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Bioactive constituents from cinnamon, hemp seed and *polygonum cuspidatum* protect against H_2O_2 but not rotenone toxicity in a cellular model of Parkinson's disease

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ABSTRACT

Mitochondrial dysfunction and oxidative stress are two factors that are thought to contribute to the pathogenesis of Parkinson's disease (PD), a debilitating progressive neurodegenerative disorder that results in the loss of catecholamine producing cells throughout specific regions of the brain. In this study we aimed to compare the effects of hydrogen peroxide (H_2O_2) and rotenone (a pesticide and mitochondrial complex 1 inhibitor) on cell viability and the expression of tyrosine hydroxylase (TH) in a cellular model of PD. We also sought to investigate the potential neuroprotective benefits of bioactive constituents from cinnamon, hemp seed and *polygonum cuspidatum*. To create a model, SH-SY5Y cells transfected with human TH isoform 1 were treated with varying concentrations of H_2O_2 and rotenone, in the presence or absence of bioactive constituents. The effect of these toxins and constituents on cell viability, apoptosis and protein expression was assessed using MTT viability assays and western blotting. Rotenone treatment caused a significant decrease in cell viability but a significant increase in TH in the remaining cells. H_2O_2 treatment caused a significant decrease in cell viability but had no significant effect on TH expression. Curcumin, cinnamaldehyde, caffeoyltyramide (hemp seed extract) and piceatannol glucoside (polygonum cuspidatum extract) were unable to attenuate rotenone induced cell death, however they were able to provide protection against H_2O_2 induced cell death. This is the first study to demonstrate the neuroprotective properties of cinnamaldehyde, caffeoyltyramide and piceatannol glucoside in a dopaminergic cell line in response to H_2O_2.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide, affecting 1–2% of the population over the age of 65.[1] The condition is characterised by the progressive loss of dopaminergic neurons from the substantia nigra pars compacta.[2]

There are a number of studies that link the development of PD with the exposure of certain pesticides such as rotenone.[3,4] As a result, rotenone is commonly used to create *in vivo* and *in vitro* models to study the disease.[5,6] H_2O_2 is a compound commonly used to model oxidative stress *in vitro* and *in vivo*.[7,8] As mitochondrial dysfunction and oxidative stress are thought to contribute to cell death in PD, we aimed to assess the effects of both rotenone and H_2O_2 on SH-SY5Y neuroblastoma cells.

The SH-SY5Y neuroblastoma cell line has been previously used to create a cellular model of PD.[9,10] The cells share many biochemical and functional characteristics with mature dopaminergic neurons and have the ability to differentiate into a

Abbreviations: DA, Dopamine; DMSO, Dimethyl sulfoxide; GSH, Glutathione; H_2O_2, Hydrogen peroxide; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetra hydropyridine; MTT, Methylthiazolyldiphenyl-tetrazolium bromide; PARP-1, Poly (ADP-ribose) polymerase-1; PBS, Phosphate buffered saline; PD, Parkinson's disease; ROS, Reactive oxygen species; SDS, Sodium dodecyl sulphate; TH, Tyrosine hydroxylase; tTH, Total TH.

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dopaminergic phenotype. As tyrosine hydroxylase (TH) and dopamine (DA) seem to be central to the pathogenesis of PD and dopaminergic neurons are specifically targeted in the condition, we opted to utilise a cell line that had been transfected with human TH isoform 1 (TH1).[11]

There were two main aims to be addressed in this study, firstly we aimed to compare the effects of rotenone and H_2O_2 treatment on cell viability and TH expression and once we had established these changes we would then assess the ability of a number of potentially neuroprotective compounds to protect against this toxicity.

Cinnamon is a spice commonly used in food throughout the world. The spice has been demonstrated to have anti-diabetic and anti-inflammatory effects[12,13] as well as some neuroprotective properties.[14] For instance a previous study demonstrated that treatment with cinnamon prevented the development of PD like symptoms and pathology in 1-methyl-4-phenyl-1,2,3,6-tetra hydropyridine (MPTP) treated mice,[15] however the effect of cinnamon on rotenone is yet to be investigated. Hemp seed and its oil have been used as both a food and medicine in China for at least 3000 years and hemp seed extracts have been found to demonstrate antioxidant and antiaging effects[16–18] as well as improve cognitive impairment induced by chemicals in mice.[19] In addition to all of this, epidemiological studies suggest societies that commonly use curcumin, cinnamon and hemp seed appear to demonstrate a lower incidence of PD and neurodegenerative disorders.[14,20] We included the use of curcumin within our study as a positive control as this substance has been shown previously to provide protection against rotenone and H_2O_2 toxicity.[10,21,22] *Polygonum cuspidatum* is widely distributed in the world and has been shown to possess antiviral, antimicrobial, anti-inflammatory, neuroprotective, and cardioprotective properties,[23] however these properties are yet to be investigated using a cellular model of PD.

In this study we assessed the effect of rotenone and H_2O_2 on SH-SY5Y cell survival and TH protein expression. We also evaluated the protective effects of curcumin, cinnamaldehyde, and constituents isolated from hemp seed (caffeoyltyramide) and *polygonum cuspidatum* (piceatannol glucoside).

2. Materials and methods

2.1. Cell culture

TH1 transfected SH-SY5Y cells (kindly provided by Associate Professor Phil Dickson, University of Newcastle, Australia) were cultured in Dulbecco's Modified Eagles Medium nutrient mixture F-12 Ham (Sigma Aldrich, USA) supplemented with 10% foetal calf serum (Assay Matrix, AUS) and 1X Antibiotic-Antimycotic (Gibco, USA (Penicillin, Streptomycin, Fungizone® antimycotic)) in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were seeded and allowed to adhere for 2 days. Media was replaced with 2% foetal calf serum media 1.5 h before treatments began and cells were left to adjust.

2.2. Treatments

Caffeoyltyramide and piceatannol glucoside were isolated and purified as described previously from hemp seed and *Polygonum cuspidatum*[24,25] respectively. Their purity (>98%) was confirmed by high performance liquid chromatography and their structures were well identified using spectroscopic techniques.

Concentrated stocks of rotenone, curcumin, cinnamaldehyde, caffeoyltyramide and piceatannol glucoside were prepared using Dimethyl Sulfoxide (DMSO) (Sigma Aldrich, USA) while stocks of H_2O_2 were prepared using PBS. The compounds were added to the media in the wells to obtain the desired treatment concentrations. Cells were pre-treated with curcumin, cinnamaldehyde, caffeoyltyramide or picceatannol glucoside 1 h before rotenone or H_2O_2 exposure. All controls received the same concentration of vehicle with the DMSO not exceeding 0.5% (v/v) in any experiment.

2.3. MTT cell viability assay

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) powder (Sigma-Aldrich, USA) was used as a means to assess cell viability as previously described.[26] Briefly, at the conclusion of the 24 h treatment duration, MTT was added to culture medium at a final concentration of 0.5 mg/ml and the plate was mixed gently for 1 min before 2 h incubation in a 37 °C, 5% CO_2 incubator. After the incubation, media was removed and 100 μL of DMSO was added to each well for 10–15 min while shaking. The intensity of the purple colour produced in each well was measured colourimetrically using a plate reader at 595 nm. The values of absorbance are expressed as a proportion of the controls.

2.4. Trypan blue assay

The Trypan Blue (Sigma Aldrich, USA) assay was used as another means of visually assessing cell viability to support the MTT assay findings. Cells were seeded into 12 well plates and treated as described above. When the treatment protocol had ended the media was removed from the wells and 30 μL of Trypan Blue was added and left for 30 s. After 30 s an image of the plate was taken using 'Cell Pad'. While not quantitative this protocol provided a means of visually assessing the viability of cell cultures.

2.5. SDS PAGE and western blots

Whole cell lysates were used for western blotting experiments to analyse total TH (tTH) and Poly-ADP ribose-polymerase (PARP-1) protein levels. Cells were seeded in a 24 well plate and treated as described above. At the end of the treatment protocol media was removed and 110 μL of 2% SDS stop buffer with inhibitors (50 mM Tris HCL (pH 6.8), 2% SDS, 2 mM EDTA, 1 mM Na orthovanadate, 1 mM Na fluoride, 10 mM Na pyrophosphate) was added. The lysed cells were collected and heated for 10 min at 100 °C. Samples were then frozen and stored at -20 °C for later analysis.

Samples were prepared for electrophoresis by dilution with sample buffer (20% DTT, 40% glycerol, 50 mM Tris, bromophenol blue, pH 6.8). Samples were run on an 8 or 10% SDS-polyacylamide gel and transferred to nitrocellulose membrane (0.45 μm and 0.2 μm, GE Healthcare, UK). To minimise non-specific binding membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.075% tween 20 (TBST) for 1.5 h at room temperature. Membranes were then incubated with anti-PARP-1 (1:1000, Santa Cruz Biotechnology catalogue no. sc-7150) or anti-total TH (tTH; 1:7,000, Sigma-Aldrich catalogue no. T2928) antibodies for 1 h at room temperature. Blots were washed and exposed to appropriate secondary antibody for 1 h at room temperature. Blots were then exposed to enhanced chemiluminescence detection reagent for 1 h and developed using a LAS 4000 imaging system (GE Healthcare, UK). Later, membranes were washed and then immunoblotted with β-actin antibody (1:10,000, Sigma-Aldrich, catalogue no. A3854) as a marker of the total protein loaded per lane. The density of the protein bands was quantified using ImageQuant TL software (GE Healthcare, UK). Quantitation of tTH and PARP-1 were normalised relative to β-actin levels.

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