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Research Article

Effects of Electroacupuncture on Behavioral deficits, Hippocampal Neuronal Death and Oxidative Stress in Rats with Parkinson's Disease

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Abstract

We aimed to study electroacupuncture (EA) for its potential neuroprotection against oxidative stress, death of hippocampal neurons and behavioral deficits in rats with Parkinson's disease (PD). PD was induced by rotenone (2 mg/kg, subcutaneous injection) in male Wistar rats. EA group, control group, miR-331-5p antagomir group, model group and EA + miR-331-5p antagomir group were set up. The novel object recognition tests together with shuttle box tests were conducted to examine the cognitive function, and the pain sensitivity was measured by the tail flick test. In comparison to the control group, the cognitive function and thermal pain threshold significantly declined in the model group. The model group had decreased superoxide dismutase (SOD) and superoxide dismutase (SOD) activities, decreased brain-derived neurotrophic factor (BDNF) expression and increased malondialdehyde (MDA) level in the hippocampus. More hippocampal neuronal loss was detected from the model group. EA increased the hippocampal GPx plus SOD activity and decreased the MDA content. EA or miR-331-5p antagomir raised the quantity of alive hippocampal neurons and the BDNF expression. EA can prevent rotenone-induced non-motor disorders through antioxidation and neuroprotective functions.

Keywords: Electroacupuncture, miR, Neuroprotection, Oxidative stress, Parkinson's disease

Introduction

Belonging to a multi-system neurodegenerative illness, Parkinson's disease (PD) manifests motor or non-motor symptoms (NMSs) as the features. Known as a most frequently occurring NMS in PD patients, cognitive impairment results from neuronal injury in memory-related cerebral regions such as the hippocampus [1]. Studies have been extensively carried out on the hippocampal function and structure in PD sufferers, with the hippocampal volume decrease accompanied by cognitive impairment reported in some cases [2]. With an estimated prevalence of 68-95%, PD is characterized by pain as an additional common NMS, reducing patients' quality of life [2]. Pain in PD has a multifactorial etiology, and there has been no proper treatment yet at present.

Electroacupuncture (EA) is a noninvasive complementary therapy with application to relieve PD symptoms for a long time [3]. Through the normalization of basal ganglia neurotransmitters [4,5], EA stimulation at a high frequency (100 Hz) suppresses neuroinflammatory responses [6], alleviates oxidative stress as well as increases neurotrophic factor levels [7,8], thereby improving the movement function of diversified PD animal models. However, the exact mechanism of EA in relieving the motor symptoms of PD has not been clearly explained. For PD, miR-331-5p, as an exosomal miRNA, has become a potential biomarker. Its elevated expression may play a crucial role in the pathological progression of PD [9,10]. However, no reports are available on the role or function of miR-331-5p in the progression of PD.

Antagomir is an oligonucleotide that specifically inhibits the function of miRNA and can regulate the expression of target genes by binding and suppressing miRNA activity [11]. Therefore, we intended to test the role of miR-331-5p antagomir or EA in the progression of rotenone-induced PD in rats from the aspects of oxidative stress, neural damage and neural function. Additionally, EA has never been combined with miR-331-5p inhibition in the context of PD until now. This study may thus provide a novel strategy for addressing the multifaceted pathophysiology of PD.

Material and Methods

Ethical Approval

This study has been approved by the animal ethics committee of our hospital on June 28th, 2022 (Approval No. CMC202206003), and great efforts have been made to minimize the animals' suffering.

Reagents

Dimethyl sulfoxide (DMSO), ethanol and xylene sourced from Merck (Germany). Rotenone was provided by Sigma (USA). ELISA kit for brain-derived neurotrophic factor (BDNF) was bought from Wuhan Boster Biological Technology Co., Ltd. (China).

Laboratory Animals

The Laboratory Animal Research Center of Cangzhou Medical College (China) supplied male Wistar rats (n=70, SPF grade, 200-220 g) for breeding with a 12/12 h dark/light cycle, controlled humidity (50-55%) and temperature (22±2°C) as well as freely offered water plus standard food.

Experimental Design

An EA group, a miR-331-5p antagomir group, a model group, an EA + miR-331-5p antagomir group, and a control group were set (n=14). Seven rats of each group were used for inhibitory passive avoidance memory test, novel object recognition test and tail flick test. The remaining seven rats of each group were sacrificed for hippocampus-related experiments. Subcutaneous injection for 5 consecutive weeks with 98% sunflower oil plus 2% DMSO (1 mL/kg) was performed on rats in the control group once daily. In the model group, the rats were daily given 2 mg/kg rotenone (5 consecutive weeks of subcutaneous injection) once. In the EA group, the rats received 5 consecutive weeks of EA stimulation (100 Hz) at 1 h before subcutaneous injection of 2 mg/kg rotenone, once daily. Briefly, the acupoints Baihui (GV20, located at the midpoint of the line connecting the apexes of both ears) and Dazhui (GV14, located just underneath the cervical spinous process) were separately punctured using a stainless-steel EA needle (diameter: 0.25 mm) by 5 mm in depth. Next, a Han's acupoint nerve stimulator (HANS, Neuroscience Research Institute, Peking University) was employed to produce bi-directional square-wave electrical pulses (duration: 0.2 ms, 100 Hz), which were delivered for 30 min per day to the rats for 5 consecutive weeks (6 days weekly). The stimulation intensity gradually rose to 3 mA from 1 mA to 2 mA, which lasted for 10 min per time. The rats remained under an awake and unrestricted state in cages during EA. In the miR-331-5p antagomir group, at 1 h prior to 2 mg/kg rotenone subcutaneous injection, 10 mg/kg miR-331-5p antagomir was subcutaneously administered once daily to the rats for 5 consecutive weeks. In the EA + miR-331-5p antagomir group, at 1 h previous to 2 mg/kg rotenone injected subcutaneously, the rats received 5 consecutive weeks of EA stimulation (100 Hz) and miR-331-5p antagomir (10 mg/kg, subcutaneous injection) once daily.

For the purpose of PD induction in rats, rotenone at 10 mg/mL in concentration was prepared by means of dissolution in DMSO first and then dilution using sunflower oil (98% sunflower oil plus 2% DMSO), which was once daily administrated at 2 mg/kg *via* subcutaneous injection for 5 weeks. The preparation of fresh solution was conducted at an interval of 3-4 days [12]. The rats from the control group merely received DMSO together with sunflower oil, and the death rate was zero.

Inhibitory Passive Avoidance Memory Test

The rats were assessed by passive avoidance memory test on the passive avoidance memory using the shuttle box apparatus manufactured by Borj Sanat Azma (Teheran, Iran). The apparatus consisted of brightly illuminated and dark chambers of the identical size (40x40x30 cm), a stainless-steel bar floor (diameter: 2.5 mm, and spacing: 1 cm) in connection with an electric shock generator, and an opaque guillotine door (7x9 cm) utilized to separate the chambers. 24 h before the test, each rat was habituated to the apparatus for 5 min. There were two sessions (training and testing) completed in two days in a row. Specifically, the brightly illuminated chamber equipped with an elevated guillotine door was applied to accommodate the rat that was permitted to access the dark chamber during the training session. Then with the guillotine door installed therein dropped, the rat was subjected to 3 s of constant current shock (50 Hz, 1 mA). Finally, the rat was placed back to the home cage after being taken out from the dark chamber 30 s later. After 120 s, the aforementioned steps were repeated again. The training was stopped in the case of 2-min residence of the rat in the brightly illuminated chamber, and successful passive avoidance learning was recorded. In contrast, the shock was applied in the same way again when the rat went back to the dark chamber within 2 min. Each rat received training three times at most. Twenty-four hours after training, the memory retention test was performed without applying electric shock. The rat entered the brightly illuminated chamber again, and step-through latency (300 s at most) was acquired by recording the latency of passing through the guillotine door [13].

NOR Test

A black cubic box (60×60×20 cm) was used as an open field to carry out the NOR test. Specifically, the rat located in the box was allowed for 5 min exploration of two exactly the same objects on the first day. 24 h later, the rat entered the box containing the two familiar objects

again, one of which was substituted using a "novel" object. The rat explored the novel object and familiar object for 5 min, with the time for probing the two different objects recorded. The duration from the rat sniffing the object or staying within 2 cm surrounding the object was determined as the object exploration time. Data were calculated based on the formula below [14]: (time of probing the novel object/[time of probing the novel object + time of probing the familiar object] x 100%).

Tail Flick Test

A tail flick apparatus provided by Borj Sanat Azma (Iran) was adopted for the tail flick test to measure rats' pain threshold. Briefly, the rat was released in a Plexiglas restrainer, with its tail fixed in a groove below the radiant heat source. Tail flick latency was defined as the latency to withdraw the tail from the light beam. As for the setting of light intensity, the baseline brightness of 4.5-5.5 s was provided for the whole animals through illuminating on a light spot at 8 cm away from the tail tip. Tissue injury was minimized by a cut-off period lasting for 10 s. Tail withdrawal time of each rat was recorded three times, with an interval of 5 min and the average value obtained [13].

Sample Acquisition and Processing

When the behavioral tests were completed, the rats had a deep and permanent anesthesia before being quickly decapitated in a non-stressful environment. The hippocampal tissue was extracted from the brains for homogenization using ice-cold phosphate-buffered saline (PBS, 0.1 M) after being harvested on ice. After that, the homogenate was subjected to 10-min centrifugation (4°C and 3000 rpm). The resulting supernatant was aspirated and then preserved at -80°C for later biochemical assays.

Malondialdehyde (MDA) Content Detection

MDA content in the hippocampus was measured for lipid peroxidation quantification. Specifically, hippocampal samples (50 µL) were mixed with thiobarbituric acid (6%, 100 µL) and trichloroacetic acid (20%, 150 µL) prior to incubation at 95°C for 20 min in a boiling water bath. Sample centrifugation at 5000 rpm was executed for 5 min subsequent to cooling on ice. Next, a clean microplate was utilized to transfer the clarified supernatants in a volume of 200 µL, and the 535 nm wavelength was examined by a spectrophotometer (Bio-Tek, USA) to obtain the absorbance. Finally, MDA level was calculated as nmol/mg protein according to the 1,1,3,3-tetraethoxypropane-prepared standard curve.

Activity Determination of Glutathione Peroxidase (GPx)

The RANSEL assay kit (Randox Laboratories Ltd., UK) was applied to detect the GPx activity (in U/mg protein).

Activity Assay for Superoxide Dismutase (SOD)

The SOD activity was measured using the Naoyuki Taniguchi's method according to the capability of SOD to repress the reduction of nitroblue tetrazolium (NBT) dye triggered by superoxide anions resulting from hydroxylamine hydrochloride autoxidation. A reaction mixture containing NBT (24 µM), sodium carbonate (50 mM, pH 10.2), Triton X-100 (0.03%, 30 µL), and EDTA (0.1 mM) was prepared in order to assess the SOD activity. The reaction was started in a brain homogenate (10 µL)-containing cuvette added with the reaction mixture and hydroxylamine hydrochloride (1 mM). The absorbance at 560 nm was measured to calculate the reduction rate of NBT complex, with the results expressed as IU/mg protein.

Determination of BDNF Level

The mouse BDNF PicoKine™ double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Boster Biological Technology, Ltd., China) was used to measure the BDNF level in the hippocampus (pg/mg protein [15]). A multifunctional microplate reader (Bio-Tek, USA) was applied to test the 450 nm wavelength to acquire the absorbance.

Histological Analysis

Brain neurons were subjected to the cresyl violet staining for histological assays and to quantify neuronal injury. In brief, the rats were given Nesdonal overdose (100 mg/kg) to induce anesthesia. They were subsequently transcardially perfused with 0.9% saline and then 4% paraformaldehyde in a volume of 50 mL and 200 mL, respectively. Later, brains were immediately removed for overnight fixation with the same fixative. After dehydration using gradient alcohol, brain samples were subjected to xylene transparentization plus paraffin embedding. Afterwards, a cryostat (Leica, Germany) was utilized to prepare paraffin-embedded samples into 5-µm sections in the coronal plane. The serial brain sections underwent 0.1% cresyl violet staining following translocation to slides. Then a light microscope (BX-50 F, Olympus, Japan) for observation (40x) and a digital camera for photography were used for the sections. The hippocampal CA1, CA3, and dentate gyrus (DG) regions were selected for manual counting of Nissl-stained cells in three fields of each section. The average of three measurements from every region was taken as the final value.

Statistical Analysis

Statistical analysis and plot drawing were accomplished by GraphPad Prism 8.0 software (GraphPad Software, USA). Data were described by mean ± standard error of the mean (SEM). Comparisons among groups were performed through one-way analysis of variance, with

the Tukey's post-hoc test for analysis. P<0.05 denoted a difference with statistical significance.

Results

Passive Avoidance Test Results

The initial latency showed insignificant differences among the control group, model group, EA group, miR-331-5p antagomir group and EA+miR-331-5p antagomir group (4.32±0.61, 4.52±0.76, 3.81±0.69, 3.78±0.53 and 3.56±0.98 s) (P>0.05). The model group presented significantly shortened step-through latency by contrast to the control group (P<0.05), as well as to the EA and miR-331-5p antagomir groups (P<0.05), whereas it was significantly increased in the EA + miR-331-5p antagomir group in comparison to that in the model group (P<0.01) *(Table 1)*.

NOR Test (Learning Memory) Results

Compared to the model group, significant increases in DI were observed in the control group (P<0.05), together with in the EA group, miR-331-5p antagomir group and EA + miR-331-5p antagomir group (P<0.01) *(Table 2)*.

Tail Flick Test Results

The model group exhibited a significantly shorter tail flick latency than the control group (P<0.05) and the EA, miR-331-5p antagomir and EA + miR-331-5p antagomir groups (P<0.01, P<0.05, P<0.01) *(Table 3)*.

Oxidative Stress in the Hippocampus

The model group, compared with the control group, had a significantly higher MDA level and significantly lower SOD and GPx activities (MDA: P<0.05, SOD: P<0.05, GPx: P<0.05). The EA, miR-331-5p antagomir and EA + miR-331-5p antagomir groups displayed a significantly reduced MDA level along with significantly enhanced SOD and GPx activities compared to the model group (MDA: P<0.05, SOD: P<0.05, GPx: P<0.05) *(Table 4)*.

BDNF Level in the Hippocampus

In comparison to that in model group, the BDNF level in the hippocampus was significantly elevated (P<0.01), and climbed in the EA group, miR-331-5p antagomir group and EA + miR-331-5p antagomir group (P<0.05, P<0.05, P<0.01) *(Table 5)*.

Table 1. *Step-through latency (n=7)*

Indicator	Control Group	Model Group	EA Group	MiR-331-5p Antagomir Group	EA + MiR-331-5p Antagomir Group
Step-through latency (s)	239.31±20.17	47. 22±7.36*	98. 14±8.62#	101.28±10.52#	150.58±11.98##

**P<0.05 vs. control group, *P<0.05, **P<0.01 vs. model group*

Table 2. *DI values (n=7)*

Indicator	Control Group	Model Group	EA Group	MiR-331-5p Antagomir Group	EA + MiR-331-5p Antagomir Group
DI (%)	78.12±6.16	44. 25±4.31***	62. 12±8.29#	64.26±10.31#	76.21±8.21#

**P<0.05 vs. control group, #P<0.05, ##P<0.01 vs. model group*

Table 3. *Tail flick latency (n=7)*

Indicator	Control Group	Model Group	EA Group	MiR-331-5p Antagomir Group	EA + MiR-331-5p Antagomir Group
Tail flick latency (s)	4.43±0.48	2. 94±0.31*	6. 06±0.58#	6.22±0.63#	8.25±0.53##

**P<0.05 vs. control group, #P<0.05, ##P<0.01 vs. model group*

Table 4. *Oxidative stress levels in the hippocampus (n=7)*

Indicator	Control Group	Model Group	EA Group	MiR-331-5p Antagomir Group	EA + MiR-331-5p Antagomir Group
MDA (nmol/mg)	1.68±0.15	5. 21±0.62*	3. 04±0.58#	2.98±0.61#	2.25±0.31#
GPx (U/mg)	64.43±7.14	28.64±3.12*	48.98±6.15#	46.05±6.27#	69.68±7.28#
SOD (U/mg)	60.23±7.19	28.64±3.11*	39.68±4.15#	41.23±4.21#	59.02±7.18#

**P<0.05 vs. control group, #P<0.05, ##P<0.01 vs. model group*

Table 5. BDNF level in the hippocampus (n=7)					
Indicator	**Control Group**	**Model Group**	**EA Group**	**MiR-331-5p Antagomir Group**	**EA + MiR-331-5p Antagomir Group**
BDNF (pg/mg)	68.27±7.12	32.63±4.14**	48.62±4.12#	51.26±5.21#	71.13±8.39##
*P<0.05 vs. control group, #P<0.05, ##P<0.01 vs. model group					

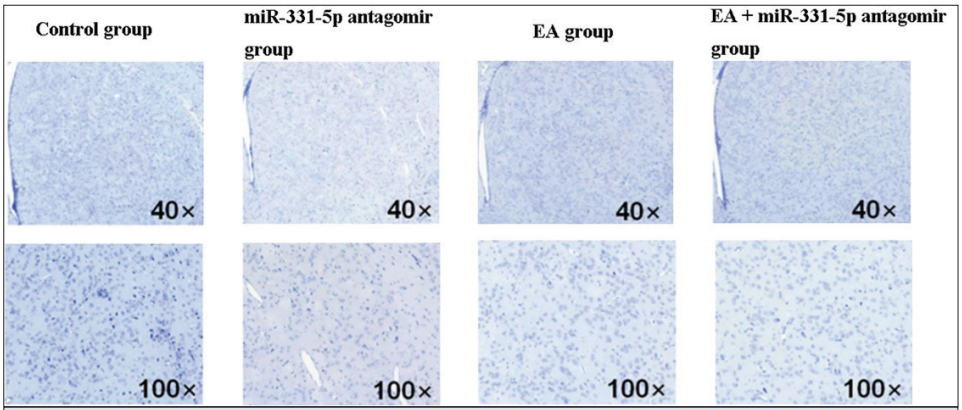

Fig 1. Light microscopic images for histological changes of sections of hippocampal CA1, CA2, CA3 and DG regions subjected to cresyl violet staining

Histological Changes of the Hippocampus

Cresyl violet staining was performed on the histological changes of the hippocampus. The results showed that compared with those in the control group, EA group, miR-331-5p antagomir group and EA + miR-331-5p antagomir group, significant drops in Nissl-stained cell counts in the hippocampal CA1, CA2, CA3, and DG regions were observed in the model group *(Fig. 1)*.

Discussion

As a complementary and alternative therapy with the most extensive application, EA has been adopted to treat PD for a long period of time [16]. As revealed by increasingly more basic studies, EA is capable of alleviating central (vascular dementia, animal models of ischemic stroke, PD, Alzheimer's disease, spinal cord injury, traumatic brain injury, *etc.*) and peripheral (such as post-surgical injury or lipopolysaccharide injection) neuroinflammation [17]. For example, Cai et al.[18] reported that EA reduced cognitive impairment through anti-neuroinflammation in PD animal models. EA ameliorates intestinal motility disorders by regulating the 5-HT4R-mediated cAMP/PKA signaling in Thy1-αSyn mice with PD [19]. In addition, EA has protective effects on dopaminergic neurons from the murine substantia nigra of PD model, which may be related to its regulatory effects on ferroptosis-induced oxidative stress and apoptosis [20]. The present research was designed to probe into the neuroprotective role of EA in rotenone-induced cognitive impairment and neuronal injury. The results revealed that EA with antioxidant properties could efficiently relieve rotenone-triggered non-motor deficits plus neuronal injury. Hence, antioxidants may become a useful therapeutic approach for rotenone toxicity by lowering neuroinflammation and oxidative stress in the neurons.

One of the predominant symptoms in the rotenone-induced PD model is motor impairment. Rotenone neurotoxicity is associated with non-motor deficiencies involving depression, sleep disorders, and cognitive impairment in addition to motor dysfunctions [21,22]. Current studies have revealed through assessment by the passive avoidance and NOR tests that rotenone can induce memory impairment. This study yielded identical findings to earlier findings that rotenone exposure impairs memory *via* disruption of the blood brain barrier (BBB) mediated by microglia [23]. According to these research, oxidative stress created by rotenone disrupts the BBB and causes neuroinflammation, neuronal damage, and cognitive deficits [24]. In this study, it was found that the 5-week administration of EA alone or EA plus miR-331-5p antagomir could prevent memory impairment in the passive avoidance memory test. The NOR test showed that EA also relieved rotenone-induced cognitive disorder by increasing DI. As consistently denoted in reports, EA can mitigate amyloid-beta pathology and cognitive impairment in Alzheimer's disease by a novel mechanism involving activation of transcription factor EB [25]. Geng et al.[26] found that EA took advantage of the TRPC1 and SIRT1/AMPK signaling pathways to ameliorate mitochondrial dysfunction besides neuronal injury in PD mice. Therefore, EA becomes a crucial participant in preventing cognitive impairment. EA's ability to reduce rotenone-induced neuroinflammation may possibly be

responsible for its memory-enhancing properties due to its antioxidant effects.

With 40-85% of patients experiencing pain, it serves as a most ubiquitous NMSs in PD [27]. Research has indicated that malfunction in the mitochondrial electron transport chain (ETC) increases free radicals together with oxidative stress to participate in neuropathy [28]. This study demonstrated *via* the tail flick test that rotenone, a recognized inhibitor of mitochondrial complex I, enhanced the thermal pain sensitivity. These results demonstrated that various forms of pain are produced by mitochondrial malfunction and oxidative stress, and they also suggested that antioxidant medications are probably conducive to preventing and curing pain [29]. For example, activation of Nrf2 mediates the antiallodynic effect of EA on the rat model of type I complex regional pain syndrome by reducing local oxidative stress and inflammation [30]. In this study, it was uncovered that EA alone or EA plus miR-331-5p antagomir significantly increased the pain threshold of animals treated with rotenone, and also interestingly prolonged tail flick latency by contrast to the control group.

Rotenone represses mitochondrial ETC complex I to impair adenosine triphosphate production while strengthening reactive oxygen species (ROS) generation [31]. The process where ROS induces oxidation of polyunsaturated fatty acids is defined as lipid peroxidation [32]. Lipid peroxidation produces MDA, a genotoxic byproduct capable of creating stable microtubules through the coupling with DNA bases plus proteins, which can cause neuroinflammation and cell death. The results of the biochemical tests in this study showed that rotenone raised the MDA level in the hippocampus. Furthermore, rotenone dramatically reduced the activities of GPx and SOD in the rat hippocampal regions. The primary antioxidant enzymes that are crucial in scavenging and getting rid of free radicals are SOD and GPx [33]. Since rotenone inhibits mitochondrial complex I and destroys the antioxidant system, it can therefore cause an accumulation of free radicals and oxidative damage. EA alone or EA plus miR-331-5p antagomir significantly decreased the MDA level while enhancing the hippocampal SOD and GPx activities in rats, consistent with the findings of Li et al.[20] that EA reduced the MDA level in PD models. By the deactivation of free radicals and ROS as well as the boosting of endogenous antioxidant enzymes, EA may lessen lipid peroxidation.

It is well known that rotenone induces motor disorders by selectively degenerating dopaminergic neurons from the substantia nigra pars compacta of the PD model. The PD-associated cognitive impairment often has a correlation with hippocampal neuronal injury [34]. According to recent studies, rotenone also causes hippocampal neuronal apoptosis in the PD model in addition to memory impairment [35]. It was found by histological analysis through the present research that in the hippocampal CA1, CA3, and DG regions, rotenone exacerbated neuronal loss. By triggering microglia, rotenone contributes to the neuroinflammatory response and disrupts the BBB, which may result in neuronal death and memory loss [24]. In addition, EA alone or EA plus miR-331-5p antagomir mitigated the apoptosis of neurons from the CA1, CA3 and DG regions of hippocampus.

Concerning the cognitive function, BDNF functions in neuronal survival, neurogenesis and synaptic plasticity as an essential neurotrophin [36]. Cognitive impairment may be the result of neuronal death caused by decreased levels of BDNF [37]. In this study, the hippocampal BDNF expression decreased in rats administered with rotenone, consistent with previous findings, revealing that rotenone can lower the hippocampal BDNF expression [38]. Nevertheless, the exact chemical mechanism behind the reduction in BDNF expression after rotenone therapy remains unclear. Moreover, it was uncovered by the present research that EA could ameliorate the rotenone-induced reduction in hippocampal BDNF expression. This signifies that EA achieves a neuroprotective effect possibly by increasing the hippocampal BDNF expression.

Nevertheless, this study is limited. Whether there is a synergistic relationship between EA and miR-331-5p antagomir is not studied, which still needs further validation.

In conclusion, EA had a positive impact on non-motor deficits resulting from rotenone-induced neurotoxicity. EA reduced rotenone-induced thermal hyperalgesia and memory impairment. Furthermore, EA reduced the hippocampal MDA (lipid peroxidation product) content and prevented neuronal injury in rats treated with rotenone. These findings demonstrate that the antioxidant properties of EA partly mediate its ability to ameliorate memory impairment and neuronal injury induced by rotenone.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (Y. Yue) on reasonable request.

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Competing Interests: There is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The article, tables and figure were not written/created by AI and AI-assisted technologies.

Authors' Contributions: Y. Y. designed and supervised the study,

and significantly revised the paper. D. L. performed this study and drafted the paper. Both authors have approved the submission and publication of the paper.

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