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ATP depletion is the major cause of MPP^+ induced dopamine neuronal death and worm lethality in α-synuclein transgenic *C. elegans*

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Abstract: Objective To investigate the toxic effect of environmental neurotoxin MPP^+ to *C. elegans* and identify the mechanisms that cause the toxicity. **Methods** Human α-synuclein transgenic *C. elegans* was used as the animal model, the toxic effect of MPP^+ to dopamine (DA) neurons and the lifespan of worms was tested. The worms were feed with OP50 to determine whether ATP increase can rescue the worm from toxicity. ATP level and aberrant protein accumulation were analyzed in the MPP^+ treated worms with or without OP50 addition. **Results** We found that MPP^+ induced DA cell death and worm lethality, which could be prevented by OP50 treatment. OP50 exerted the protective effect by up-regulating ATP level, even though it also induced accumulation of α-synuclein. Despite the undefined role of protein aggregation to the cell death, our results showed that the toxicity of MPP^+ was mainly caused by the ATP depletion in the α-synuclein transgenic *C. elegans*. **Conclusion** MPP^+ could induce DA neuronal death and worm lethality in α-synuclein transgenic *C. elegans*; Compared with the aggregation of α-synuclein, the major cause of MPP^+ toxicity appeared due to ATP depletion.

Keywords: Parkinson's disease; MPP^+; ATP; α-synuclein; *C. elegans*

1 Introduction

Parkinson's disease (PD) is a severe neurodegenerative disorders involved nearly 2% of the population beyond 65 years[1]. It is characterized by dopaminergic (DA) neuron degeneration in the substantia nigra pars compacta, with the aggregate called Lewy body formation[2,3]. The etiology of this disease is not fully understood. Environmental factors like drug abusing, malnutrition, injury and so on combined with genetic background contribute to the pathological progress. Despite the etiological difference, most PD cases show defects in mitochondrial Complex-1 activity and dopamine deficiency[4]. Mitochondrial Complex-1 inhibitor 1-methyl-4-phenylpyridinium (MPP^+), a metabolic product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), could induce the symptoms similar to those of PD in animals and human[5,6]. However, mechanisms underlying the MPP^+ toxicity need further investigation.

One outcome of Complex-1 inhibition is ATP depletion. ATP as the most important product of electron transport is necessary for maintaining normal cellular function. The 26S proteasome system and the heat shock system which response for protein homeostasis are both ATP dependent[7,8]. Therefore, ATP depletion may decrease the proteasome activity and heat shock response[9,10], which lead to the slowing down of protein turnover and finally cause a wide range of cell damage[11,12]. Among all the cell types in the brain, DA neuron is the most ATP consumer, which make it very sensitive to ATP depletion[13]. Except for the ATP depletion, MPP^+, the Complex-1 inhibitor also induces aberrant protein accumulation and oxidative stress[14-16]. Till now, there is no consensus about the mechanism of MPP^+ toxicity. Recent data showed that MPP^+ caused neuroblastoma cell death through up-regulating the expression/aggregation of α-synuclein, while other results from isolated rat brain cells suggested that ATP depletion was the primary cause

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Article ID: 1673-7067(2007)06-0329-07
CLC number: Q189; Q253
Document code: A
Received date: 2007-08-03

for MPP^+ toxicity[17].

Alpha-synuclein is a native unfolded protein and it is prone to form aggregates *in vitro* and *in vivo*. It has been proved that overexpression of the α-synuclein, including the wild type and its point mutations (A53T, A30P, and E46K), contributes to genetic PD cases[18-20]. But until now, the role of α-synuclein in the pathological progress of PD remains a puzzle and debatable. Some studies showed that overexpression of α-synuclein in DA neurons of aged *C.elegans* caused aggregates and apoptosis; in contrast, other evidences supported that the formation of aggregates protected cells from apoptosis[21,22].

In this study, we use human α-synuclein transgenic *C.elegans* as the animal model, because no α-synuclein or its homolog has been found in *C.elegans*. We evaluated roles of ATP depletion and protein accumulation (or aggregation) under MPP^+ treatment. Our results showed that MPP^+ caused DA cell death and worm lethality in α-synuclein transgenic *C.elegans*; uptaking OP50 can rescue the worm from MPP^+ neurotoxicity by elevating the ATP level. However, in this transgenic *C.elegans*, the toxicity of MPP^+ is dependent on ATP depletion but not on the accumulation/aggregation of α-synuclein.

2 Materials and methods

2.1 *C.elegans* strains *C.elegans* was maintained with standard method[23]. TA401, TA402, TA403, and TA404 were gifts from Prof. Takeshi Iwatsubo (Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, University of Tokyo). TA401 is a transgenic line expressing wild-type human α-synuclein, whereas TA402, TA403, and TA404 are transgenic lines expressing the A53T, A30P, and E46K point mutations, respectively, of human α-synuclein. The expression of α-synuclein is driven by the DAT-1 promoter, which only expressed in DA neurons. The TA strains' DA neurons were tagged with GFP, which could be detected under fluorescent microscope.

2.2 ATP test Worms (n = 200) were collected from 6 × 100 mm nematode growth media (NGM) plates with M9 buffer (22 mmol/L KH_2PO_4, 22 mmol/L Na_2HP_4, 85 mmol/L NaCI, 1 mmol/L MgSO4). Age-synchronized worms were obtained by treating gravid adults with 2% sodium hypochlorite and 0.5 mol/L NaOH to isolate embryos. The embryos grew for about 12 h at 20 °C to L1 stage. Then the larvae were incubated with 1 mmol/L MPP^+ for different time spans at 20 °C. The worms were collected to a 1.5-mL tube and centrifuged to the bottom. After the worm pellet was washed with M9 buffer twice, 200 μL lysis buffer from ATP detection kit was add to each tube and then ultrasonicated. The lysate were centrifuged at 12 000 × g for 5 min at 4 °C. The supernatant was transferred to a new 1.5-mL tube for ATP test with the ATP detection kit purchased from Beyotime (China).

The relative ATP level was calculated according to the following formula: relative ATP level = ATP value/protein value. The protein value of the sample was measured at 562 nm with Multiscan MK3 manufactured by Bio-Rad (Finland).

2.3 Western blot MPP^+-treated stage synchronized worms (n = 200) were collected with M9 buffer, and washed twice after centrifuging at 3 000 × g for 5 min. Worm pellet was ultrasonicated in 200 μL lysis buffer (100 mmol/L Tris, pH 6.8, 2% SDS, and 15% glycerol). The lysate was centrifuged at 12 000 × g for 5 min at 4 °C. Supernatant were transferred to a new 1.5 mL tube, mixed with 50 μL 5 × loading buffer and boiled for 10 min. For α-synuclein detection, a 1:2 000 dilution of rabbit anti-α-synuclein primary antibody purchased from Pierce (USA) and a 1:4 000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody purchased from Pierce (USA) were used.

2.4 Fluorescence microscope Twenty to thirty MPP^+-treated stage synchronized worms (n = 200) were transferred to the slides coated with gelatin, and then covered with coverslips. Samples were detected under an OLYMPUS IX81SIF driven by the DPManager software.

2.5 Life span test Worms (n = 200) were collected from six 100-mm NGM plate. Age-synchronized worms were obtained by treating gravid adults with 2% sodium hypochlorite and 0.5 mol/L NaOH to isolate embryos. The embryos grew for about 12 h at 20 °C to L1 stage. Then the larvae were incubated with 1 mmol/L MPP^+ for different time-span at 20 °C in dark. Then the larvae were calculated into living group and dead group.

3 Results

3.1 MPP^+ induced dramatic DA neurons loss in *C.elegans* As MPP^+ is selectively neurotoxic to DA neurons in several rodent and primate animal models, we first test whether the DA neurons of α-synuclein transgenic *C.elegans* could be also targeted by MPP^+. As shown in Fig.1, 1 mmol/L MPP^+ induced dramatic loss of DA neurons in *C.elegans*. For normal hermaphrodite worm, there are eight DA neurons in all: two pairs of CEP neurons and a pair of ADE

neurons in the head (Fig. 1A), and a pair of PDE neurons in the tail. Similar to the response to 6-OHDA[24], different sets of DA neurons in worms showed different sensitivity to MPP^+ (ADE > CEP > PDE) (Fig. 1B-D). Four hours after MPP^+ treatment, ADE neurons began to disappear while the CEP neurons were still present (Fig. 1B). Both ADE and CEP neurons were lost after 6-h MPP^+ treatment (Fig. 1C). More than ninety percent of the head DA neurons including their axons disappeared, but quite a part of PDE neurons could still be detected in the tail at 8 h post-treatment (Fig. 1D). Furthermore, different from classic apoptosis in phenotype, both the axons and cell bodies crashed to small dots which "floated" around after MPP^+ treatment.

3.2 MPP^+ induced high worm lethality in a time dependent manner Besides the DA neuron loss, MPP^+ also caused dramatic increase of lethality in transgenic *C.elegans* in a time dependent manner (Fig. 2). Studies have demonstrated that MPP^+ was specifically transported by dopamine transporter in DA neuron, concentrated in mitochondria to function as Complex-1 inhibitor and finally caused DA neuron death[14]. We found that, in addition to the toxicity to DA neutron, MPP^+ also induced high worm lethality which was apparently not associated with the loss of DA neurons, as it is known that *C.elegans* could live without DA neurons. This indicated that there must be other toxic targets for MPP^+ in transgenic *C.elegans*.

3.3 DA cell loss and worm lethality could be rescued by up-regulating the ATP uptake Results from isolated rat brain cells showed that MPP^+ displayed toxicity primarily by ATP depletion[14], so we wonder that whether increasing the ATP level could rescue the cell loss and worm lethality in transgenic *C.elegans*. Normally *C.elegans* was feed with OP50, a unique *E.coli* strain, which is reported to elevate the ATP level instantly after digested by *C.elegans*[23]. We fed *C.elegans* with OP50 to up-regulate the ATP level. As a result, most DA neurons maintained intact even with MPP^+ treatment for 24 h in the presence of OP50, in comparison with that without OP50 (Fig. 3A). Meanwhile, OP50 also markedly rescued the worm from death (Fig. 3B). Furthermore, the ATP level was elevated dramatically in the first hour of OP50 uptake (Fig. 3C). The decreased ATP level at 7 h and 15 h of each group reflected energy consume,

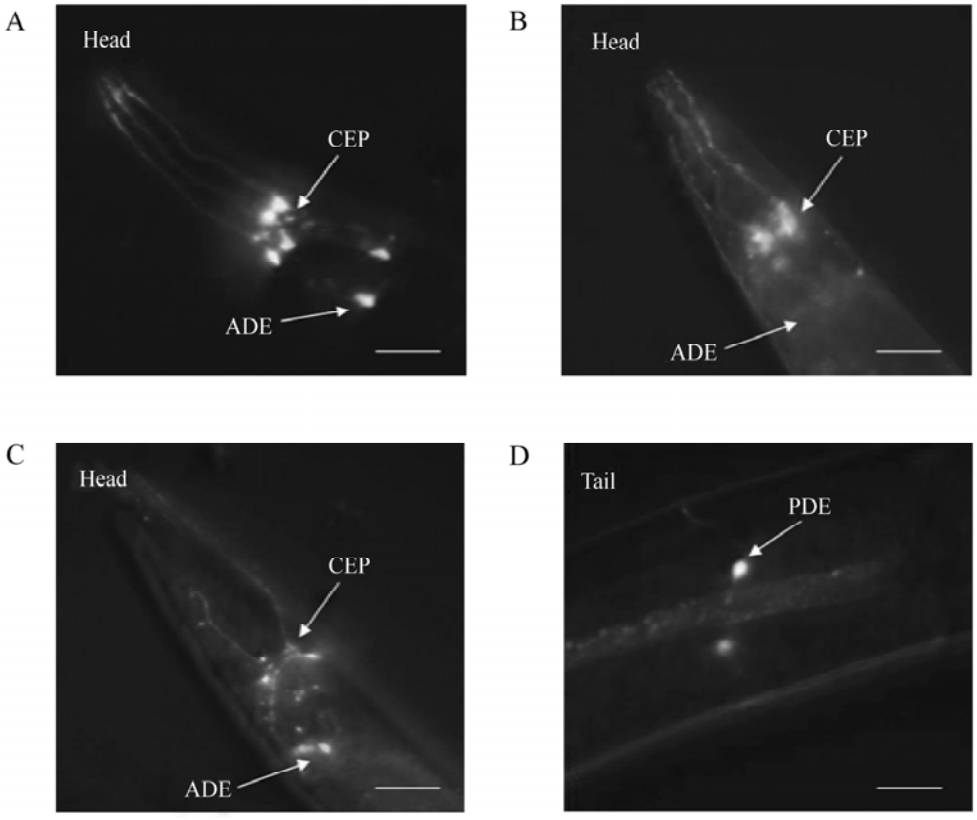

Fig. 1 MPP^+ (1 mmol/L) dramatically induced cell fragmentation and DA neuron loss of *C.elegans*. Worms kept in M9 buffer were treated with 1 mmol/L MPP^+ for indicating time. A: Untreated normal DA neurons tagged with GFP in head. B: Loss of ADE neurons in head after treatment with 1 mmol/L MPP^+ for 4 h. C: Both ADE and CEP neurons in head were lost after treatment with 1 mmol/L MPP^+ for 6 h. D: PDE neurons remained intact after treatment with 1 mmol/L MPP^+ for 8 h. Scale bar, 50 μm.

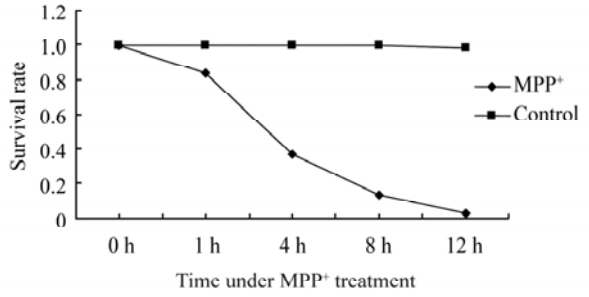

Fig. 2 MPP+ induced high lethality in transgenic *C.elegans* in a time dependent manner. MPP+ group was kept in the M9 buffer without food after hatch, the final concentration of MPP+ was 1 mmol/L. Control group was kept in M9 buffer after hatched without MPP+. In each group, the worms number was around 200 (n = 200). The indicated values are the representative of several different experiments.

which meant that the incipient ATP level was critical for worm and neuron vitality.

3.4 The toxicity of MPP+ was independent on the accumulation/aggregation of α-synuclein In neuroblastoma cells, MPP+ toxicity is dependent on the expression/aggregation of α-synuclein[17]. In contrast, we did not detect accumulated α-synuclein under MPP+ treatment alone in our *C. elegans* model, even though most of the worms in this group died (Fig. 4A, MPP+ group). However, we detected strong accumulation/aggregation of α-synuclein in the presence of OP50 (Fig. 4A, MPP+/OP50 group and OP50 group). Similar to the results from neuroblastoma cells[17], MPP+ could enhance the accumulation/aggregation of α-synuclein (Fig.

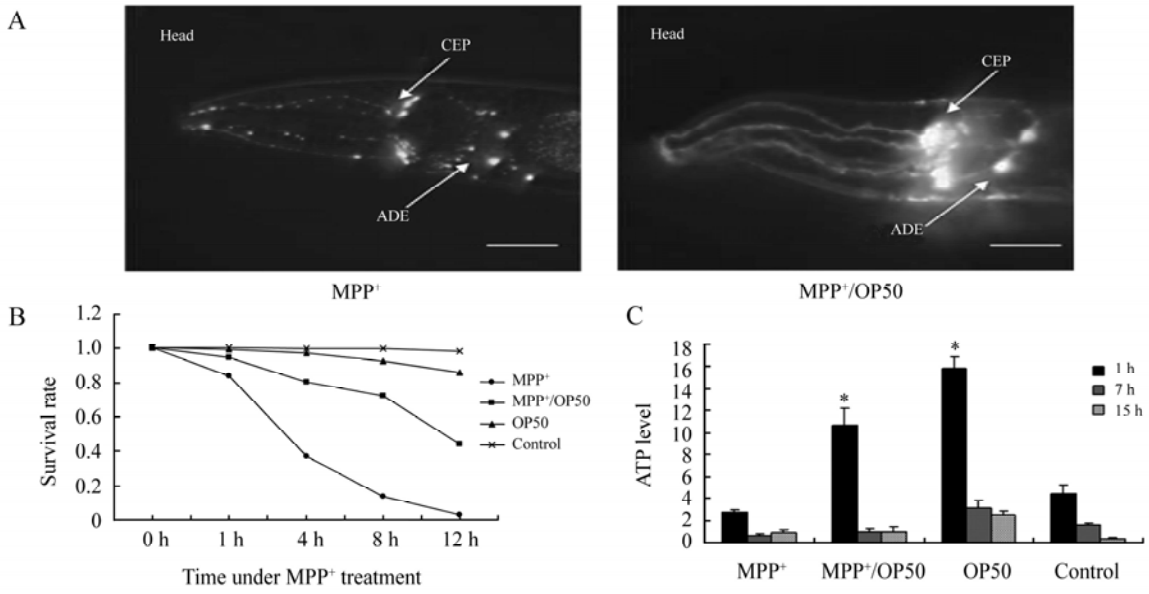

Fig. 3 DA cell loss and worm lethality could be rescued by up-regulating the ATP level by OP50. A: Twenty four hours after MPP+ treatment, most of the DA neurons in head were lost in the MPP+ group, DA neurons in head remained intact in the presence of OP50 under MPP+ treatment (MPP+/OP50 group). B: Worm lethality was rescued in MPP+/OP50 group compared with MPP+ group. OP50 group were fed with OP50 without MPP+ treatment, Control group were kept with neither OP50 nor MPP+. Data shown is the representative of several different experiments (n = 200). C: OP50 dramatically increased the ATP level in the first hour. In each group, the worms number was around 200 (n = 200). The indicated values are means±SD of three separate experiments. *P < 0.01 *vs* MPP+ group. Scale bar in panel A, 50 μm.

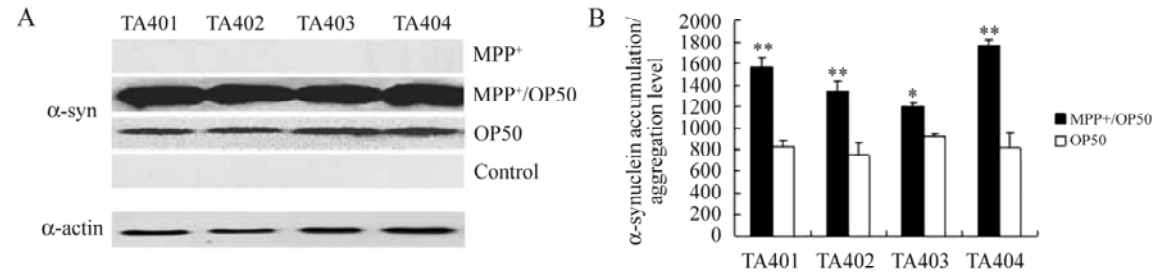

Fig. 4 The toxicity of MPP+ was independent on the accumulation/aggregation of α-synuclein. A: The accumulation/aggregation levels of α-synuclein in the worms treated with OP50 (MPP+/OP50 group and OP50 group) or without OP50 (MPP+ group and control group) were detected by Western blot with anti-α-synuclein antibody. B: MPP+ enhanced accumulation/aggregation of α-synuclein when worms were fed with OP50. The densitometry of corresponding bands from MPP+/OP50 group and OP50 group shown in (A) was analyzed by Quantity One software (Bio-Rad). The data shown are the representative of three different experiments. The indicated values are means±SD of three separate experiments. *P < 0.05, **P < 0.01 *vs* OP50 group.

4B, MPP^+/OP50 group *vs* OP50 group). However, it should be noted that the vitality of worm and DA neuron was barely interfered in the MPP^+/OP50 group, in which the α-synuclein aggregated most seriously (Fig. 3). This indicated that the accumulation/aggregation of α-synuclein may contribute to the MPP^+ toxicity, but maybe not the major cause for the toxic effect. What's more, it remains controversial that whether the aggregation was toxic or functioned as a passive protection for the cells. Therefore, the present data implicates that the toxicity of MPP^+ to the transgenic worm appears independent on the accumulation/aggregation of α-synuclein.

4 Discussion

MPTP may present in synthesized drugs, pesticide and chemical material. Patients exposed to this toxin has been reported to develop PD[6]. In mouse model, MPP^+, the metabolic product of MPTP *in vivo*, could induce PD-like syndrome and has been used in PD research for a long time[5]. Nowadays, *C.elegans* is used as a new animal model in PD research, for its transparent body and clear genetic background[25]. But until recently, there was no report to investigate whether MPP^+ can induce PD like syndrome in *C.elegans*. In our study, we used α-synuclein transgenic *C.elegans* to investigate the toxicity of MPP^+ to *C.elegans.* The results showed that MPP^+ caused wide range DA cell death and high worm lethality, which made the MPP^+-treated *C.elegans* to be an ideal model for PD research.

Although both in patients and in animal models MPP^+ can cause PD like syndrome, the mechanisms for its toxicity remains controversial. Some studies proved that ATP depletion was the primary reason and the oxidative stress was the secondary impact for its toxicity[14]. While other researches believed that MPP^+ toxicity was dependent on the accumulation/aggregation of α-synuclein, as siRNA against α-synuclein could fully rescue the toxicity[17]. Our results showed that in human α-synuclein transgenic worm, compared with the accumulation/aggregation of α-synuclein, ATP depletion contributes the most to MPP^+ toxicity, as up-regulation ATP level by OP50 uptake could rescue the worm from DA neurons loss and lethality. We also found that MPP^+ toxicity to transgenic *C.elegans* was not dependent on the accumulation/aggregation of α-synuclein, as OP50 itself could induce α-synuclein accumulation/aggregation without any toxic syndrome. What's more, the enhanced accumulation/aggregation of α-synuclein induced by OP50 uptake under MPP^+ treatment did not lead to more severe toxicity, but in the contrary protected the worm from the lethality. Collectively, these results implicate a crucial role for ATP depletion in MPP^+ toxicity via an α-synuclein transgenic animal model, in which α-synuclein accumulation/aggregation is not the necessary inducement.

It has been reported that MPP^+ is a DA specific toxin as it could only entered DA neurons by dopamine transporter. As *C.elegans* could live without DA neurons, so MPP^+, which was thought to be the DA neurons specific toxin, should not be lethal to *C.elegans*. Unexpectedly, in this study, MPP^+ caused high lethality in α-synuclein transgenic *C. elegans,* which reminded us DA neurons may not be the sole target for MPP^+, other cell types might also be damaged under MPP^+ treatment and finally lead to worm death. Better illustration of MPP^+ target will be helpful for PD research.

It is also very interesting for us to find that OP50 treatment alone could induce the accumulation/aggregation of α-synuclein. The following provide a possible explanation for this phenomenon. In the absence of food resource, *C.elegans* larval development will be arrested at L1 stage after hatch, during L1 arrest, worms nearly shut down all the protein synthesis and the autophagy will be activated to maintain the basic energy consuming[26]. This may be the reason why there was no protein accumulation in those groups without OP50. L1 arrest is a reversible process. When the arrested worms were afforded with food, they would perform further development. And protein synthesis would be up-regulated instantly after food supply, which might cause the protein accumulation of α-synuclein temporarily. However, this hypothesis needs further investigation.

Another question remained to be addressed is the role of α-synuclein accumulation/aggregation in the pathological progress of PD[27]. Although many studies demonstrated that α-synuclein (wide type or mutant) overexpression resulted in aberrant protein aggregation, oxidative stress and ultimately caused cell damage in various cultured cells[18]. However, increasing evidences implicate that large molecular aggregation could be a protective mechanism for the cells to be insulted[28]. At present, it is not entirely clear whether the accumulation/aggregation of α-synuclein is the causative reason for protection or it is just the byproduct of protecting pathway. But the present work provide addi-

tional evidence that the aggregation of α-synuclein in this transgenic *C.elegans* appears not the major cause of MPP^+ toxicity.

Our study highlights that MPP^+ can induce DA neurons death and worm lethality in human α-synuclein transgenic *C.elegans*, which could be used as a PD model. Compared with α-synuclein accumulation/aggregation, the primary reason for its toxicity to *C.elegans* is ATP depletion, because up-regulating ATP level by food uptake can rescue the worm from MPP^+ toxicity. It is hoped that further study of other potential targets for MPP^+ and the role of α-synuclein accumulation/aggregation in this *C.elegans* model will provide new thoughtful theory for etiology of PD and help to develop useful methods for its clinic therapy.

Acknowledgements: We appreciate Prof. Iwatsubo (University of Tokyo) for supplying the α-synuclein transgenic worms. The α-actin antibody was kindly provided by Prof. Bing Sun (Shanghai Institute of Pasteur). This research was supported by a grant from the Health Center of Chinese Academy of Sciences.

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ATP 损耗是 MPP+ 引起 α-synuclein 转基因线虫多巴胺能神经元死亡和虫体死亡的主要原因

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摘要：目的 揭示环境神经毒素 MPP+ 对线虫的毒性影响并探讨其毒性机理。**方法** 以人源 α-synuclein 转基因线虫作为动物模型，用 MPP+ 处理该线虫，观察 MPP+ 对线虫多巴胺能神经元和其生存能力的影响。通过供给 OP50 以提高线虫体内 ATP 的水平，对比分析 ATP 水平、蛋白质异常沉积等重要指标，探讨二者在 MPP+ 引起的转基因线虫的病变中所起的作用。**结果** MPP+ 能够引起线虫多巴胺能神经元和线虫虫体的死亡；尽管进食 OP50 也会引起 α-synuclein 的沉积，但进食 OP50 能够提高体内 ATP 的水平并缓解 MPP+ 的毒性。虽无直接证据证明 α-synuclein 沉积对神经细胞的影响，但结果提示，在该转基因线虫中，与蛋白质的异常沉积相比，MPP+ 导致的 ATP 损耗是其毒性作用的最主要诱因。**结论** MPP+ 可以引起 α-synuclein 转基因线虫多巴胺能神经元的死亡和虫体的死亡，其毒性的主要原因是 ATP 损耗而不是 α-synuclein 的异常聚集（沉积）。

关键词：帕金森病；MPP+；ATP；α-synuclein；线虫