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Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research https://doi.org/10.1016/j.apjtm.2017.09.014

Neuron-protective effect of subanesthestic-dosage ketamine on mice of Parkinson's disease

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ARTICLE INFO

Article history:
Received 12 Jul 2017
Received in revised form 2 Aug 2017
Accepted 5 Sep 2017
Available online 18 Sep 2017

Keywords:
Parkinson's disease
Ketamine
Subanesthestic-dosage
MPTP
Autophagy

ABSTRACT

Objective: To discuss the neuron-protective effect and possible mechanism of subanesthestic-dosage ketamine on Parkinson's disease mice induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

Methods: A total of 30 mice were divided equally into three groups, model control group (MC group), ketamine treatment group (KT group), and blank control group (BC group), respectively. The Parkinson's disease mice of MC group and KT groups were established by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (20 mg/kg/d), while mice in KT group were treated by intraperitoneal injection of subanesthestic-dosage ketamine (8 mg/kg). Differences on behaviors and the number of nigra dopaminergic neurons of mice in each group were compared through the behavioral test and tyrosine hydroxylase immunohistochemistry experiments after the treatments. Furthermore, Western blot was used to test the expression of autophagy-related gene LC3-Ⅱ, Beclin1, Parkin, PINK1, and mTOR.

Results: Compared with the BC group, the neuroethology scores were lower and the amount of TH positive cells were less both in MC and MT groups; In KT group, the neuroethology scores were higher and the amount of tyrosine hydroxylase positive cells were significantly more than that in MC group ($P < 0.05$). Moreover, expression levels of autophagy-related proteins LC3-II, Beclin1, Parkin, and PINK1 were higher, while the mTOR expression level was lower than that in MC group.

Conclusions: The subanesthestic-dosage ketamine has some protective effects on the coordinating ability of movement and cognitive ability of Parkinson's disease mice induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. This is probably due to that the autophagy activity of cells is activated by subanesthestic-dosage ketamine and that the neurons are protected.

1. Introduction

Parkinson's disease (PD) is a neurological disease with long-term degenerative disorder of central nervous system that mainly affected the motor system. It has four essential characteristics, such as static tremor, rigidity, bradykinesia and postural unsteadiness [1], and the primary pathogenesis is that the level of dopamine is reduced by progressive death of neurons in the substantianigra pars compacta (SNpc) [2]. At present, genetic studies suggested that PD morbidity was closely associated with genetic factor, environment, abnormal accumulation of proteins, oxidative stress, and other factors [3–6], but the exact cause and pathogenesis was still not clear. Levodopa drugs are mainly used to improve PD's symptom clinically, but long-term use of levodopa can cause adverse reaction such as disorders in movement and cognition or 'on-off' phenomenon [7]. Therefore, it is the focuses of current research that seeking for the new neuron-protective drugs to treatment PD.

As a commonly-used antalgic anesthetic, ketamine is also the non-competitive antagonist of the *N*-methyl-D-aspartic acid receptor. For its complex pharmacological effects, different dose

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Peer review under responsibility of Hainan Medical University.
Foundation project: This study was supported by National Natural Science Foundation of China (No. 81600940) and the Science of Technology project of Henan, China (No. 142102310246).

and usage have different functions on the neuronal system [8]. The latest research showed that subanesthestic-dosage ketamine could promote the synthesis and transport of dopamine, protect neurons of PD rats and reduce disorders in the cognitive function after surgeries [9]. Currently, there are few studies on the effects of long-term use of subanesthestic-dosage ketamine on PD patients. In this study, C57BL/6 mice were treated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intraperitoneal injection to establish the PD models, and intervened by subanesthestic-dosage ketamine at the same time. Then, according to the results of behavioral tests and biochemical experiments, the influences of subanesthestic-dosage ketamine on behavior, cognition, and nigra dopaminergic neuron of PD mice were discussed.

2. Materials and methods

2.1. Animals

A total of 30 6–8 weeks old male C57BL/6 mice at SPF level (provided by Laboratory Animal Center of Henan Province, license number: SCXK (Yu) 2015-0004) were selected. The mice were randomly divided into three groups, model control group (MC group), ketamine treatment group (KT group), and blank control group (BC group), with 10 in each group.

2.2. Experiment methods

2.2.1. Modeling method

MPTP was used to establish the PD mice model. First day, each mouse in KT group received intraperitoneal injection of ketamine 8 mg/kg, while the same amount of normal saline was injected to each mouse in MC group. In the following 7 d, 8 mg/kg ketamine was injected to each mouse in KT group and the same amount of normal saline was injected to each mouse in MC group (the same as first day), 20 min later, each mouse was injected with 20 mg/kg MPTP in the two groups. For the BC group, 8 mg/kg normal saline was injected to each mouse for 8 d. Then 1–2 h later after the last injection, the behavioral tests and biochemical experiments were conducted in succession.

2.2.2. Behavioral tests

(1) Rotarod test: The duration that each mouse stayed on the rotating bar at different rotate rate was record, which was used to assess the behavior ability (balance ability). The rotate rate was set as 19 r/min, 26 r/min, and 38 r/min respectively. The maximum duration was limited with 5 min. The test was conducted 3 times at an interval of 1 min for each time and the average was calculated. (2) Swimming experiment: This experiment was used to test the limb coordination ability of mice. Each mouse was placed in an organic-glass water tank (20 cm × 30 cm × 20 cm), with 10 cm-deep water at the temperature of 22–25 °C. Standard for evaluation: 3 score (swimming without interruption), 2.5 score (swimming with occasional floating), 2 score (floating for half of the testing time), 1.5 score (swimming occasionally), and 1 score (occasionally swimming using their hind limbs and floating around). The test was conducted for 3 min at an interval of 2 min for each time, and then the average scores were taken. (3) Water meze test: Place navigation test was conducted to evaluate the learning and memorizing ability. Each mouse was put in to the water meze from the first quartile facing to the pool wall, and the time (Escape Latency, EL) used to find the platform was record during 2 min. This test was conducted twice a day, total 3 d, the average time was obtained from the 6 times.

2.3. Immunohistochemistry

After the behavioral tests, 30 mice were sacrificed and brain tissues were taken out and fixed in paraformaldehyde (4%) for 48 h. Then, paraffin imbedding, slicing, and conventional dewaxing were performed. The samples were hatched for 10 min in H_2O_2 (3%), and then rinsed by PBS. After the tissue antigen recovery, the tissues were kept with fetal bovine serum (5%) for 30 min, and hatched overnight by rabbit-anti TH monoclonal antibody (1:300) at 4 °C. Then, the secondary antibody was incubated (1:500) for 40 min. DAB colored, and conventional sealing were performed. Five blocks were randomly selected from each group, 3 consecutive slices at the same area of each block were selected, and 3 pictures were randomly selected from each slice. The average number of SNpc TH-positive neurons in each group was calculated.

2.4. Western blot

The total brain tissue protein was extracted by tissue protein extraction kits, and concentration was detected by BCA. The expression of mTOR, LC3-II, Beclin1, Parkin and PINK1 were detected by Western blot. Primary antibody: mTOR polyclonal antibody, LC3 polyclonal antibody, Beclin1 polyclonal antibody, Parkin polyclonal antibody, PINK1 polyclonal antibody (1:1 000), and rabbit anti-mouse β-actin antibody (Abnova) (1:2 000). Second antibody: HRP goat anti rabbit IgG or HRP goat anti mouse IgG (1:3 000). And relative expression quantity of each protein was calculated by Image J 2x.

2.5. Statistical analysis

All data was analyzed by SPSS 19.0. Measurement data was shown as average ± standard deviation (mean ± SD) and K−S was used to check whether the measurement data was consistent with the normal distribution. If yes, independent *t*-test was used to analyze the differences between two groups, and one-way ANOVA was taken to analyze the differences among three groups. If not, rank-sum test was applied to analyze the difference. $P < 0.05$ was considered that the difference was statistically significant.

3. Results

3.1. Behavior test results

The results of behavior tests were shown in Table 1. Compared with BC group, there were various degrees of neurobehavioral changes in the mice of MC and KT groups after MPTP intraperitoneal injection, such as tremor, hollow back, hypotonia, hypokinesia, which was the typical parkinsonian symptom. There were significant differences in the behavioral abilities among BC group, MC group and KT groups. Compared with MC group, mice in KT group were with significantly longer stay on the rotating bar, shorter average latency in the water meze test, and higher score in the swimming test. The results

Table 1
Behavior test results.

Group	Rotrod test (s)			Water meze test (s)	Swimming test (score)
	19 rpm	26 rpm	38 rpm		
MC group	157.2 ± 12.0*#	109.8 ± 14.2*#	78.8 ± 12.0*#	68.3 ± 6.2*#	1.8 ± 0.3*#
KT group	235.4 ± 12.9	200.9 ± 20.8	167.3 ± 16.5	34.5 ± 6.5	2.4 ± 0.5
BC group	247.4 ± 14.2	209.6 ± 18.3	178.8 ± 16.2	22.3 ± 6.1	2.7 ± 0.4
F	140.076	69.203	132.538	145.606	9.742
P	0.000	0.000	0.000	0.000	0.001

* Compared with BC group, the difference was statistically significant ($P < 0.05$). # Compared with KT group, the difference was statistically significant ($P < 0.05$).

Table 2
Comparison of relative expression level of autophagy proteins (the ratio of β-actin).

Group	mTOR	LC3-II	Beclin1	Parkin	PINK1
MC group	2.43 ± 0.10*#	0.52 ± 0.06*#	0.54 ± 0.06*#	0.35 ± 0.03*#	0.37 ± 0.05*#
KT group	1.36 ± 0.15*	0.86 ± 0.09*	0.78 ± 0.05*	0.64 ± 0.07*	0.69 ± 0.05*
BC group	1.02 ± 0.15	0.94 ± 0.07	0.89 ± 0.07	0.72 ± 0.06	0.78 ± 0.06
F	30.541	86.756	70.344	126.721	145.065
P	0.000	0.000	0.000	0.000	0.000

* Compared with BC group, the difference was statistically significant ($P < 0.05$). # Compared with KT group, the difference was statistically significant ($P < 0.05$).

proved that subanesthestic-dosage ketamine improved coordinating ability of movement, learning and memory ability of PD mouse.

3.2. Immunohistochemical results

There were a large number of TH-positive neurons in SNpc in BC group. The numbers of TH-positive neurons in SNpc in BC group, KT group and MC group were 59.8 ± 4.2, 55.7 ± 3.8 and 41.3 ± 3.0, respectively. There were significant differences in the number of TH-positive neurons among the three groups ($P < 0.001$). Compared with BC group, the number of TH-positive neurons in MC group and KT group was significantly decreased, and the number of TH-positive neurons in KT group was significantly more than that in MC group. The results indicated that ketamine prevented TH-positive neurons from being attacked by MPTP.

3.3. Expression of autophagy-related proteins

Compared with BC group, the expression level of LC3-II, Beclin1, Parkin, PINK1 in MC and KT groups were significantly lower, and the mTOR was markedly higher ($P < 0.05$). Compared with MC group, the expression level of LC3-II, Beclin1, Parkin, PINK1 in KT group was higher and the mTOR protein was lower, and the difference was statistically significant ($P < 0.05$) (Table 2). The results indicated that ketamine improved the expression levels of LC3-II, Beclin1, Parkin, and PINK1 and reduced the expression of mTOR which could be speculated that the subanesthestic-dosage ketamine could enhance the autophagy.

4. Discussion

PD was one of the most common neurodegenerative disorder in the elderly. In the research of PD-forming mechanism and prevention, C57BL/6 mice induced by MPTP was the most common PD animal models [10]. MPTP could cross the blood brain barrier and turn into the neurotoxic metabolite 1-methyl-4-phenyl-pyridinium (MPP^+) with the neutralization of monoamine oxidase B, then MPP^+ suppressed the autophagy activity of cells and increased mitochondria oxidative damage and resulted in neuron death or apoptosis [11,12]. In this study, the amount of nigra dopaminergic neurons was significantly decreased while mice treated by MPTP and the mice showed typical PD characteristic, which illustrated that MPTP could cause neuron death and PD symptom.

As an anesthetic drug commonly used in clinical, Ketamine functioned well on analgesia and anesthesia. There was no consistent conclusion at present about the effect of ketamine on the cognitive function. William *et al.* [13] found that heavy use of ketamine for a long time had neurotoxicity on differentiation and development of neural stem cells of rat, eventually damaging their neurological function. Bosnjak *et al.* [14] had proved that ketamine time- and dose-dependently induce human neurotoxicity at supraclinical concentrations via ROS-mediated mitochondrial apoptosis pathway and that these side effects can be prevented by the antioxidant agent Trolox. On the contrary, Dorandeu *et al.* [15] showed that ketamine have anti-epilepsy and neuron-protective functions in clinical studies. Besides, Kakinohana [16] also found that ketamine had neuron-protective effect on PD mouse models induced by MPTP and 6-OHDA. The statistical results of Hudetz *et al.* [17] showed that subanesthestic-dosage ketamine could promote synthesis of dopamine and improve the recognition ability. Some Chinese scholars also suggested that subanesthestic-dosage ketamine could suppress inflammatory or decrease the expression level of apoptosis-related gene, which reducing neuron apoptosis or protecting the neuron [18,19]. In this study, the coordinating ability of movement of mice in KT group was better than that in MC group. In the immunohistochemistry experiment, the number of nigra TH-positive neurons in KT group was significantly more than that in MC group, which indicated that subanesthestic-dosage ketamine could suppress dopaminergic neuron apoptosis in PD mice induced by MPTP, and prevent nerve tissues from being damaged, reduce disorders of PD mice in movement and cognitive function.

Mitochondria dysfunction was the typical pathological feature of PD neuron cells, which could cause selective death of neurons in substantianigra of midbrain. Autophagy pathway was the necessary channel to remove the abnormal mitochondria and protein aggregates. To explore whether subanesthestic-dosage ketamine had neuron-protective function, the expression levels of autophagy-related protein: mTOR, LC3-II, Beclin1, Parkin, and PINK1 were detected. Parkin could reduce peroxides which generated during the dopamine metabolism, remove damaged mitochondria and prevent apoptosis [20]. PINK1 could promote survival of neurons through proteasome and autophagy [21], and the signaling pathway of PINK1-Parkin was essential for mitophagy [22]. The expression level of LC3-II and Beclin1 could reflect the autophagy activity [23,24]. As a negative regulatory factor, mTOR interacted with autophagy proteins and affected the forming of autophagosome [25]. In this study, the result of Western blot showed that, compared with MC group, expression level of mTOR in mice brain tissues in KT group was significantly decreased, while that of LC3-II, Beclin1, PINK1, and Parkin were increased. This indicated that subanesthestic-dosage ketamine could up-regulate the expression of LC3-II, Beclin1, PINK1 and Parkin, down-regulate the expression of mTOR, and enhance autophagy. Therefore, subanesthestic-dosage ketamine could remove damaged or superfluous proteins and organelles by activating mitophagy or cell autophagy signals, degrade proteins aggregated abnormally, and offer protection against neurotoxicity, consequently affecting the changes in the behavior, morphology and cognition function of PD mice.

In conclusion, this study showed that subanesthestic-dosage ketamine had neuron-protective effects on PD mice, the mechanism may be that it could activate mitophagy or cell autophagy signals to protect dopaminergic neurons and improve the coordinating ability of movement and cognition function. Therefore, subanesthestic-dosage ketamine may become a new choice for PD. However, the curative effects and mechanism of subanesthestic-dosage ketamine on PD still need further study. The time and dose dependent effect of Ketamine indicates that it has two side for neurological function, so the best dosage and use regimen of ketamine should be verified for further study.

Conflict of interest statement

All the authors declared no conflicts of interest with regard to this study.

Acknowledgments

Thanks for the support of National Natural Science Foundation of China (No. 81600940) and the Science of Technology project of Henan, China (No. 142102310246).

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