Effects of zinc oxide nanoparticles on neuronal response of pyramidal neuron of the CA1 hippocampus in rat model of parkinson's disease

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Abstract
Due to the nano size, zinc oxide nanoparticles (ZoN) easily pass through the blood-brain barrier and can enter the brain cells and have different effects on different parts of the nervous system. In the present study, the effect of zinc oxide nanoparticles on the electrical activity of pyramidal cells in the CA1 region of the hippocampus in Parkinson's disease-like rats has been investigated. In this experimental study, adult male Wistar rats as model were randomly divided into five groups: Substantia nigra pars compacta (SNC) lesion (the lesions were induced by (Intra Peritoneal (IP)) injection of Rotenone 2mg/kg/19day48h) and four groups of ZoN (lesions plus 0.5, 1, 1.5 and 3 mg/kg IP of ZoN. Spontaneous neural activity was recorded for all groups in the CA1 region of the hippocampus. The results revealed that IP injection of ZoN (1, 1.5 and 3 mg/kg) decreased neuronal spontaneous activity in the rat model of PD. The current study results suggested that acute IP injection of ZoN decreased neuronal response in the CA1 region of the hippocampal in a rat model of PD.

Keywords: Electrophysiology, Parkinson's disease, Rats, Rotenone, Zinc Oxide Nanoparticles

1. Introduction
Protein aggregation is a common histopathological feature in Parkinson's disease (PD) and Alzheimer's disease (AD). PD is characterized by accumulation of misfolded protein α-synuclein (α-syn) in components called Lewy bodies (LB) in dopaminergic neurons, leading to severe motor dysfunction. Alzheimer's disease is characterized by the abnormal accumulation of amyloid-β (Aβ) plaques and tau neurofibrillary tangles, resulting in brain damage that affects critical cognitive processes. Emerging clinical and experimental results support the hypothesis that pathological α-syn, Aβ, and tau are prion-like peptides/proteins that can induce the proliferation of endogenous monomers and cause the cell-to-cell spread of proteinopathy [10].

In pathological conditions, native α-syn undergoes a misfolding process from a soluble, random conformation to an insoluble, fibrillar form. When α-syn aggregates are misfolded, they localize to mitochondria, causing mitochondrial fragmentation and reduced membrane potential. Aβ peptide accumulation is thought to be the result of inefficient production of mitochondrial reactive oxygen species (ROS) and metal dyshomeostasis due to oxidative stress. In AD, the microtubule-associated protein tau is known to undergo abnormal hyperphosphorylation, leading to the binding of tau with prion-like activity. Although the mechanism and effects of tau crosslinking are still poorly understood, there is a promise that tau is an effective therapeutic target [6].

Similar pathological mechanisms in both diseases increase the production of ROS, leading to a cascade of oxidative stress. By increasing cellular stress, microglial reactions, and increased expression of inflammatory cytokines, both diseases significantly increase neuroinflammation, which is thought to lead to cell death and further protein/peptide accumulation. Biological mechanisms affecting PD and AD as described are misfolded protein aggregation, oxidative stress, inflammation, and cell death [9]. Despite decades of clinical trials using traditional therapies, the highly successful treatment of oxidative stress and protein misfolding in neurodegenerative diseases has been elusive. Fighting amyloidosis in both AD and PD with small molecules, peptides, and monoclonal antibodies in particular has had little success. This opens the door to nanomaterials with attractive physicochemical properties, stability, and multifunctionality to improve the understanding and treatment of diseases [8]. Nanoparticles (NPs) can enhance the transport of therapeutics across the blood brain barrier (BBB) during pathological conditions in PD and AD. Characteristics of diseased BBB include increased vascular permeability, reduced expression of tight junctions and BBB transporters, and accumulation of blood-derived cells and debris in perivascular spaces.
Such pathological conditions disrupt concentration gradient-based diffusion and reduce the function of carrier-mediated transport (CMT) and receptor-mediated transport (RMT) [9].

Zinc oxide nanoparticles are one of the most widely used nanoparticles in the fields of industry, medicine, and health. Due to their small size, nanoparticles have a greater ability to cross biological barriers and easily enter the brain.

The aim of the present study is to investigate the effect of zinc oxide nanoparticles on the electrical activity and neural response of pyramidal neurons in the CA1 region of the hippocampus in Parkinson's disease-like rats.

2. Methods
2.1. Study animals
In the present study, Adult male Wistar albino rats (220±20 g) were prepared and all experimental protocols were approved by the Ethics Committee of the Shahid Chamran University of Ahvaz (Ahvaz, Iran) and tested according to the NIH guidelines for the care and use of laboratory animals (International Institute for Health Publications No. 23-80; revised in 1978). In this section, mice were exposed to controlled humidity (50 ± 6%) and light conditions (12 hours light/dark cycle; light). The room temperature was set at 23±2 °C and food and water were freely available.

2.2. Study drugs
Rotenone (Sigma Aldridge), was dissolved in dimethyl sulfoxide (DMSO) and diluted with polyethylene glycol (PEG). Zinc oxide nanoparticles (manufactured by Tecnan, Spain, with a purity of 99.9% and a size of 20-30 nm) in the required doses daily and 30 minutes before the start of the experiment, dissolved in 0.9% physiological serum and with the help of an ultrasonic bath was dispersed and before each injection, the composition was again mixed for 1 minute by a shaker. The animals in the control group received saline.

2.3. Experimental procedure
In this research, the single unit recording method was used in anesthetized animals from the hippocampal CA1 region pyramidal neurons. The experiments were performed in a completely quiet room at a normal temperature of 25 ± 1°C. A total of 40 male Wistar rats were used in this experiment. There were five groups (n = 8) as follows: 1. Substantia nigra pars compacta (SNc) lesion was made by IP rotenone injection of 2mg/kg/19day/48h, 2. lesions + 0.5 mg/kg of ZnO (IP), 3. lesions + 1 mg/kg of ZnO (IP), 4. lesions + 1.5 mg/kg of ZnO (IP), 5. lesions + 3 mg/kg of ZnO (IP). The recovery period for the lesion group was seven days. After recovery, they were prepared for 120 mins single-unit recording, after baseline recording (15 minutes), ZnO or IP saline was injected and recording continued for 105 minutes thereafter. The change in firing activity of the recorded neurons after drug injection was calculated and interpreted as an indicator of the effect of the drug on the electrical properties of the neurons. The experimental design and groups of animals are shown in Fig. 1.

2.4. Induced Parkinson's disease model
To create Model D, the animals were first anesthetized with ketamine (78 mg/kg, IP, Alfasan, Netherlands) and xylacin (3 mg/kg, IP, Alfasan, Netherlands) and then the SNc was destroyed by rotenone injection. One week after surgery, the animals were prepared for electrophysiological testing and single-unit recording. A histological specimen confirming the degradation of SNc of PD was presented (Fig. 2).

2.5. Animal preparation and stereotactic surgery
Due to the fact that the use of ketamine for anesthesia blocks NMDA receptors and thus changes electrophysiological records. For this purpose, a substance that does not block brain receptors should be used to anesthetize animals. Urethane is a good material for this purpose. Animals were anesthetized with urethane (1.5 g/kg, IP; Sigma Aldrich, Germany) at supplemental doses (0.1 g/kg) every hour it needed to maintain a deep and stable level of anesthesia, as indicated by immobility. Response to strong tail pinching rats underwent tracheostomy to reduce respiratory impulses and maintain a stable airway waiting to be recorded. For this purpose, the hair on the front of the neck was shaved and an incision was made. The muscles and smooth tissue of the neck were then removed toward the trachea. A slit was made in the trachea and a polyethylene tube was placed in the lower part of the trachea and tightened with sutures. The animal was then gently placed in a stereotactic device (Stoelting, USA). The animals’ skins were cleaned to reveal the surface of the skull, and the Bregma spot was designated as a reference for stereotactic. A hole of 2 mm in diameter was created above the CA1 region (AP -3.8 mm, ML ± 2.2
mm, DV -2.4 mm) of the hippocampus. Body temperature was maintained at 36-37 °C for the entire experiment with a heating pad.

Fig. 1. Experimental design.
2.6. Extracellular single-unit recording and data acquisition

Extracellular recording of individual neurons was performed using tungsten microelectrodes (coated with Parylene, shaft diameter 127 μm, tip impedance 5 MΩ, Harvard device). The microelectrode was stereotactically transferred to the CA1 region of the hippocampus. Thereafter the electrode was moved slowly in the layer of pyramidal neurons using a microelectrode driver till a specific spike activity is recorded with a signal-to-noise ratio of >2 separations of the background activity. Spike signals were amplified (×10000 gain; 300 Hz, and 10 kHz for low and high filters, respectively) and displayed continuously on a storage oscilloscope as signals. The spike frequency was calculated and transmitted online in time bins of 1000 ms for the entire recording time by online sorter software (Spike; Science Beam, Tehran, Iran). The action potentials of the baseline activity were separated using a windows discriminator, which produced output pulses for single units based on the spike height, which calculated the number of spikes per unit of time. In this experiment, the recording time for data gathering was 7200 s with bin size 1000 ms constantly stored on the hard disk and the average frequency was computed by computer [3]. According to the results, pyramidal neurons in the CA1 region are known based on their spontaneous frequency of 8 or less [3]. Recording continued for about 15 minutes due to the identification of a pyramidal neuron with a constant firing frequency and a constant spike amplitude and waveforms as the baseline. After 15 minutes, the drug was injected and the recording continued for about 105 minutes. In the present study, the discharge of each neuron over a 60-second time interval was calculated using a data acquisition program to generate Peri-Stimulus Time Histograms (PSTHs) with a time interval of 15 minutes before injection to 105 minutes after drug injection. Data were analyzed offline using Windows Home Analysis software. In order to identify patterns of neural response to saline, ZoN 0.5, 1, 1.5, and 3 mg/kg were administered, and the entire perception period was cut into 60-second time buckets. Increasing or decreasing neuronal activity was considered as twice the standard time deviation from baseline activity for three consecutive points as a stimulatory or inhibitory response.

2.7. Histological confirmation

At the end of the electrophysiological recordings, the brains of the animals were removed and fixed in a 10% formalin solution. Then, 20 μm sections were removed from near the electrode and the incisions were stained using hematoxylin and eosin (H&E). Finally, a microscope (Japan; Olympus EX51) was used to determine the recording location in the CA1 region of the hippocampus and the results were compared with references (Fig. 3).

Fig. 2. SNC lesions area by rotenone (A. Control, B. Rotenone).
2.8. Statistical analysis

Data were recorded before and 105 minutes after the IP administration of drugs. The obtained data were analyzed using SPSS software version 20. To evaluate the data, paired t-test was used to evaluate the effect of the drug on nerve firing rate before and after drug injection. In addition, GraphPad Prism version 6.07 was used to plot the effect of the drug on the number of stimulatory, inhibitory, and ineffective neurons. The data were presented as Mean ± Standard Error of the Mean (SEM). P<0.05 was considered statistically significant.

3. Results

Aiming at the effect of saline on the electrical firing of pyramidal neurons in the CA1 hippocampus, 0.2 ml of saline was injected into the IP after basal recording and nerve firing was recorded for 105 minutes. From the obtained results, paired t-test was performed and the stimulatory response of neurons in the lesion group to saline injection did not show a significant increase in the frequency of neurons after injection compared to baseline activity (Figs. 4 and 5).

![Fig. 4. Effects of saline on the mean firing frequency of pyramidal neurons in the CA1 region of the hippocampus compared to the baseline (t=-1.03, df=12; P>0.05).](image-url)
In the present study, 12 neurons were recorded from 8 rats, of which saline was able to stimulate 2 neurons, inhibit 3, and also had no effect on 7 of them. Specifically, the effect of saline on neuronal stimulation was 38 to 54 minutes after IP injection. Also, the mean increase in the activity of pyramidal neurons in the CA1 region of the hippocampus showed that intraperitoneal saline injection was associated with a 20 to 50% increase in activity in 2 neurons and a 50 to 65% decrease in activity in 3 neurons. The results of injection of ZoN 0.5 mg/kg after lesion showed that the t-test of paired samples did not show a significant decrease in the frequency of neurons after injection compared to basal activity ($t = -0.854$, df = 18; $P < 0.05$) (Figs. 6 & 7). In this group, 13 neurons from 8 rats were recorded and it was observed that ZoN 0.5 mg/kg had no effect on 7 neurons, inhibited 3 neurons, and stimulated 3 neurons. After injection of 0.5 mg ZoN, stimulation of neuronal firing began within 34 to 53 minutes.
Fig. 7. Histogram of electrophysiological recording pattern of spike CA1 neurons in the baseline as well as after ZoN 0.5 mg/kg injection.

Then, the group receiving ZoN 1mg/kg after the lesion showed a significant decrease in the firing frequency of neurons in area A after injection compared to the basal neuronal activity in the t-test of paired samples (t = -1.632, df = 16; P <0.05). (Figs. 8 & 9). Specifically, in this group of 8 mice, 13 neurons were recorded and it was found that 1mg of ZoN had a stimulatory effect on 6 neurons, had an inhibitory effect on 4 neurons, and had no effect on 3 neurons. After the injection of 1mg ZoN, stimulation was observed in the stimulation period of 45 to 56 minutes.

Fig. 8. Effects of ZoN 1mg/kg on mean neuronal firing frequency (t = -1.632, df = 16; P <0.05).
Fig. 9. Histogram of electrophysiological recording pattern of spike CA1 neurons in baseline as well as after ZoN 1mg/kg injection.

The results of injection of 1.5 mg/kg ZoN after lesion showed that a significant decrease in neuronal firing frequency was observed compared to baseline activity in a paired t-test ($t = -2.423$, df = 18; $P < 0.005$) (Fig. 10 and 11). In this group of 8 mice, 12 neurons were recorded that 1.5mg of ZoN had a positive effect on 5 neurons, a negative effect on 5 neurons, and no effect on 2 neurons (Fig. 12). The onset of ZoN stimulation was observed in a period of 41 to 58 minutes.

Fig. 10. Effects of ZoN 1.5mg/kg on mean neuronal firing frequency ($t = -2.423$, df = 18; $P < 0.005$).
The results of injection of 3 mg/kg ZoN after lesion showed that a significant decrease in neuronal firing frequency was observed compared to baseline activity in a paired t-test ($t = -2.7142$, df = 17; $P < 0.005$) (Figs. 12 and 13). In this group of 8 mice, 13 neurons were recorded that 3 mg of ZoN had a positive effect on 6 neurons, a negative effect on 5 neurons, and no effect on 2 neurons (Fig. 14). The onset of ZoN stimulation was observed in a period of 43 to 55 minutes.
Fig. 13. Histogram of electrophysiological recording pattern of spike CA1 neurons in the baseline as well as after ZoN 3 mg/kg injection.

Fig. 14. Scatterplot representing the response of pyramidal neurons to saline and ZoN injection.
4. Discussion
In the present study, saline and ZoN were injected at 0.5, 1, 1.5, and 3 mg/kg in rotenone-induced Parkinson’s disease mice, and ZoN was found to decrease spontaneous activity at 1, 1.5 & 3 mg/kg. The pyramidal neurons become CA1, while the other values had no significant effect.
Sarbigi et al. (2019) reported that rotenone alters the electrical activity of the hippocampus and its associated behavioral changes. Rotenone mice showed a significant reduction in standing movements during the 3 weeks compared to control animals [8].
Zinc oxide nanoparticles cause a significant decrease in long-term memory retrieval in the passive avoidance learning model in rats [5]. Oxide nanoparticles are able to disrupt spatial memory in male Wistar rats in the Mauritius water maze [11].
Zinc ion is an antagonist of glutamate NMDA receptors. Zinc oxide nanoparticles reduce the activity of the NMDA receptor and as a result disrupt the memory function [7]. Researches have shown that this ion is able to enter the neuron through different routes, including: glutamate receptors and L-type voltage-dependent calcium channels. Zinc ion in small amounts causes learning and memory, but when its amount exceeds a certain limit [2]. Also, research has shown that zinc oxide nanoparticles attack beta cells of the pancreas, causing the destruction of these cells and the reduction of insulin hormone in the body. With the decrease of insulin, and 3β-GSK 4, the phosphorylation of JNK 5 protein kinases in the cell is reduced and causes an increase in neurofibrillary tangles and amyloid plaques, followed by atrophy and neuronal death in different areas of the brain, including the hippocampus [1]. These nanoparticles increase the production of active oxygen species and cell death by affecting the mitochondrial organelle in the cell. Exposure to different amounts of zinc oxide nanoparticles causes oxidative stress, lipid peroxidation, cell membrane damage and DNA damage [4].

5. Conclusion
The results of the present study and according to the studies, it seems that ZoN can at least partially reduce the activity of pyramidal neurons in the CA1 region of the hippocampus in mice with Parkinson’s disease and can be a suitable option for the treatment of diseases Nervous including Parkinson’s and Alzheimer’s to be used.

Conflicts of Interest
The author declares no conflict of interest.

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