Original paper

Folia Neuropathologica

Exercise improves the body function and protects the neuronal injury in Parkinson's disease rats by activating calpain 1 and kallikrein 6

Jing Guo[1], Jinsuo Yang[1], Jiang Fu[2], Alok Shiomurti Tripathi[3,4]

[1]Department of Neurology, Baoji Gaoxin Hospital, Baoji, China, [2]Department of Neurology, Zhuji People's Hospital, Zhuji, China, [3]Department of Pharmacology, Amity Institute of Pharmacy, Amity University, Noida (U.P.), India, [4]Department of Pharmacology, P. Wadhwani College of Pharmacy, Yavatmal (MS), India

Folia Neuropathol 2022; 60 (3): 301–307 *DOI: https://doi.org/10.5114/fn.2022.118794*

Abstract

Parkinson's disease (PD) is a neurodegenerative disease, which alters body and cognitive functions. The present study evaluates the effect of exercise on body function and neuronal injury against a 6-hydroxydopamine hydrobromide (6-OHDA) induced PD rat model and postulates a possible molecular mechanism of its action. Parkinson's disease was induced by administration of (20 µg/5 µl at the rate of 1 µl/min) 6-OHDA and exercise training was given to mice by motorized rodent treadmill for a period of 14 days after the confirmation of PD. Behavioural changes were observed by apomorphine-induced rotation and motor function was assessed using the rotarod apparatus. The effect of exercise was observed on the level of neurochemicals and the expression of calpain-1 (CAPN1) and kallikrein 6 (KLK6) was estimated in brain tissue of PD rats using western blot assay. A more significant improvement in the motor and cognitive function was observed in the PD + exercise group than in the PD group of rats. Exercise attenuates the altered level of γ-aminobutyric acid (GABA), dopamine (DA) and glutamate in brain tissue of PD rats. Intracellular concentration of Ca^+ ion was reduced significantly in brain tissue of the PD + exercise group compared to PD rats. Moreover, exercise activates the expression of KLK6 and CAPN1 protein in brain tissue of PD rats. In conclusion, data of the study reveal that exercise protects neuronal injury by reducing intracellular concentration Ca^+ ion and activates KLK6 and CAPN1 in brain tissue of PD rats and thereby improves motor and cognitive functions.

Key words: *exercise, Parkinson's disease, kallikrein 6, neurochemicals, motor function.*

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder; loss of dopaminergic neuron occurs in substantia nigra and reduces the dopamine level in striatum [1]. Bradykinesia, rigidity, and tremor are classical symptoms of PD. There are several pathogenic pathways involved in the development of PD including inflammation and α-synuclein (α-syn) [18]. Aggregation of α-syn in an abnormal level on the glia, neurites and neuronal cells contribute to neurodegeneration in PD, which leads to multiple system atrophy (MSA) [11]. Cytoplasmic inclusion and neuronal nuclear proteins are the major features of PD. Impairment of clearance of this protein contribute to the pathogenesis of PD as dysregulation of autophagy-lysosomal pathway (ALP) and ubiquitin-proteasomal pathway (UPS) affects the clearance of α-syn. Calpain-1 (CAPN1) and kallikrein-6 (KLK6) are the non-lysosomal proteases which regulate the level of α-syn through its cleavage [15]. Aggregation of α-syn within the non-amyloid-β component (NAC) region causes PD, and CAPN1 and KLK6 are the cleavage enzymes which break this protein aggregation [8]. The literature suggests that fragmentation of this pro-

Communicating author:
Jiang Fu, Department of Neurology, Zhuji People's Hospital, Zhuji, 311800, China, phone: +86-0575-81782173, e-mail: vox0_0@sina.cn

tein ceases the aggregation of α-syn and is less toxic to neurons [10]. Oligodendrocytes and neurons are reported to express KLK6, which degrades α-syn extracellularly [15]. CAPN1 is localized in cytosol with α-syn, expressed neuronally, which degrades fibrillar α-syn. Moreover, several studies suggest that reduced activity of these enzymes causes an increase in aggregation of α-syn and contributes to the pathogenesis of PD [12].

Conventional therapies available for the management of PD slow the progression of disease and improves the motor function. Exercise has been reported to result in a significant improvement of multiple organ function, which is also beneficial for PD patients [14]. A preclinical study reveals that exercise also improves a nigrostriatal DA level in PD, which improves the control over the movement [6]. Exercise improves the production of neuroprotective molecules which protects the dopaminergic neurons [19]. Regular exercise promotes motor function in PD rats by regulating the neurotransmission of glutamate in the corticostriatal region. The literature suggests that exercise also protects dopaminergic neuron by enhancing the neurotrophic factors i.e. glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) [5]. BDNF regulates neuroprotection by reducing neuronal inflammation and stimulation of its level is regulated by CAPN 1 and KLK6 [2]. Thus, the present report evaluates the protective effect of exercise on PD.

Material and methods

Animal

We used healthy Sprague-Dawley rats (female, age: 6 weeks, weight: 190-210 g) housed under controlled conditions (temperature: 25 ±2°C, humidity: 55 ±5%) with a 12 h light/dark cycle as per the guideline. All the protocol based on animals were approved by the institutional animal ethical committee of P. Wadhwani College of Pharmacy, India (650/02/C/CPCSEA/22/2019).

Chemicals

6-OHDA was procured from Merck Limited, Guangzhou, China. ELISA kits, Fura 2-AM dye and antibodies used for western blot assay were procured from ThermoFisher Scientific Ltd., USA.

Experimental development of Parkinson's disease model

All the rats were separated into four different groups (n = 10) such as the sham group, the exercise group, the PD group, and the PD + exercise group. PD was induced in rats, after anesthesia placed them on the stereotaxic frame. The skull of rats was drilled to form a hole of the coordinates: 0.0 mm anterior to the coronal suture, 3.0 mm lateral to the midline, depth 6.0 mm deep from the surface of the brain, where the 26-gauge needle was implanted into striatum. 6-OHDA was administered 20 µg/5 µl at the rate of 1 µl/min. Further, the needle was withdrawn slowly from the surface. However the sham operated groups were administered a physiological salt solution.

Treadmill exercise

Exercise training was given to mice by motorized rodent treadmill for a period of 14 days after the confirmation of PD. Training was provided for 40 min every day with an inclination of 0° at a speed of 8 m/min, i.e. the first 5 min at a speed of 2 m/min, the next 5 min at 5 m/min and the last 30 min at a speed of 8 m/min. Sedentary mice were regularly transported to the training room without exercise, so that these mice were exposed to the same environment. Behavioural, biochemical, and neuronal activities were determined 48 h after the exercise session to avoid misinterpretation of data.

Apomorphine-induced rotation

Lesions over the dopaminergic system were assessed by observing the behaviour in the PD rat model. The extent of dopamine depletion relates to the number of rotations under apomorphine in PD rats. Apomorphine 3 mg/kg administered s.c. induces the contralateral rotation and monitor it for a period of 60 min.

Assessment of motor function

The rotarod apparatus was used to determine the motor coordination and balance. Animals were mounted on the rod of the rotarod, which rotates at a speed of 18 RPM and latency period was recorded for the rats to fall.

Assessment of cognitive function

Morris water maze test was performed for the assessment of the cognitive function as per a previously reported study [13]. The apparatus used in the report had the following dimensions: 120 cm circular pool, depth of 30 cm and height of 50 cm. The pool was separated into four quadrants and a platform was kept in each quadrant for first 5 days at a constant place. Assessment of spatial memory was performed by giving the trial for 5 consecutive days and on 6th day after removing the platform, time spent in the target quadrant was determined by using DigBehav System.

Preparation of brain tissue homogenate

All the animals were sacrificed using cervical dislocation and isolated brain tissue was homogenized in 0.1 M phosphate buffer (pH = 7.4). Brain tissue homogenate was centrifuged for a period of 15 min at 3000 rpm and supernatant was used for the estimation of biochemical parameters.

Estimation of dopamine, glutamate, and γ-aminobutyric acid

The level of glutamate, γ-aminobutyric acid (GABA), and dopamine in brain tissue homogenate was measured using the respective assay kits as per the directions given by the manufacturer of kits.

Estimation of intracellular influx of Ca^+ ion

The brain was isolated from each animal and blood vessels and membranes of the brain were removed from the brain. D-Hank's solution was used three times to wash the striatal tissue of the brain. Pancreatin (0.1%) was added to the chopped striatal tissue and the digestion was stopped by transferring the tissue to an ice cold stop solution. Fura-2/AM (with the final concentration of 5 mol/l) was added to the cell suspension at 37°C for 45 min and the cell suspension was centrifuged for 5 min at 800 RPM to separate out the supernatant. Fluorescence intensity was estimated at 510 nm to determine the intracellular influx of Ca^+.

Estimation of kallikrein 6

Kallikrein 6 activity was estimated in the isolated brain tissue using ELISA as per the directions of the manufacturer of the kit.

Western blotting

The expression levels of calpain-1 and kallikrein 6 were assessed by western blotting of brain homogenates. A BCA assay kit was used to quantify the protein level in each homogenate, and the proteins were separated *via* 10% (w/v) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes, which was blocked with 5% (w/v) blocking solution (non-fat milk) and incubated in blocking buffer with primary antibodies (ThermoFisher Scientific Ltd., USA) such as anti-kallikrein 6 (dilution 1 : 500) and anti-calpain-1 (dilution 1 : 1000) overnight at 4°C. Goat secondary antibodies conjugated with horseradish peroxidase were added, and a chemiluminescence kit was used to detect the proteins.

Statistical analysis

Results were expressed as mean ± SEM (n = 10). The statistical analysis was performed using one-way ANOVA followed by Dunnett test for multiple comparisons (GraphPad Prism software, ver. 6.1; USA). The level of statistical significance was set at $p < 0.05$.

Results

Exercise attenuates behavioural changes in PD rats

The exercise effect was observed on the behavioural changes such as apomorphine-induced rotation and motor function in 6-OHDA induced PD rats as shown in Figure 1A, B. Total net rotation was observed in 6-OHDA induced PD rats by apomorphine-induced rotation as shown in Figure 1A. There was a significant increase in the number of rotations in the PD group compared to the sham and exercise group of rats. However, number of rotations were reduced in exercised exposed PD rats compared to the PD group. The effect of exercise was observed on the number of falls per min in 6-OHDA induced PD rats using the rotarod apparatus (Fig. 1B). There was an increase in the number of falls per min in the PD group than the exercise and sham group of rats, treatment with exercise reduces the number of falls per min in PD rats.

Exercise attenuates cognitive dysfunction

Cognitive function was estimated in exercise treated 6-OHDA induced PD rats using Morris water maze. Percentage of time spent in the target quadrant and the number of crossings were lower in the PD group than the sham and exercise alone group. There was a significant improvement in percentage of time spent in the target quadrant and number of crossings in exercise treated PD rats. Escape latency was enhanced significantly ($p < 0.01$) in the PD group compared to the sham and exercise group and treatment with exercise improves escape latency in PD rats (Fig. 2).

Exercise attenuates level of GABA, DA and glutamate

The level of DA, GABA and glutamate was estimated in brain tissue of exercise treated 6-OHDA induced PD rats. The level of DA and GABA is reduced significantly, and the glutamate level is enhanced in brain tissue of the PD group compared to the exercise and sham treated group of rats. There was an increase in the level of

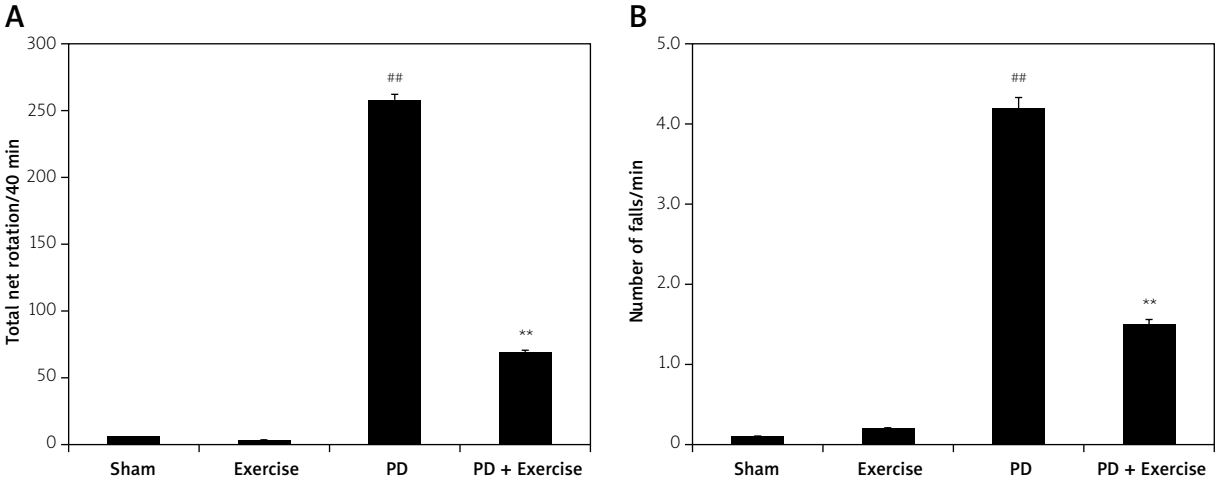

Fig. 1. Effect of exercise on the behavioural changes in 6-OHDA induced PD rats. **A**) Exercise reduces apomorphine-induced rotations in 6-OHDA induced PD rats. **B**) Exercise reduces the number of falls in 6-OHDA induced PD rats using the rotarod apparatus. Mean ±SEM (n = 10); $^{\#\#}p < 0.01$ compared to the sham and exercise group; $^{**}p < 0.01$ compared to the PD group.

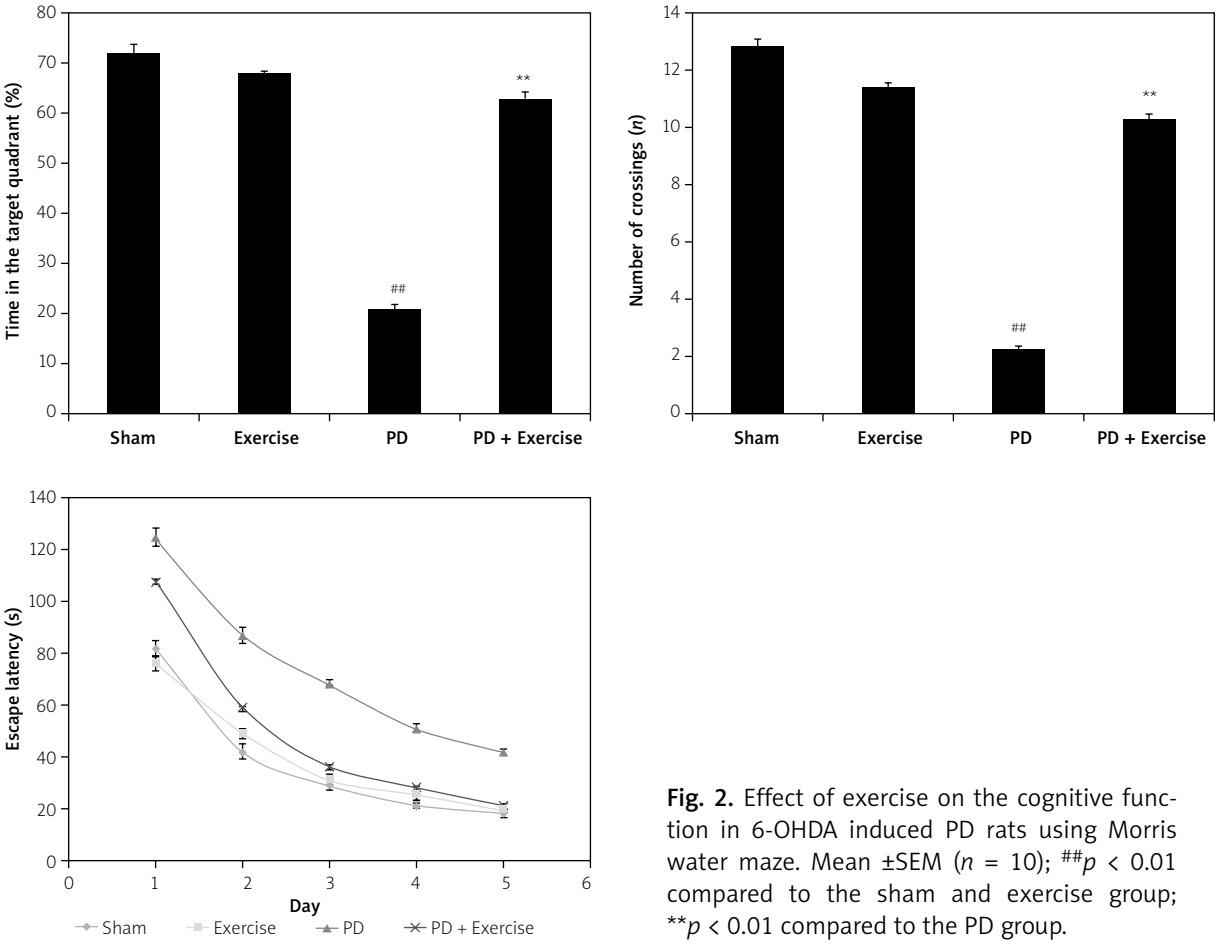

Fig. 2. Effect of exercise on the cognitive function in 6-OHDA induced PD rats using Morris water maze. Mean ±SEM (n = 10); $^{\#\#}p < 0.01$ compared to the sham and exercise group; $^{**}p < 0.01$ compared to the PD group.

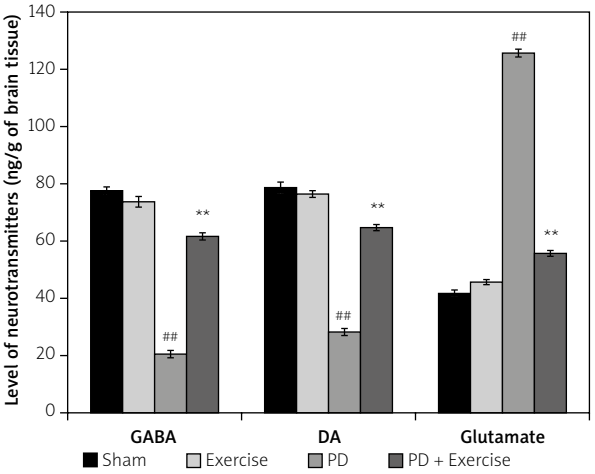

Fig. 3. Effect of exercise on the level of GABA, DA and glutamate in brain tissue of 6-OHDA induced PD rats. Mean ±SEM (n = 10); $^{\#\#}p < 0.01$ compared to the sham and exercise group; $^{**}p < 0.01$ compared to the PD group.

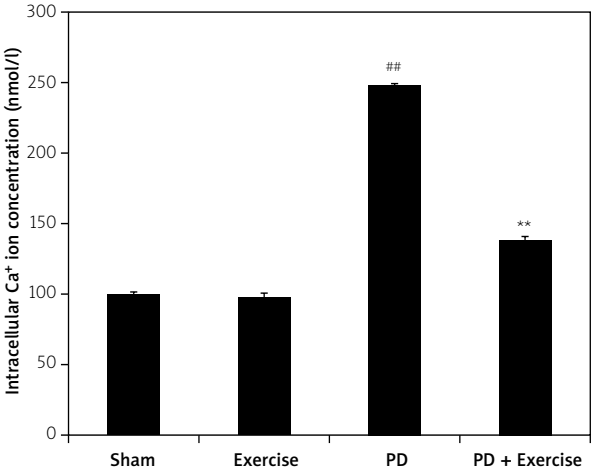

Fig. 4. Effect of exercise on the intracellular concentration of Ca^+ ion in the brain tissue of 6-OHDA induced PD rats. Mean ±SEM (n = 10); $^{\#\#}p < 0.01$ compared to the sham and exercise group; $^{**}p < 0.01$ compared to the PD group.

GABA and DA and reduction in the level of glutamate in brain tissue of the PD + exercise group compared to the PD group of rats (Fig. 3).

Exercise attenuates intracellular influx of Ca^+ ion

The intracellular concentration of Ca^+ ion was determined in the brain tissue of exercise treated-OHDA induced PD rats as shown in Figure 4. There was a significant ($p < 0.01$) increase in intracellular concentration of Ca^+ ion in brain tissue of the PD group compared to the sham and exercise group of rats and treatment with exercise reversed the concentration Ca^+ ion in the brain tissue of PD rats.

Exercise attenuates calpain1 and kallikrein 6

The effect of exercise was observed on the expression of KLK6 and CAPN1 proteins and the level of KLK6 in brain tissue of 6-OHDA induced PD rats. The level of KLK6 was estimated in brain tissue of 6-OHDA induced PD rats using ELISA as shown in Figure 5A. There was a significant decrease in the level of KLK6 in the PD group compared to the exercise and sham group of rats and exercise reversed the level of KLK6 in the PD rats. Expression of CAPN1 and KLK6 protein was estimated in brain tissue of exercise treated PD rats by western blot assay (Fig. 5B). There was a significant decrease in expression of KLK6 and CAPN1 protein in brain tissue of the PD group compared to the sham and exercise group of rats. However, exercise attenuates the expression of CAPN1 and KLK6 protein in brain tissue homogenate of 6-OHDA induced PD rats.

Discussion

Parkinson's disease is a degenerative disorder of dopaminergic neurons characterized by bradykinesia, rigidity, and tremor [18]. Deposition of α-syn around the neurons, which disturb the neuronal homeostasis leads to its degeneration [7]. A conventional drug available for the management of PD regulates symptomatic relief, however its effective management needs development of a new therapeutic approach. Thus, the present report evaluates the effect of exercise on development of PD and its effect on deposition of α-syn. The effect of exercise was observed on the change in the behaviour and cognitive function in PD rats. Moreover, the effect of exercise was determined on the level of neurochemicals, intracellular level of Ca^+ and expression of KLK6 and CAPN1 in brain tissue of PD rats.

Dopamine analogue like 6-hydroxydopamine reported to be observed in the brain tissue of patients suffering from PD [17]. 6-OHDA is reported to cause a loss of dopaminergic neurons by increasing oxidative stress as it promotes production of ROS [16]. A PD animal model was produced by administration of a unilateral injection of 6-OHDA, which clinically matched disease [9] and present report supports it. PD also involves alteration of neurotransmitters including dopamine, glutamate and GABA in the brain. These neurochemicals are also reported to be altered in 6-OHDA induced PD rats [3] and the present study supports it. Effective man-

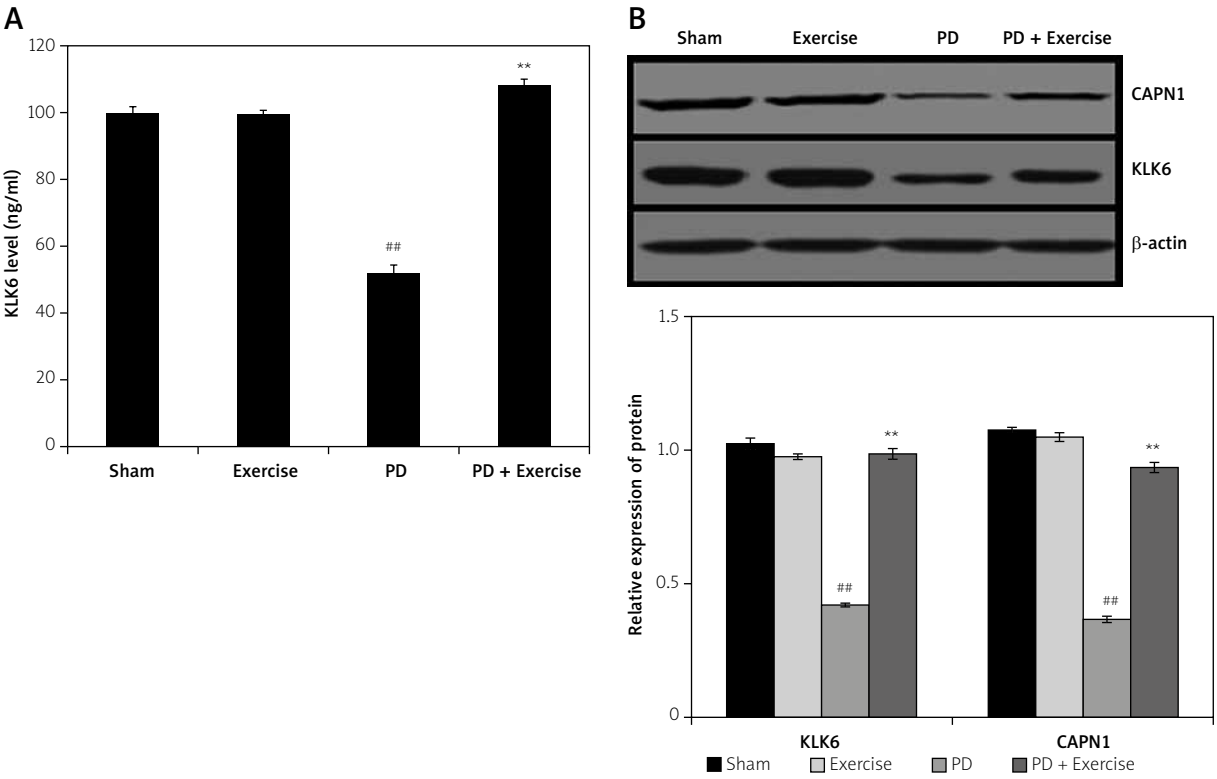

Fig. 5. Effect of exercise on the expression of CAPN1 and KLK6 in the brain tissue of 6-OHDA induced PD rats. **A**) Exercise attenuates the level of KLK6 in brain tissue of 6-OHDA induced PD rats using the ELISA method. **B**) Exercise attenuates relative expression of KLK6 and CAPN1 proteins in brain tissue homogenate of 6-OHDA induced PD rats using western blot assay. Mean ±SEM (n = 10); $^{\#\#}p < 0.01$ compared to the sham and exercise group; $^{**}p < 0.01$ compared to the PD group.

agement of PD is reported to be achieved by regulating these altered neurotransmitters and exercise balances the level of neurotransmitters in PD too, which is also suggested by the present study.

Cognitive dysfunction is always observed in neurodegenerative disorders including PD, however there are several reports revealing that neurodegeneration in PD is also associated with cognitive dysfunction [4] and the present report also suggests it. Prevention of PD also improves the cognitive function in PD and presented that exercise improves cognitive dysfunction and also improves symptoms of PD too.

KLK6 belongs to a family of kallikrein which is a serine protease enzyme abundantly present in the brain tissue. The literature suggested that KLK6 degrades α-syn, which prevents its deposition around the neuron [10]. Pathogenesis of PD suggests that deposition of α-syn around the neurons, which alters the permeability of cellular membrane, leads to neuronal injury [7]. Moreover CAPN1, a cytosolic enzyme, degrades α-syn protein which prevents its deposition around neurons. The literature suggests that activation of KLK6 and CAPN1 enzyme promotes degradation of α-syn around the neurons, which prevents neurodegeneration and activation of them are beneficial for the prevention of PD [14]. The present study suggests that activity of CAPN1 and KLK6 is reduced in PD and exercise enhances activity of CAPN1 and KLK6, which prevents neurodegeneration in PD rats.

Conclusions

In conclusion, data of the study reveal that exercise protects neuronal injury which ameliorates the activity of KLK6 and CAPN1 leads to reduction in deposition of α-syn around neurons in PD rats. Moreover, exercise improves the cognitive and body functions in PD rats.

Acknowledgments

All the authors of the manuscript are thankful to P. Wadhwani College of Pharmacy, India and Zhuji People's Hospital, China for providing the necessary facility to conduct the presented work.

Ethics approval statement

All the protocols were approved by the institutional animal ethical committee of P. Wadhwani College of Pharmacy, India (650/02/C/CPCSEA/22/2019).

Disclosure

The authors report no conflict of interest.

References

1. Alexander GE. Biology of Parkinson's disease: pathogenesis and pathophysiology of a multisystem neurodegenerative disorder. Dialogues Clin Neurosci 2004; 6: 259-280.
2. Bathina S, Das UN. Brain-derived neurotrophic factor and its clinical implications. Arch Med Sci 2015; 11: 1164-1178.
3. Calabresi P, Ghiglieri V, Mazzocchetti P, Corbelli I, Picconi B. Levodopa-induced plasticity: a double-edged sword in Parkinson's disease? Philos Trans R Soc Lond B Biol Sci 2015; 370: 20140184.
4. Fang C, Lv L, Mao S, Dong H, Liu B. Cognition deficits in Parkinson's disease: mechanisms and treatment. Parkinsons Dis 2020; 2020: 2076942.
5. Hirsch MA, van Wegen EEH, Newman MA, Heyn PC. Exercise-induced increase in brain-derived neurotrophic factor in human Parkinson's disease: a systematic review and meta-analysis. Transl Neurodegener 2018; 7: 7.
6. Hou L, Chen W, Liu X, Qiao D, Zhou FM. Exercise-induced neuroprotection of the nigrostriatal dopamine system in Parkinson's disease. Front Aging Neurosci 2017; 9: 358.
7. Jellinger KA. Basic mechanisms of neurodegeneration: a critical update. J Cell Mol Med 2010; 14: 457-487.
8. Kasai T, Tokuda T, Yamaguchi N, Watanabe Y, Kametani F, Nakagawa M, Mizuno T. Cleavage of normal and pathological forms of alpha-synuclein by neurosin in vitro. Neurosci Lett 2008; 436: 52-56.
9. Konnova EA, Swanberg M. Animal models of Parkinson's disease. In: Stoker TB, Greenland JC (Eds.). Parkinson's Disease: Pathogenesis and Clinical Aspects [Internet]. Chapter 5. Codon Publications, Brisbane (AU) 2018.
10. Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of α-synuclein: from structure and toxicity to therapeutic target. Nat Rev Neurosci 2013; 14: 38-48.
11. Lee HJ, Ricarte D, Ortiz D, Lee SJ. Models of multiple system atrophy. Exp Mol Med 2019; 51: 1-10.
12. Miners JS, Renfrew R, Swirski M, Love S. Accumulation of α-synuclein in dementia with Lewy bodies is associated with decline in the α-synuclein-degrading enzymes kallikrein-6 and calpain-1. Acta Neuropathol Commun 2014; 2: 164.
13. Nunez J. Morris water maze experiment. J Vis Exp 2008; 19: 897.
14. Oliveira de Carvalho A, Filho ASS, Murillo-Rodriguez E, Rocha NB, Carta MG, Machado S. Physical exercise for Parkinson's disease: clinical and experimental evidence. Clin Pract Epidemiol Ment Health 2018; 14: 89-98.
15. Pampalakis G, Sykioti VS, Ximerakis M, Stefanakou-Kalakou I, Melki R, Vekrellis K, Sotiropoulou G. KLK6 proteolysis is implicated in the turnover and uptake of extracellular alpha-synuclein species. Oncotarget 2017; 8: 14502-14515.
16. Perese DA, Ulman J, Viola J, Ewing SE, Bankiewicz KS. A 6-hydroxydopamine-induced selective parkinsonian rat model. Brain Res 1989; 494: 285-293.
17. Tansey MG, McCoy MK, Frank-Cannon TC. Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention. Exp Neurol 2007; 208: 1-25.
18. Tansey MG, Wallings RL, Houser MC, Herrick MK, Keating CE, Joers V. Inflammation and immune dysfunction in Parkinson disease. Nat Rev Immunol 2022; 1-17.
19. Vecchio LM, Meng Y, Xhima K, Lipsman N, Hamani C, Aubert I. The neuroprotective effects of exercise: maintaining a healthy brain throughout aging. Brain Plast 2018; 4: 17-52.