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·Original Article·

Curcumin protects nigral dopaminergic neurons by iron-chelation in the 6-hydroxydopamine rat model of Parkinson's disease

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Abstract: Objective Curcumin is a plant polyphenolic compound and a major component of spice turmeric (*Curcuma longa*). It has been reported to possess free radical-scavenging, iron-chelating, and anti-inflammatory properties in different tissues. Our previous study showed that curcumin protects MES23.5 dopaminergic cells from 6-hydroxydopamine (6-OHDA)-induced neurotoxicity *in vitro*. The present study aimed to explore this neuroprotective effect in the 6-OHDA-lesioned rat model of Parkinson's disease *in vivo*. **Methods** Rats were given intragastric curcumin for 24 days. 6-OHDA lesioning was conducted on day 4 of curcumin treatment. Dopamine content was assessed by high-performance liquid chromatography with electrochemical detection, tyrosine hydroxylase (TH)-containing neurons by immunohistochemistry, and iron-containing cells by Perls' iron staining. **Results** The dopamine content in the striatum and the number of TH-immunoreactive neurons decreased after 6-OHDA treatment. Curcumin pretreatment reversed these changes. Further studies demonstrated that 6-OHDA treatment increased the number of iron-staining cells, which was dramatically decreased by curcumin pretreatment. **Conclusion** The protective effects of curcumin against 6-OHDA may be attributable to the iron-chelating activity of curcumin to suppress the iron-induced degeneration of nigral dopaminergic neurons.

Keywords: 6-hydroxydopamine; curcumin; Parkinson's disease; dopaminergic neurons; iron

1 Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the selective loss of dopaminergic neurons in the substantia nigra (SN), resulting in a clinical syndrome characterized by stiffness, tremor, slowness of movement, and postural instability. However, the etiology of PD has yet to be fully uncovered. Increasing evidence suggests that iron plays a key role. Iron catalyzes the formation of highly toxic free radicals through the Fenton reaction, resulting in impaired mitochondrial function and autoactivity of proteases and phospholipases, leading to cell death[1]. Increased iron levels have been found in the SN of PD patients, as well as in 6-hydroxydopamine (6-OHDA)-induced animal models of PD[2-6]. Recently, antioxidant and iron-chelating strategies have been the focuses of PD treatment[7,8], among which flavonoids are considered to be the most promising candidates because of their well-reported biological properties.

Flavonoids are plant polyphenolic compounds that can protect against oxidative stress by scavenging free radicals[9] and chelating iron[10]. The polyphenolic flavonoid curcumin (1,7-bis[4-hydroxy 3-methoxy phenyl]-1,6-heptadiene-3,5-dione) (Fig. 1) from *Curcuma longa* exhibits

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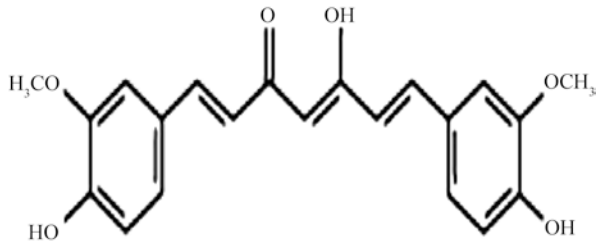

Fig. 1. Structure of curcumin.

anti-inflammatory[11], antioxidant[12], and iron-chelating activities[13]. It was reported that curcumin attenuates the incidence of colon cancer and is anti-atherogenic, and these effects are associated with its antioxidant activity[11]. Curcumin has cytoprotective effects against MPP^+ neurotoxicity in PC12 cells via its anti-apoptotic and antioxidant properties through the Bcl-2–mitochondrion–reactive oxygen species–inducible nitric oxide synthase pathway[14]. Our previous work has also demonstrated that curcumin attenuates 6-OHDA-induced cytotoxicity by anti-oxidation and nuclear factor kappa B modulation in MES23.5 cells[15], which suggests that curcumin has a neuroprotective effect on dopaminergic neurons in PD.

6-OHDA is a neurotoxin commonly used to produce PD models. Previous studies have shown that iron content is increased in the SN of 6-OHDA-induced PD models[2,16,17], while iron chelators are neuroprotective against PD. Curcumin is known to have this iron-chelating activity *in vitro*[18-20]. However, whether it has neuroprotective effects on dopaminergic neurons in PD animal models through iron-chelating activity remains unknown. Therefore, the present study aimed to investigate the effects of curcumin on the degeneration of dopaminergic neurons and on iron levels in the SN of 6-OHDA-treated rats.

2 Materials and methods

2.1 Materials Curcumin, 6-OHDA, apomorphine, dopamine, and rabbit anti-tyrosine hydroxylase (TH) antibody were all from Sigma Chemical Co. (St. Louis, MO). Goat anti-rabbit IgG was from ZSGB-BIO Co. (SP-9001; Beijing, China). All other chemicals and reagents were of the highest grade available from local commercial sources.

2.2 Animals The animals were from Qingdao Institute for Drug Control. All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Guidelines for the Use of Animals in Neuroscience Research. Female Wistar rats about postnatal 50 days, weighing 200–220 g were housed under a 12:12 h light/dark cycle, with free access to food and water. Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted on a stereotaxic frame. One burr hole (2.5 mm in diameter) was drilled into the skull above the left medial forebrain bundle (MFB) region, according to the coordinates of Paxinos and Watson. Two microinjections of 6-OHDA (3.6 mg/mL dissolved in saline containing 0.2 mg/mL *L*-ascorbate) were made into the left MFB[21] at the following two coordinates (in mm, relative to anterior fontanelle): (1) tooth bar (TB), −2.3; anteroposterior (AP), −4.4; mediolateral (ML), 1.2; ventral (V), −7.8; (2) TB, +3.4; AP, −4.0; ML, 0.8; and V, −8.0, at volumes of 2.5 and 3.0 μL respectively, at a rate of 1.0 μL/min. After injection, the microinjection needle was left in place for a further 5 min before being slowly extracted[16]. Control animals received injections of saline containing 0.2 mg/mL *L*-ascorbate.

The rats were divided into three groups: (1) sham control (saline injection into the left MFB + oral gum arabic twice a day for 24 days), (2) 6-OHDA + curcumin pretreatment [intragastric curcumin (200 mg/kg) twice a day for 24 days in total, with 6-OHDA lesioning on day 4 of curcumin treatment], and (3) 6-OHDA treatment (the same as group 2, except that gum arabic was administered instead of curcumin). Rats were tested for their rotational behavior in response to apomorphine (0.05 mg/kg, s.c.) in "rotameter" bowls for 30 min. Rotational behavior was tested three times at intervals of 3 weeks. Rats reaching a level of at least 7 rotations/min were regarded as PD model rats.

2.3 High-performance liquid chromatography with electrochemical detection The rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and then decapitated. Both sides of the striatum were carefully isolated and transferred into liquid nitrogen for storage. Samples were prepared as previously described[22]. Separation was

achieved on a PEC18 reverse-phase column. The mobile phase contained (in mmol/L) 20 citromalic acid, 50 sodium caproate, 0.134 EDTA-2Na, 3.75 sodium octane sulfonic acid, 1 disecbutylamine and 5% (*v/v*) methanol; the flow rate was 1 mL/min. A 2465 electrochemical detector (Water Corp., Milford, MA) was operated in screen mode. The levels of dopamine and its metabolites were expressed as ng/mg wet weight of brain tissue.

2.4 TH immunohistochemical staining of SN Immunohistochemistry was performed as previously described[22]. Briefly, the sections were warmed to room temperature, rinsed in 0.01 mol/L PBS for 10 min, and then placed into 99.7% methanol containing 0.3% hydrogen peroxide for 30 min to eliminate endogenous peroxidase. After three washes in PBS, sections were incubated in 10% normal goat serum (in PBS) for 15 min at 37°C, and then incubated overnight with rabbit anti-TH (1:10 000) at 4°C in a humidified chamber. The sections were washed in PBS and incubated with biotinylated goat anti-rabbit IgG for 30 min at 37°C, followed by amplification with streptavidin peroxidase for 30 min at 37°C. Next, sections were rinsed with PBS and incubated for 10–15 min with DAB. Sections without the primary antibody incubation showed negative staining. Sections were viewed under a 10× objective lens (Olympus) and the images were captured by a video camera (Olympus) at a final magnification of 400×. The analyzed area was within the central SN pars compacta (SNpc). The average number of positive cells throughout the entire rostrocaudal extent of the SNpc was calculated due to the relative sparseness of such cells[16]. All counts were carried out by an experimenter unaware of the animal groups.

2.5 Perls' iron staining Iron staining was by the Perls' Prussian blue reaction: sections were fixed in 4% formaldehyde for 5 min and washed for 30 s in Milli-Q water before staining, then incubated for 30 min in a freshly-prepared solution of equal parts 2% HCl and 2% potassium ferrocyanide. After washing with 0.01 mol/L PBS, sections were immersed in a solution containing 99% methanol and 1% hydrogen peroxide for 20 min to quench endogenous peroxidase activity and then incubated in DAB solution. Immunocytochemical staining was analyzed as for the TH staining above. One section was chosen from every three sections throughout the SNpc per brain.

2.6 Data analysis All experiments were done in triplicate. Data are expressed as mean ± SEM, and analyzed using one-way analysis of variance followed by the Student-Newman-Keuls test. P <0.05 was considered statistically significant.

3 Results

3.1 Curcumin protected against 6-OHDA-induced depletion of dopamine and its metabolites in the rat striatum Twenty-one days after 6-OHDA lesioning, the levels of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) decreased on the lesioned side of the striatum, while curcumin partly restored the levels (Fig. 2). The homovanillic acid (HVA) level was, however, unchanged. In addition, no changes were found on the contralateral side of the striatum in each group (data not shown).

3.2 Curcumin protected against 6-OHDA-induced decrease of TH-positive neurons in rat SN The TH-positive neurons in the normal SN were multipolar, with darkly stained somata and dendrites (Fig. 3A, D). On day 21 after 6-OHDA lesioning, the TH-positive neurons markedly

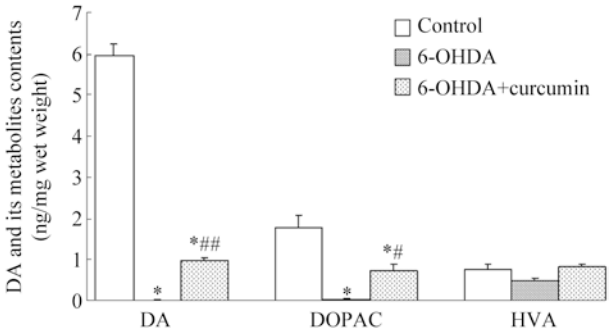

Fig. 2. Effects of curcumin on dopamine (DA) and its metabolites in the 6-OHDA-lesioned striatum. The DA and 3,4-dihydroxyphenylacetic acid (DOPAC) levels in the 6-OHDA group decreased compared with controls (n = 6, $^{*}P$ <0.01 *vs* control). Curcumin pretreatment partly restored the DA and DOPAC levels (n = 6, $^{\#}P$ <0.05, $^{\#\#}P$ < 0.01 *vs* 6-OHDA group). The homovanillic acid (HVA) level was unchanged.

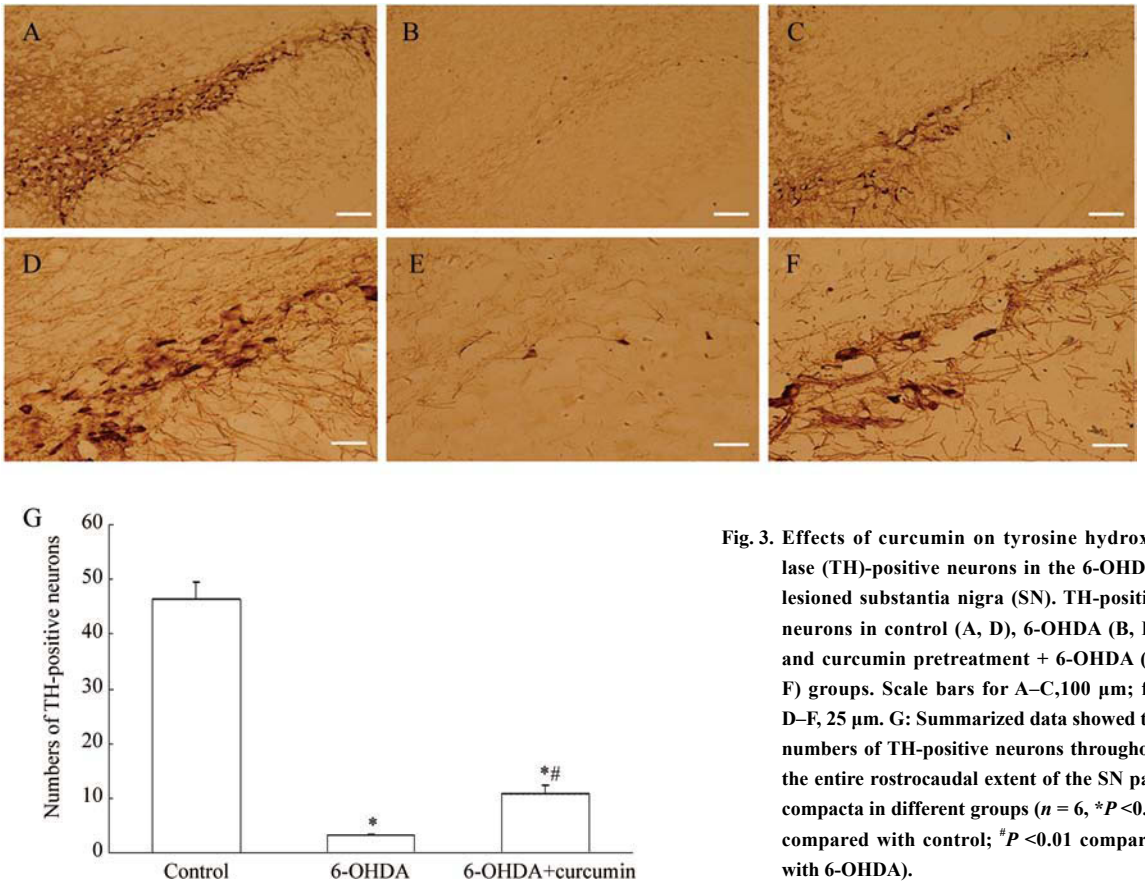

Fig. 3. Effects of curcumin on tyrosine hydroxylase (TH)-positive neurons in the 6-OHDA-lesioned substantia nigra (SN). TH-positive neurons in control (A, D), 6-OHDA (B, E), and curcumin pretreatment + 6-OHDA (C, F) groups. Scale bars for A–C,100 μm; for D–F, 25 μm. G: Summarized data showed the numbers of TH-positive neurons throughout the entire rostrocaudal extent of the SN pars compacta in different groups (n = 6, $^{*}P$ <0.01 compared with control; $^{\#}P$ <0.01 compared with 6-OHDA).

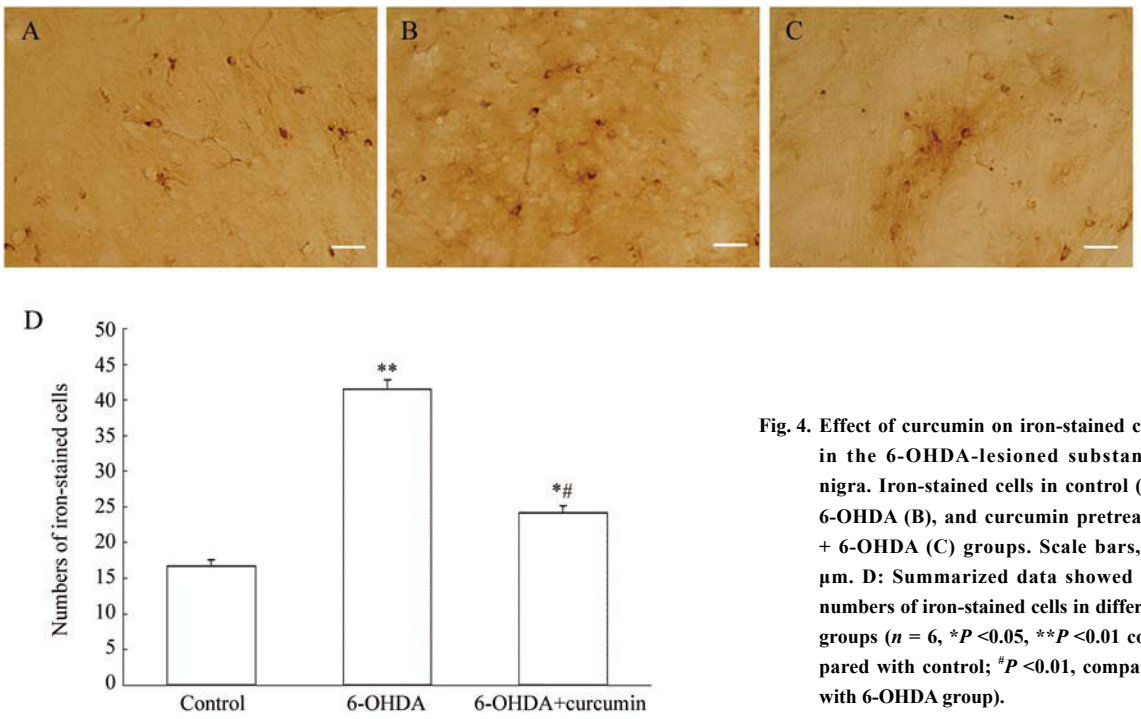

Fig. 4. Effect of curcumin on iron-stained cells in the 6-OHDA-lesioned substantia nigra. Iron-stained cells in control (A), 6-OHDA (B), and curcumin pretreated + 6-OHDA (C) groups. Scale bars, 25 μm. D: Summarized data showed the numbers of iron-stained cells in different groups (n = 6, $^{*}P$ <0.05, $^{**}P$ <0.01 compared with control; $^{\#}P$ <0.01, compared with 6-OHDA group).

decreased on the lesioned side (Fig. 3 B, E, G), but were significantly restored by curcumin pretreatment (Fig. 3 C, F, G). However, in the SN on the unlesioned side, both the morphology and the number of TH-positive neurons remained unchanged (data not shown).

3.3 Curcumin inhibited 6-OHDA-induced increase of iron-stained cells in the SN On day 21 after 6-OHDA lesioning, the number of iron-positive cells increased on the lesioned side compared with control. However, a marked decrease of iron-positive cells was found in the curcumin pretreatment group compared with the 6-OHDA treatment group (Fig. 4). Besides, no significant difference was found in the SN on the unlesioned side in each group (data not shown).

4 Discussion

Curcumin has been shown to possess anti-inflammatory[11], powerful antioxidant[12], iron-chelating[13] and free radical-scavenging[9] properties. The present study reported the neuroprotective activity of curcumin in a 6-OHDA-induced model of PD. We showed that curcumin pretreatment reversed the 6-OHDA-induced decrease in the levels of dopamine and its metabolite DOPAC in the striatum, indicating that curcumin could restore the dopamine content. However, the HVA level did not change in the 6-OHDA treatment group compared with the sham control group. This might be due to compensation by the dopamine system under conditions of dopamine depletion. The released dopamine increased its metabolism into HVA, thereby increasing the HVA content.

6-OHDA injures dopaminergic neurons both *in vivo* and *in vitro* by three main mechanisms: reactive oxygen species, hydrogen peroxide formation, and direct inhibition of the mitochondrial respiratory chain[23]. 6-OHDA-treated animals exhibit the major hallmarks of PD, including loss of dopaminergic neurons in the SN and a decrease in TH-positive neurons. Here, we counted the TH-positive neurons in the SN and found that curcumin inhibited the 6-OHDA-induced decrease, indicating that curcumin could directly protect dopaminergic neurons against 6-OHDA.

However, the precise mechanisms underlying the degeneration of nigral dopaminergic neurons in PD remain elusive. Iron overload and iron-induced hydroxyl radical formation by the Fenton reaction in the presence of H_2O_2 have been indicated as causative factors in PD. Here, we further showed that the numbers of iron-positive cells markedly decreased after curcumin pretreatment compared with the 6-OHDA treatment group. Previous work has shown that the iron-containing cell types in the SN are glia and neurons[16,23,24]. In addition, iron chelators protect dopaminergic neurons against PD by eliminating excess iron[25]. These findings suggest that the iron-chelating activity of curcumin plays an important role in its protective effects on dopaminergic neurons. In fact, several studies have shown that curcumin binds iron[13,20] with an affinity similar to other iron chelators such as nitrilotriacetic acid[20]. Proton donation from the phenolic group is responsible for the "superb antioxidant" properties of curcumin[19].

In conclusion, the present data showed that curcumin attenuated the toxic effects of 6-OHDA on the SN – striatum system. The neuroprotective action of curcumin may be mediated by a reduction of oxidative stress due to its iron-chelating property. This finding may lead to the development of promising drugs for the prevention and treatment of PD.

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