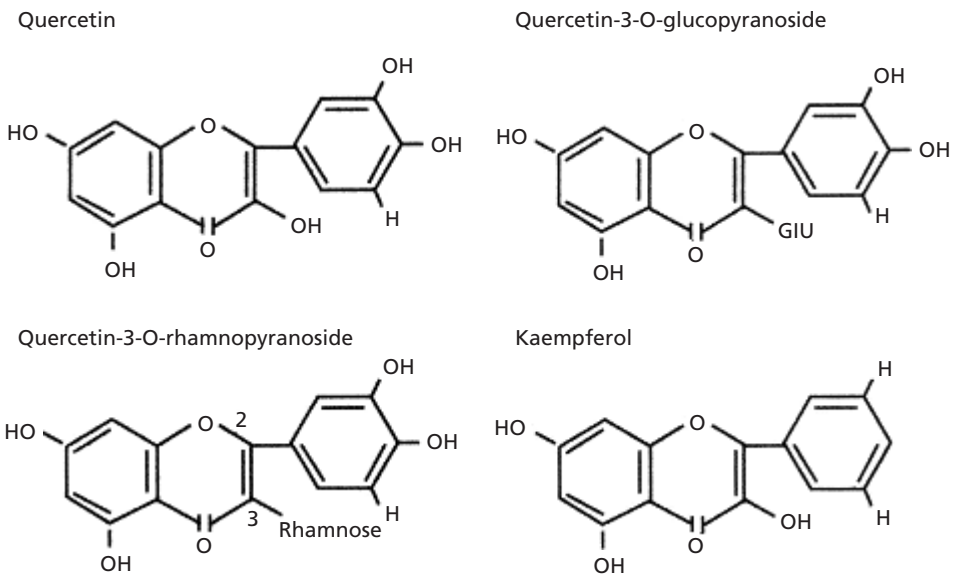

Figure 1 Phenolic constituents of stdEM-X drink.

across the groups (see Results). After lesioning, all rats were given tap water only for the remaining 7 days of the study. All scientific procedures were carried out with the approval of the Home Office, UK.

Surgery

On the morning of the fourth day of treatment, rats were anaesthetized with etorphine–methotrimezapine (small animal Immobilon; 0.04 mL/rat, i.m.; G-Vet, UK) and 6-OHDA (8 μg dissolved in 4 μL of 0.1% ascorbic acid–saline solution) was injected onto the medial forebrain bundle (stereotactic co-ordinates: −2.2 mm AP, +1.5 mm ML from bregma and −7.9 mm DV from dura with the ear bars 5 mm below the incisor bars), as described (Datla et al 2001a). One week post 6-OHDA lesioning, rats were killed by cervical dislocation and the brains immediately removed. A coronal section was made at the level of the hypothalamus and forebrain and hindbrain parts were separated.

TH immunostaining

The hindbrain containing the SNpc was fixed for 7 days in 4% paraformaldehyde, then cryoprotected with 30% sucrose solution for 2–3 days and used for the tyrosine hydroxylase (TH) immunostaining as described (Datla et al 2001a). Briefly, 20-μm fixed coronal free-floating sections from the hindbrain containing the SNpc were incubated with polyclonal rabbit anti-TH (1:3000; Chemicon, Harrow, UK) followed by biotinylated anti-rabbit IgG and avidin–biotin complex (Vector Laboratories, UK). The TH immunocomplex was then visualized with diaminobenzidine and H_2O_2. Images of TH positive cells (TH+ cells) were captured by a Xillix CCD digital camera and counted automatically (Image Proplus; Datacell, Yateley, UK). The number of TH+ cells in the SNpc on the unlesioned side was compared with the lesioned side by averaging the number of cells at 5 different levels (Figure 2), as described (Datla et al 2001a).

Dopamine and metabolite estimations by HPLC-electrochemical detection

From the forebrain, the lesioned and unlesioned striata were dissected out, snap frozen and stored at −80 °C until analysis. Striatal dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were estimated by HPLC-electrochemical detection as described elsewhere (Datla et al 2001a).

Data analysis

The statistical software package SPSS was used to assess any significant effects of the 6-OHDA lesion, dilEM-X and stdEM-X treatments by analysis of variance with 6-OHDA lesion as within-subject factor and dilEM-X and stdEM-X treatments as between-subject factors. Where significant effects were found, post-hoc comparisons within group (paired) or between groups (unpaired) were made using Student's *t*-test accordingly. A *P* value of less than 0.05 was taken as being significant.

Results

Each rat, on average, drank 38 mL of EM-X drinks or tap water per day. Neither the food/fluid intake nor weight

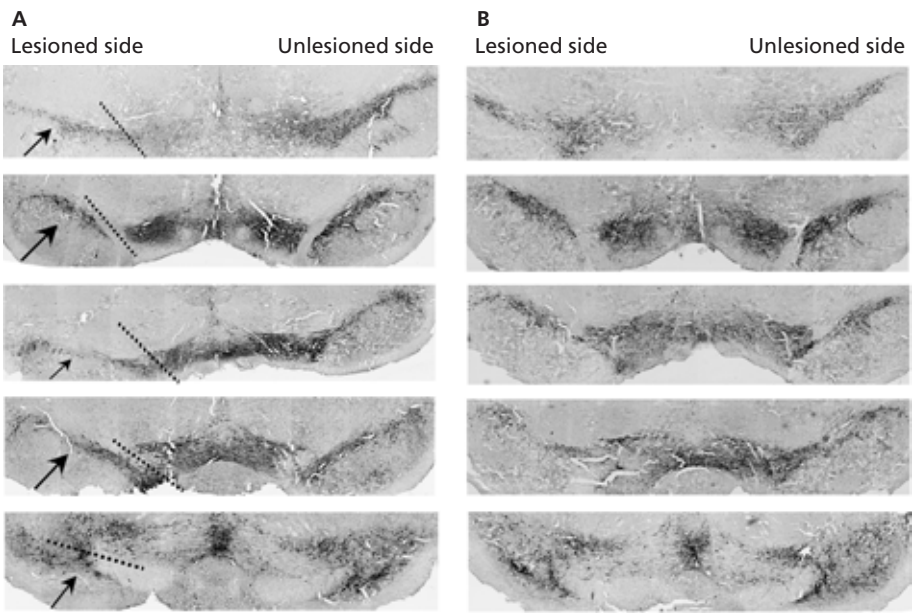

Figure 2 Tyrosine hydroxylase positive (TH+) cells in different levels of the rat substantia nigra pars compacta after immunostaining (arrow head). The coronal sections in each column were taken at −4.8, −5.1, −5.4, −5.7, −6.0 mm from the bregma according to Paxinos & Watson (1986). The dotted line indicates the area of cells counting. A. Vehicle treatment. B. stdEM-X treatment.

gain in the EM-X-treated rats was significantly different from the control rats. This is consistent with the result of a 7-day supplementation study that reported minimal changes in weights of experimental and normal rats during the study period (Aruoma et al 2002).

TH+ cells in the SNpc

The number of TH+ cells on the unlesioned side of the brains of vehicle- and both EM-X- treated groups was comparable (Figure 3). 6-OHDA lesioning significantly reduced the number of TH+ cells (analysis of variance; $F_{(2,14)} = 58.169$; $P < 0.001$) on the lesioned side of the brain in the vehicle-treated rats. EM-X treatments had no effect on the TH+ cells on the unlesioned side but stdEM-X treatment significantly prevented the loss of TH+ cells due to 6-OHDA administration (analysis of variance; $F_{(2,14)} = 14.008$; $P < 0.001$). Post-hoc comparison showed that the number of TH+ cells on the lesioned side in the stdEM-X-treated group was significantly higher than the corresponding side of the vehicle (6-OHDA + tap water)-treated group ($P < 0.01$; unpaired Student's t-test; Figure 3), whereas dilEM-X had no effect on the extent of the 6-OHDA lesion (Figure 3).

Striatal dopamine, DOPAC and HVA levels

Similar to TH+ cells data, analysis of variance revealed that lesioning with 6-OHDA significantly reduced striatal levels of dopamine ($F_{(1,15)} = 59.767$; $P < 0.001$), DOPAC ($F_{(1,15)} = 39.619$; $P < 0.001$) and HVA ($F_{(1,15)} = 48.978$; $P < 0.001$; Figure 4). However, the loss of dopamine due to the 6-OHDA lesion was attenuated dose-dependently by the EM-X treatment ($F_{(2,15)} = 17.273$; $P < 0.001$), with 100% protection seen after stdEM-X and a smaller but significant protection from the dilEM-X (Figure 4A). Similarly, the levels of HVA were also restored to control levels with stdEM-X ($F_{(2,15)} = 7.467$; $P < 0.01$), although the dilEM-X failed to have any effect (Figure 4C). Unlike striatal dopamine and HVA levels, stdEM-X treatment produced only a small but significant restoration of DOPAC levels in the lesioned striata when compared with control levels (Figure 4B). The levels of dopamine in the unlesioned striata were slightly but significantly lower in the stdEM-X-treated group, but not the dilEM-X-treated group, when compared with the corresponding vehicle-treated group ($P < 0.05$, unpaired Student's t-test). Unlike dopamine, no significant changes were seen in the levels of DOPAC or HVA with EM-X treatments. Finally, there was no change in the dopamine turnover with the EM-X treatments (data not shown).

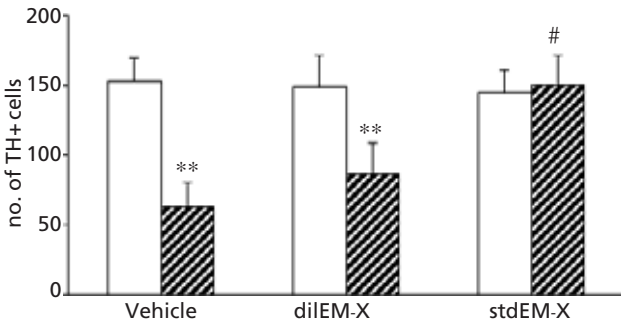

Figure 3 Effect of EM-X treatment on the number of tyrosine hydroxylase positive (TH+) cells in the rat substantia nigra pars compacta. Open and filled columns represent mean + s.e.m. of TH+ cells on the unlesioned and lesioned side nigra, respectively. $^{**}P < 0.01$ vs respective unlesioned side; $\#P < 0.01$ vs lesioned side of the vehicle-treated rats after Student's t-test following significant analysis of variance, n = 6 rats per group.

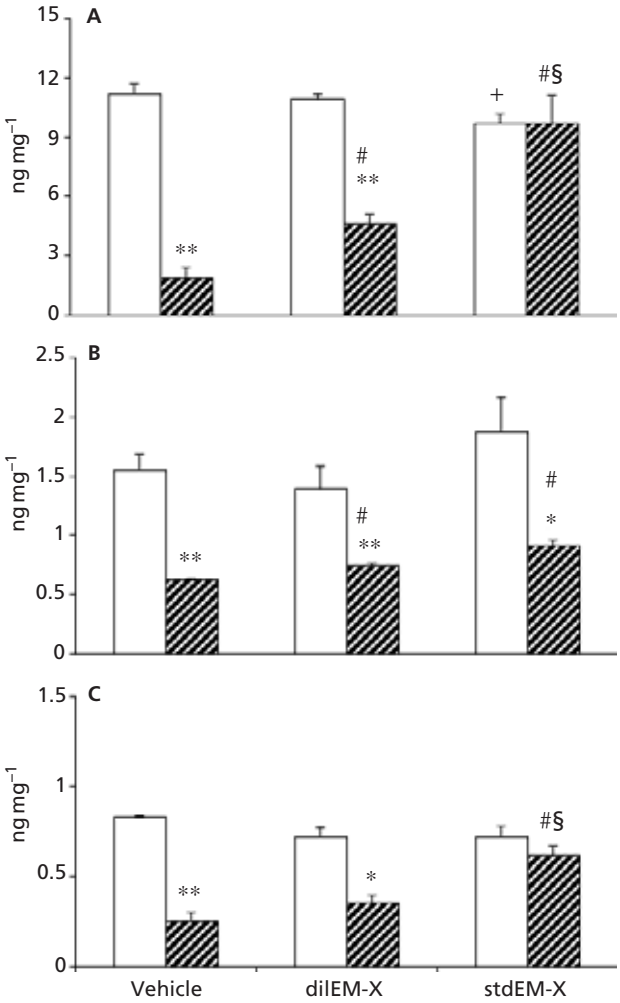

Figure 4 Effect of EM-X treatment on dopamine (A) DOPAC (B) and HVA levels (C) in the rat striata. Open and filled columns represent mean + s.e.m. (ng/mg wet striata) on the unlesioned and lesioned side, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ vs respective unlesioned side; $\#P < 0.01$ vs lesioned side of the vehicle-treated rats; $^{+}P < 0.05$ vs unlesioned side of the vehicle-treated group; $\S P < 0.001$ vs lesioned side of dilEM-X-treated group after Student's t-test following significant analysis of variance, n = 6 rats per group.

Discussion

stdEM-X pretreatment significantly prevented the loss of nigral dopaminergic neurons and the reduction in striatal dopamine content normally observed after the administration of 6-OHDA in this rat model of Parkinson's disease. dilEM-X failed to have any effect on numbers of surviving TH+ cells in but did produce a small but significant restoration of striatal dopamine levels.

In this animal model the infused 6-OHDA is taken up by the dopamine transporter and metabolized by dopaminergic neurons, generating highly damaging reactive hydroxyl radicals. Hydroxyl radicals are implicated in causing the oxidation of lipids, proteins and even DNA, thus altering the normal function of the neurons, leading to death (Olanow & Tatton 1999). Indeed, 6-OHDA, an auto-oxidation product of dopamine, is known to be present in trace amounts in the brains and in urine of Parkinson's patients (Curtius et al 1974; Andrew et al 1993).

From examination of the chemical make up of the EM-X drink it would appear most logical that the observed neuroprotection is due to the antioxidant properties of stdEM-X. Three lines of indirect evidence strengthen this hypothesis. Firstly, the cellular non-enzymatic antioxidants ascorbic acid, α-tocopherol and flavonoid antioxidants quercetin and kaempferol are present in the stdEM-X drink (Figure 1). Secondly, treatment with EM-X prevented the increased oxidation of brain liposomes (Deiana et al 2002) and lipids in the kidney and liver that occurred after infusion of ferric-nitrilotriacetic acid to rats (Aruoma et al 2002). And thirdly, EM-X treatment inhibits hydrogen peroxide and TNF-mediated release of IL-8 in epithelial A549 cells in-vitro (Deiana et al 2002). However, involvement of other antioxidant mechanisms for the observed neuroprotection cannot be ruled out at this stage, especially in the light of recent observations that stdEM-X treatment can also increase the cellular antioxidant enzyme superoxide dismutase (Ke et al 2001).

Based on evidence from in-vitro studies, it is reasonable to assume that the antioxidant flavonoids, quercetin and kaempferol, and non-flavonoids vitamin E, saponins and lycopenes present in EM-X drink may play a key role in the neuroprotection seen in this study. For example, quercetin treatment was shown to protect PC12 cells (Wang & Joseph 1999) and human lymphocytes (Duthie et al 1997) from H_2O_2 toxicity, an agent similar to 6-OHDA used in this study, which induces toxicity by increasing the generation of free radicals. This free radical scavenging property of quercetin was attributed to the catechol moiety present on the right ring of quercetin (see Figure 1), as kaempferol, an antioxidant flavonoid structurally similar to quercetin, but without a catechol moiety, was ineffective in protecting PC12 cells (Wang & Joseph 1999). Thus, the antioxidant property of kaempferol may not be dependent on its free radical scavenging ability, but may depend on its metal chelating properties, as recently kaempferol treatment was shown to reduce Cu-induced lipid-peroxidation in hepatocytes as effectively as quercetin treatment, but not iron-induced lipid-peroxidation (Sugihara et al 1999). Similarly, vitamin E treatment was shown to protect hepatocytes against oxidative stress (Zhang et al 2001) and rat striatal primary cell cultures from paraquat (Osakada et al 2003), but not cortical cells from H_2O_2 toxicity (Shea et al 2002). The protective effect of vitamin E treatment was suggested to be dependent on its free radical scavenging property. Finally, saponins were shown to reduce superoxide generation in a xanthine–xanthine oxidase chemical reaction (Sur et al 2001). A treatment rich in all these antioxidant components can be expected to offer neuroprotection. Thus, dopaminergic neuroprotection seen in the study could be dependent on all or some of the antioxidant components of EM-X drink by scavenging hydroxyl radicals or chelating the iron released by 6-OHDA infusion.

A very small decrease in striatal dopamine on the unlesioned side of the brain was observed with stdEM-X treatment. Although this was significant, it is not immediately explicable. However, given that the dopamine concentrations did not translate to increased DOPAC or HVA levels, and there was no effect on turnover rates, it may be possible that stdEM-X treatment had an effect on dopamine synthesis. Further studies examining the effects of long-term administration of EM-X are required before we can elucidate whether this drink may have any detrimental effects on dopamine levels. In this study, stdEM-X was only given for four days but recent toxicological tests in mice showed that oral treatment with concentrated EM-X (20 fold), which greatly exceeds the daily dose utilized in the clinic and this study, were not toxic (Zhong et al 1999; Higa & Ke 2001).

Conclusions

The loss of dopaminergic neurons in the substantia nigra and the dopamine content in the striata with 6-OHDA lesioning was attenuated dose dependently by 4-day treatment with EM-X drink. Thus EM-X drink might be a beneficial product for the treatment of Parkinson's disease. However, further studies are needed to evaluate the precise mechanisms of neuroprotection as it contains a variety of compounds including flavonoids, saponins, vitamins E and C and lycopenes.

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