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Research Paper

Similar potency of catechin and its enantiomers in alleviating 1-methyl-4-phenylpyridinium ion cytotoxicity in SH-SY5Y cells

Hao-lan Ruan[a], Yi Yang[b], Xiao-nan Zhu[b], Xue-lan Wang[b] and Ru-zhu Chen[b]

[a]Guangdong Institute for Drug Control, Guangzhou, China and [b]Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, China

Abstract

Objectives Previously, the flavonoid (±)-catechin was shown to exert potent neuroprotective action in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease model. The purpose of this study was to investigate whether the different enantiomers of catechin ((+)-catechin, (−)-catechin and (±)-catechin, a 50 : 50 mixture of (+)-catechin and (−)-catechin) could protect SH-SY5Y cells against 1-methyl-4-phenylpyridinium ion (MPP^+) toxicity by decreasing the generation of oxygen free radicals. The inhibitive effect of (±)-catechin on JNK/c-Jun activation was investigated.

Methods The effects of (+)-catechin, (−)-catechin or (±)-catechin in protecting against MPP^+ toxicity were evaluated and compared in SH-SY5Y cells by testing the release of lactate dehydrogenase. The generation of reactive oxygen species (ROS) was measured by immunochemistry and the phosphorylation level of JNK/c-Jun was determined by Western blotting.

Key findings In SH-SY5Y cells, (+)-catechin, (−)-catechin or (±)-catechin reduced apoptosis induced by MPP^+ and decreased ROS generation caused by MPP^+. Different enantiomers of catechin showed protective effects at similar potency. Moreover (±)-catechin decreased JNK/c-Jun phosphorylation which was increased by MPP^+.

Conclusions Catechin and its two enantiomers could protect SH-SY5Y cells against MPP^+ cytotoxicity at a similar potency. Antioxidative stress and inhibition of the JNK/c-Jun signalling pathway might have been involved in the neuroprotective mechanisms of catechin against MPP^+ cytotoxicity in SH-SY5Y cells.

Keywords catechin; JNK MAPK; MPP^+; oxidative stress; SH-SY5Y cells

Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta. Oxidative stress causes the death of mesencephalic dopaminergic neurons and is considered as an important factor in the pathological process of PD.[1–3] Reactive oxygen species (ROS) induced oxidative stress in dopaminergic neurons and caused neuron apoptosis, suggesting that antioxidants might rescue dopaminergic neurons from apoptosis effectively.[4]

Catechin is a flavonoid, one of the antioxidants rich in fruits, wine and cocoa. It has two enantiomers (+)-catechin and (−)-catechin. Previous studies reported that (+)-catechin reduced the toxicity of 6-hydroxydopamine (6-OHDA) in primary cultures of rat mesencephalic cells or PC12 cells and (−)-catechin, the enantiomer of (+)-catechin, could prevent neuronal death in the gerbil by alleviating ischaemia-reperfusion-induced damage, suggesting these two enantiomers could be beneficial for dopaminergic neuron protection.[5–7] Recently (±)-catechin, a 50 : 50 mixture of (+)-catechin and (−)-catechin, was demonstrated to prevent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration in mice. The suppression of the JNK/c-Jun and glycogen synthase kinase 3β (GSK-3β) signalling pathway might have been involved in the mechanism of this protection.[8] (+)-Catechin, (−)-catechin and (±)-catechin have different rotations, but the question as to whether there is any difference between the neuroprotective effects of these enantiomers has not been answered.

Correspondence: Ruzhu Chen, Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, China.
E-mail: ylfour@mail.sysu.edu.cn

The human neuroblastoma cell line, SH-SY5Y, can produce dopamine and expresses dopamine receptors.[9,10]It has been extensively used in research as an in-vitro model for dopaminergic neurons. 1-Methyl-4-phenylpyridinium ion (MPP^+), an active metabolite of MPTP, is a potent inhibitor of complex I of the respiratory chain. Loss of complex I function induces toxicity in dopaminergic neurons.[11] MPP^+ has been utilized extensively to produce a cell model of PD in SH-SY5Y cells, as SH-SY5Y cells express the noradrenaline transporter, which transports both dopamine and MPP^+.[12] MPP^+ produces ROS and activates the JNK/c-Jun signalling pathway which results in SH-SY5Y cell apoptosis.[13]

JNK mediates dopaminergic neuron apoptosis in PD.[14] Flavonoids, as potent antioxidants, can protect neurons from apoptosis by decreasing ROS and inhibiting the JNK pathway. Epicatechin was reported to protect mouse primary striatal neurons from oxidized low-density lipoprotein-induced apoptosis by attenuating the activation of the JNK pathway.[15] In SH-SY5Y cells (−)-epicatechin exerted a neuroprotective action against toxicity induced by H_2O_2 plus $FeSO_4$ via reducing ROS production and inhibiting JNK activation.[16] However, it remains to be ascertained whether (+)-catechin, (−)-catechin and (±)-catechin prevention against oxidative damage is related to inhibition of the JNK pathway.

In this report, using SH-SY5Y cells, we have explored the effects of (+)-catechin, (−)-catechin and (±)-catechin on reducing MPP^+ cytotoxicity and decreasing ROS evoked by MPP^+. The activation of the JNK/c-Jun signalling pathway was analysed with (±)-catechin.

Materials and Methods

Materials

SH-SY5Y cells were obtained from the American Type Cell Collection (Manassas, VA, USA). (+)-Catechin, (−)-catechin, (±)-catechin and MPP^+ were obtained from Sigma (Saint Louis, MO, USA). Dichloro-fluorescein diacetate (DCF-DA) was purchased from Applygen Technologies Inc. (Beijing, China). Rabbit anti-phospho-JNK and rabbit anti-phospho-c-Jun (Ser73) antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-β-tubulin antibody was obtained from Sigma. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG, and ECL Western blotting detection reagents were purchased from Amersham (Amersham Biosciences, GE Healthcare Biosciences, Little Chalfont, Buckinghamshire, England). Unless stated, all other chemicals were purchased from Sigma-Aldrich.

SH-SY5Y culture

For experiments, SH-SY5Y cells were seeded in 96-well plates and 6-well plates with Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% (v/v) fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and were incubated at 37°C in a humidified atmosphere containing 5% CO_2/95% air. When the cells were grown to 80% confluence the medium was replaced with DMEM containing 2% (v/v) FBS for an additional 24 h. The above conditions were applied to all of the experiments.

(+)-Catechin, (−)-catechin or (±)-catechin was dissolved in dimethyl sulfoxide (DMSO; final concentration of DMSO in culture medium was 0.1%) and added to culture medium for 1 h before 5 mM MPP^+ exposure. Meanwhile, all of the vehicle control groups were treated with the same volume of vehicle (DMSO).

Lactate dehydrogenase release assay

SH-SY5Y cell death was assessed by measuring the leakage of lactate dehydrogenase (LDH) from the broken SH-SY5Y cells in the medium, as described previously.[17] SH-SY5Y cells were seeded in 96-well plates and were incubated with (+)-catechin, (−)-catechin or (±)-catechin (100, 150 or 300 μM) for 1 h before exposing to 5 mM MPP^+. LDH release from each group was determined 24 h after treating with MPP^+. Medium (100 μl) from each well was mixed with 100 μl reaction buffer containing sodium lactate, INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium), PMS (phenazine methosulfate) and NAD (nicotinamide adenine dinucleotide). The reaction lasted for 30 min at 37°C. Optical density value was measured and normalized with control LDH release.

Determination of ROS generation in SH-SY5Y cells

The ROS generation in cells was determined with the fluorescent probe 20,70-dichlorofluorescein diacetate (DCF-DA). When DCF-DA enters a cell, it is converted into a nonfluorescent dichlorofluorescein (DCFH) by intracellular esterase. Intra-cellular ROS oxidizes DCFH-DA to a highly fluorescent compound dichlorofluorscein (DCF), which can be visualized by a fluorescence microscope. SH-SY5Y cells were seeded in 24-well plates and then incubated with (+)-catechin, (−)-catechin or (±)-catechin at 100, 150 or 300 μM for 1 h before 5 mM MPP^+ exposure. After treatment, the cells were incubated with 10 μM DCFH-DA at 37°C for 30 min and washed twice with phosphate buffered saline (PBS). Finally, the fluorescence intensity of DCF was measured. To estimate the relative amount of green fluorescent cells, all SH-SY5Y cells in each field were first counted and then the number of green fluorescent positive cells was determined. A total of 1000 cells was analysed.

Analysis of the level of phosphorylation of JNK and c-Jun in SH-SY5Y cells by Western blotting

Previous it was reported that 5 mM MPP^+ could activate JNK in SH-SY5Y cells.[18] In this report, SH-SY5Y cells were treated with 5 mM MPP^+ for 15 min, 30 min, 1, 2 or 4 h to explore the evident time for phosphorylation of JNK and c-Jun detection. To explore the impact of (±)-catechin on the JNK/c-Jun signalling pathway, the cells were pretreated with (±)-catechin (100, 150 or 300 μM) for 1 h and then treated with 5 mM MPP^+ for another 1 h. The proteins were then analysed by Western blotting.

Western blotting analysis was performed as described previously.[17] Protein concentrations were determined with Bradford reagent. In brief, protein lysates prepared from SH-SY5Y cells were separated by a 10% SDS-polyacrylamide gel electrophoresis and the resolved proteins were transferred to a polyvinylidene fluoride membrane. This membrane was then subjected to immunoblotting with a primary antibody against phosphorylated JNK, phosphorylated c-Jun or β-tubulin at

4°C overnight. After that, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham, 1 : 2000) and visualized by using the ECL Western blotting detection reagents (Amersham). Quantitation of the protein band was measured by the image biological analysis system (IBAS, an image analysis system from OPTON, Germany) in terms of calculating the optical density of each blot area.

Statistical analysis

Measurement data were expressed as means ± SEM. Comparison of more than two groups was performed by one-way analysis of variance with a post-hoc test. Differences were considered significant when $P < 0.05$. Data analyses were performed with the SPSS 10.0 software. All results represented at least three independent replications performed in triplicate.

Results

(+)-Catechin, (–)-catechin and (±)-catechin alleviated MPP+ toxicity in SH-SY5Y cells

After SH-SY5Y cells were treated with 5 mM MPP+ for 24 h, LDH release increased significantly compared with the vehicle control group (Figure 1). When the cells were pretreated with 100, 150 or 300 μM (+)-catechin, (–)-catechin or (±)-catechin before MPP+ exposure, the toxicity induced by MPP+ was alleviated dose-dependently. When compared with the MPP+-treated group, (+)-catechin at 100, 150 or 300 μM reduced LDH release by 14 ($P < 0.05$), 29 ($P < 0.01$) and 47% ($P < 0.01$), respectively; (–)-catechin reduced it by 16, 31 ($P < 0.05$) and 37% ($P < 0.01$) and (±)-catechin reduced it by 11, 15 and 29% ($P < 0.01$), respectively.

Together, these results suggested that (+)-catechin, (–)-catechin and (±)-catechin protected SH-SY5Y cells against MPP+ toxicity. At the same concentrations, the results of their effects showed no significant differences ($P > 0.05$) in the MPP+ toxicity cell model (see Figure 1).

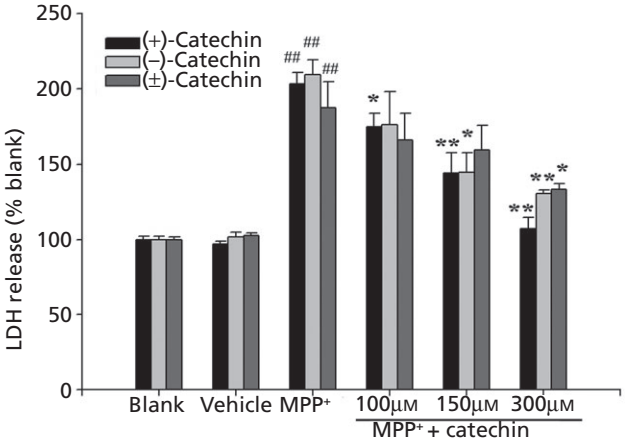

Figure 1 (+)-Catechin, (–)-catechin and (±)-catechin alleviated toxicity induced by MPP+ in SH-SY5Y cells. SH-SY5Y cells were pretreated with 100, 150 or 300 μM (+)-catechin, (–)-catechin or (±)-catechin for 1 h before incubating with MPP+ for 24 h. Lactate dehydrogenase (LDH) release was determined and calculated as described in Materials and Methods. The values presented are the mean ± SEM of six samples from three independent experiments. Data were analysed by using one-way analysis of variance. ##$P < 0.01$ vs the vehicle control; *$P < 0.05$ and **$P < 0.01$ vs MPP+ group.

(+)-Catechin, (–)-catechin and (±)-catechin reduced ROS generation caused by MPP+ in SH-SY5Y cells

After treating with 5 mM MPP+ for 24 h, the green fluorescence of SH-SY5Y cells increased significantly in the presence of DCFH-DA, which was the index of ROS generation. Pretreatment with (+)-catechin, (–)-catechin or (±)-catechin (100, 150 or 300 μM) downregulated fluorescent intensity obviously when compared with the MPP+-treated group (Figure 2a). To estimate the ratio of green fluorescence-positive cells, SH-SY5Y cells were first counted and then the numbers of green fluorescence-positive cells were determined in the same field. A total of 1000 cells from each group was analysed. The proportion of green fluorescence-positive cells in the control group was 2.16%, while that in the MPP+-treated group was 64.09% ($P < 0.01$ vs control). However, pretreatment with (+)-catechin 100, 150 or 300 μM reduced the proportion to 5.42, 3.29 and 2.9% (all $P < 0.01$, compared with MPP+-treated group), respectively. (–)-Catechin reduced the proportion to 3.91, 2.57 and 1.37% and (±)-catechin reduced it to 4.11, 5.26 and 3.06%, respectively (all $P < 0.01$) (Figure 2b). This result suggested that inhibition of MPP+-induced ROS generation in SH-SY5H cells might have been involved in the neuroprotective effects of the catechin enantiomers.

Phosphorylation of JNK and c-Jun increased by MPP+ were suppressed by (±)-catechin

The JNK/c-Jun signal pathway has been considered as an important signal pathway closely related to dopaminergic neuron apoptosis.[19,20] Hence the activation of the JNK/c-Jun signal pathway acted as an index to explore the mechanism of the neuroprotective action of catechin. After MPP+ exposure, phosphorylation of JNK1 and JNK2 (p-JNK1 and p-JNK2) began to increase in SH-SY5H cells. After cells were treated with MPP+ for one hour, p-JNK1 and p-JNK2 peaked to 135 ($P < 0.01$) and 235% ($P < 0.05$) compared with the vehicle control, respectively. Meanwhile, phosphorylation of c-Jun (p-c-Jun) increased to 199% ($P < 0.05$) vs the vehicle control and then declined gradually (Figure 3a and b). As activation of the JNK/c-Jun pathway peaked after one hour of MPP+ treatment, we selected this duration to check for (±)-catechin effect.

As can be seen from Figure 3c–d, pretreatment with 100, 150 or 300 μM (±)-catechin before MPP+ exposure reduced activation of the JNK/c-Jun pathway. p-JNK1, p-JNK2 and p-c-Jun of the MPP+ group reached 160, 251 and 210%, respectively, vs the control group. At 100, 150 or 300 μM, p-JNK1 of the (±)-catechin group was 153, 127 and 85%, p-JNK2 was 198, 146 and 125%, and p-c-Jun was 199, 160 and 111%, respectively, vs the control.

The results showed that (±)-catechin remarkably decreased the phosphorylation of JNK1/2 and c-Jun, which were activated significantly by MPP+, indicating that blockade

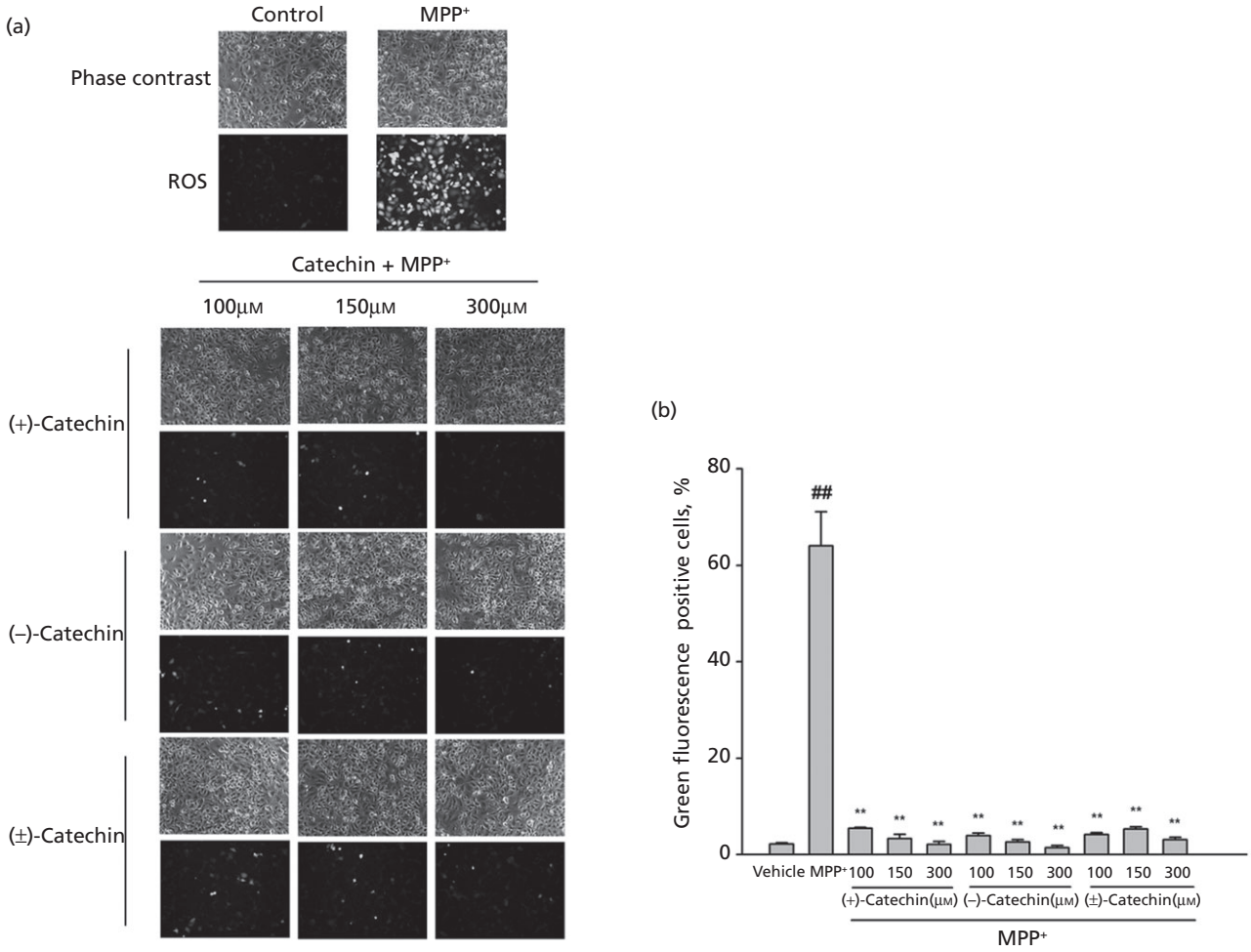

Figure 2 Intracellular ROS levels increased by MPP^+ were suppressed by (+)-catechin, (−)-catechin and (±)-catechin. (a) Images of green fluorescence-positive cells. The number of green fluorescence-positive cells indicated the presence of intracellular ROS. The images shown are representative of three independent experiments. (b) Quantification of green fluorescence-positive cells. Data were presented as mean ± SEM, $n = 3$, ##$P < 0.01$ vs vehicle group; **$P < 0.01$ vs MPP^+-treated group.

of JNK/c-Jun activation might have been involved in the mechanism of protection against MPP^+-induced toxicity in SH-SY5Y cells.

Discussion

Flavonoids are potent antioxidants and can protect neuronal cells *in-vivo* and *in-vitro*.[21] Among the various flavonoids, (+)-catechin attenuated the neurotoxin of 6-OHDA in primary cultures of rat mesencephalic cells or PC12 cells and (±)-catechin attenuated the neuronal toxicity of MPTP in mice, suggesting that both (+)-catechin and (±)-catechin might protect dopaminergic neurons in PD as potent antioxidants.[5,6] However, no studies had been reported about the neuron protection of (−)-catechin in PD. So, whether there was any difference between catechin and its two enantiomers on neuron protection in PD needed to be clarified.

In this report, we utilized SH-SY5Y cells to investigate the different effects between (+)-catechin, (−)-catechin and (±)-catechin. SH-SY5Y cells were treated with the same concentrations of (±)-catechin or its two enantiomers, respectively, before MPP^+ exposure. Results showed that the reduction of LDH release in each catechin group was similar in potency. What was more, catechin and its two enantiomers showed no difference in reducing the ROS increased by MPP^+. These findings suggested that (+)-catechin, (−)-catechin and (±)-catechin might have protected SH-SY5Y cells against MPP^+-induced cytotoxicity via their potent radical scavenging properties.

It was reported that the scavenging efficiency of catechins depended on their chemical structure. Studies on the antioxidant activity among tea catechins found that (−)-epigallocatechins gallate (EGCG) = (−)-epicatechin-3-gallate (ECG) > (−)-epigallocatechin (EGC) > (−)-epicatechin (EC), indicating that the active sites of the tea catechins that reacted with oxygen free radicals were an ortho-hydroxyl group in the B-ring and a galloyl moiety in the C-ring. The gallate group in the C-ring is the most important factor contributing to the antioxidant efficiency.[22] Though catechin and its two enantiomers lack the galloyl moiety in the C-ring, they posses the

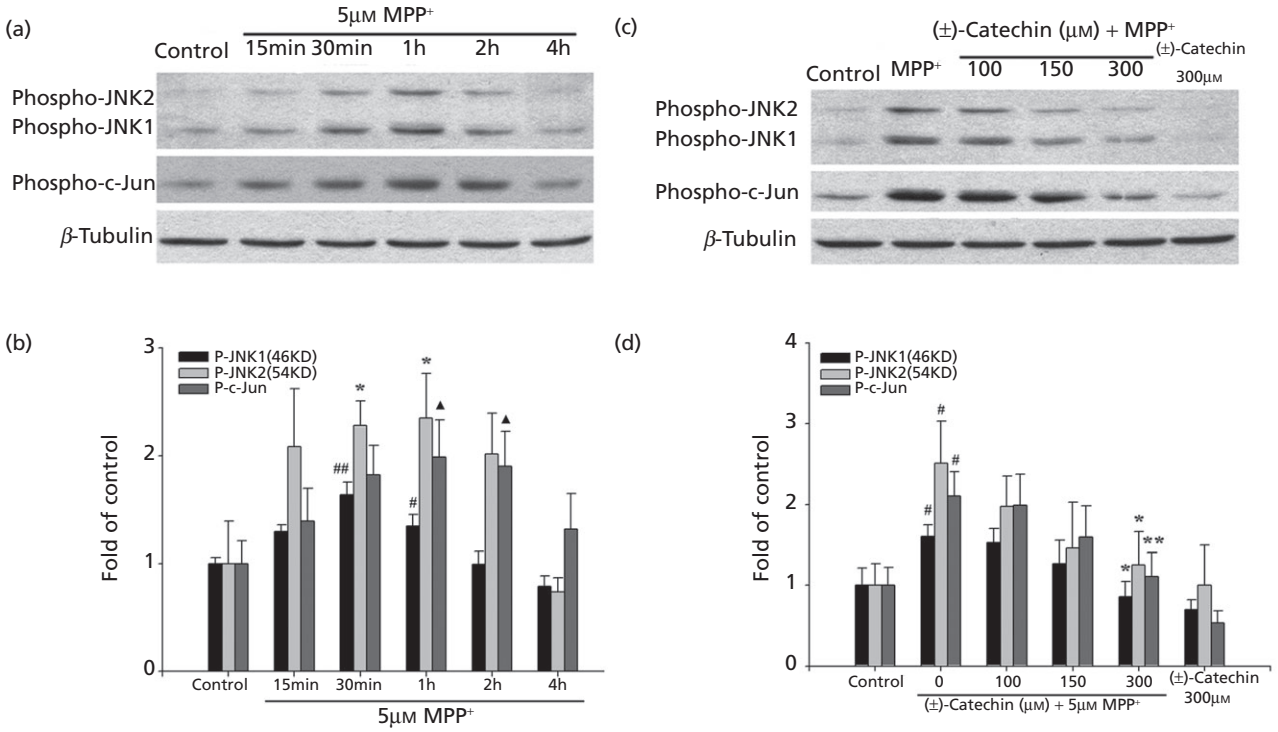

Figure 3 (±)-Catechin suppressed MPP+-activated JNK/c-Jun pathway in a dose-dependent manner. (a) p-JNK1/2 and p-c-Jun were determined by Western blotting analysis after cells were treated with MPP+ for different times. (b) Quantification of p-JNK1/2 and p-c-Jun. (c) p-JNK1/2 and p-c-Jun were determined after cells were pretreated with 100, 150 or 300 μM (±)-catechin before MPP+ exposure. (d) Quantification of p-JNK1/2 and p-c-Jun. Results were from three independent experiments. Data were analysed with one-way analysis of variance. $*P < 0.05$ and $**P < 0.01$ vs control.

same B-ring. We assumed that the same chemical structure of catechin and its two enantiomers determined their similar effects against MPP+ toxicity in SH-SY5Y cells.

ROS could activate the JNK/c-Jun signalling pathway and induce dopaminergic cell apoptosis.[4] Antioxidants could rescue dopaminergic neurons in PD by inhibiting the activation of the JNK/c-Jun signalling pathway. Flavonoids, particularly epicatechin, were able to attenuate the activation of JNK and protected neuronal cell death against damage induced by oxidative stress in primary striatal neurons.[15] Our previous work reported that (±)-catechin might protect dopaminergic neurons against MPTP toxicity in mice by suppressing the activation of the JNK signalling pathway.[8]

In this study, catechin and its two enantiomers showed similar potency against MPP+ toxicity in SH-SY5Y cells. Thus, we selected (±)-catechin for JNK/c-Jun signal pathway analysis. The results showed that JNK and c-Jun were activated significantly by treating with MPP+, but this activation subsided with (±)-catechin pre-administration, indicating that (±)-catechin might protect SH-SY5Y cells against MPP+ toxicity through suppression on JNK/c-Jun pathway activation. (+)-Catechin and (−)-catechin showed similar potency as (±)-catechin in protecting SH-SY5Y cells and reducing ROS generation, both of them were supposed to protect SH-SY5Y cells via inhibiting JNK/c-Jun pathway activation. Whether they possessed similar potency in suppressing the JNK/c-Jun pathway requires further study.

Our previous work in mice indicated that (±)-catechin might have protected dopaminergic neurons via inhibiting the JNK/c-Jun and GSK-3β signal pathway.[8] However, GSK-3β activation evoked by MPP+ in SH-SY5Y cells could not be detected in this study(data not shown). Previous studies indicated that inhibition of GSK-3β with lithium protected SH-SY5Y cells against the toxic effects of MPP+ and GSK-3β was found to be activated by MPP+ in SH-SY5Y cells that were stably transfected with α-Synuclein and transiently transfected with human dopamine transporter.[23,24] So, with SH-SY5Y cells transfected with α-Synuclein and human dopamine transporter, the effects of (+)-catechin, (−)-catechin or (±)-catechin on the activation of GSK-3β might be detected.

Accumulating evidence suggested that flavonoids may interact directly with mitogen-activated protein kinase (MAPK) signalling pathways.[25] Quercetin, a dietary flavonol, was found to inhibit specifically JNK activity and expression of c-jun mRNA, which were induced by phorbol 12-myristate-13-acetate (PMA) and tumour necrosis factor-α (TNF-α) in human endothelial cells.[26] Studies by Schroeter *et al*.[15,27] indicated that flavonoids might protect striatal neurons from oxidized low-density-lipoprotein (oxLDL)-induced apoptosis via inhibiting the activation of JNK but without preventing the increase of intracellular oxidative tress. (−)-Epicatechin could stimulate phosphorylation of ERK1/2 in mouse cortical neurons. In this report, (±)-catechin decreased ROS level in SH-SY5Y cells and (±)-catechin suppressed the activation of the JNK/c-Jun pathway. We assumed that the suppression of JNK/c-Jun was probably the result of eliminating ROS, which is the activator of the

JNK signalling pathway.[4] However, which one of the up-stream proteins of the JNK/c-Jun pathway would be the first target of catechin needs clarification.

Conclusions

We have demonstrated the similar potency of (+)-catechin, (−)-catechin and (±)-catechin in protecting SH-SY5Y cells against MPP^+ toxicity. Radical scavenging properties as well as the suppression of the JNK/c-Jun signalling pathway by catechin might have contributed to the neuroprotective effects of (+)-catechin, (−)-catechin and (±)-catechin.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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