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Bushen Zhichan Formula Affects Neurofunction and Fas/FasL Signal in Rats with Parkinson's Disease by Regulating Microglial Polarisation

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KEYWORDS Microglia. Neuron. Parkinson's Disease. Protein Expression. Signalling Pathway

ABSTRACT The researchers have assessed the effect of Bushen Zhichan Formula (BSZCF) on the neurofunction and Fas/Fas ligand (FasL) signal in rats with Parkinson's disease (PD) by regulating microglial polarisation. Sham group, PD group, BSZCF group and DBSJ (levodopa and benserazide hydrochloride tablets) group were established for random allocation. The pole-climbing time, neurological function score, tumor necrosis factor-á and interleukin-6 levels in the substantia nigra, ionised calcium-binding adaptor molecule 1 (IBA1) cluster of differentiation 86 (CD86) cell count, CD86/CD206 ratio and Fas and FasL protein expressions significantly declined, whereas the Tamm-Horsfall protein expression, hanging score, and number of $IBA1^{+}CD206^{+}$ cells in the substantia nigra significantly increased in BSZCF and DBSJ groups compared to those in the PD group ($P<0.05$). BSZCF can alleviate neurological injuries and repress dopaminergic neuron loss mediated by the Fas/FasL signalling pathway.

INTRODUCTION

As a central nervous system degenerative disease, Parkinson's disease (PD) has the feature of dopaminergic deficiency in the nigrostriatal system, with tremor, bradykinesia, abnormal gait and posture as common motor symptoms, and depression, constipation, and hypoesthesia as non-motor symptoms, thus severely threatening patients' quality of life (Mollenhauer and von Arnim 2022; Berthouzoz et al. 2023). At present, oral administration of levodopa is the 'gold standard' for clinical treatment, which, however, easily leads to various adverse reactions (for example, dyskinesia) (Cosentino et al. 2022). Drugs for reinforcing kidney and liver functions have been highlighted for the effective treatment of PD in recent years (Muhammad et al. 2022).

In traditional Chinese medicine, PD is classified as a tremor. "The deficiency of liver and kidney function" is the leading PD symptom (Chen et al. 2022). Chinese herbal compounds for nourishing the liver and kidney have exerted obvious effects on repairing damaged neurons and impeding the progression of PD (Huan et al. 2022). Bushen Zhichan Formula (BSZCF) is prepared by combining Guilu Erxian Cream Formula and Dading Fengzhu Formula. In the formula, Guilu Erxian Cream embodies the relationship among essence, qi and spirit and the concept of qi-blood generation, while Dading Fengzhu emphasises the effect of nourishing yin and suppressing yang as well as calming wind-evil. This formula is combined with drugs such as Common Macrocarpium Fruit, Rhizoma Polygonati, Herba Cistanches, and Uncaria, which jointly participate in nourishing the liver and kidney, boosting essence and supplementing marrow, calming wind-evil and smoothing collaterals, thus relieving symptoms such as tremble, stiffness and bradykinesia caused by PD. The ability of BSZCF to mitigate both the motor and non-motor symptoms of PD patients has been confirmed (Liang et al. 2022).

BSZCF can suppress the progression of PD by protecting neurons, but its specific mechanism has not yet been clarified (Liu et al. 2019). PD has a close association with neuroinflammation induced by microglial activation (Isik et al. 2023). The activated microglia are able to differentiate into M1 or M2 phenotype. The former aggravates inflammatory injuries by releasing proinflammatory factors, whereas the latter relieves inflammatory responses by releasing anti-inflammatory factors, thereby protecting nerves.

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Hence, promoting the M2 phenotype polarisation of microglia to mitigate neuroinflammation is of great significance for treating PD and improving neurological function. Fas ligand (FasL), one of the common surface molecules in the tumour necrosis family, can bind the Fas receptor to induce apoptosis. PD may be ascribed to dopaminergic neuron degeneration, death, and apoptosis, and the apoptosis of dopaminergic neurons can be mediated by the Fas/FasL signalling pathway (Sun et al. 2022). Therefore, suppressing the Fas/FasL signalling pathway may exert an effect on treating PD.

Therefore, this study aimed to investigate whether BSZCF can improve the neurological function and suppress the Fas/FasL signalling pathway in PD rats by regulating microglial polarisation.

MATERIAL AND METHODS

Laboratory Animals

Forty specific pathogen-free Sprague-Dawley rats (male, age: 8 weeks old, weight: 180-220 g) (Beijing Huafukang Bioscience Co. Ltd., China) were subjected to one week of adaptive feeding in an animal room (temperature: 22-25°C, relative humidity: 50-60%) with a normal circadian rhythm. Food and water were offered freely.

Drugs

BSZCF was composed of antler gelatin (10 g), Colla Corii Asini (10 g), Fructus Lycii (30 g), Rhizoma Polygonati (10 g), Common Macrocarpium Fruit (10 g), raw tortoise shell (20 g), Codonopsis pilosula (15 g), Fructus Cannabis (15 g), Radix Paeoniae Alba (30 g), Radix Ophiopogonis (15 g), Carapax Trionycis (15 g), Rhizoma Gastrodiae (15 g), Herba Cistanches (30 g), Uncaria (30 g), Radix Rehmanniae (25 g) and honey-fried licorice root (6 g). They were all single decoction-free granules (Tianjiang Pharmaceutical Co. Ltd., Jiangyin, China). Levodopa and benserazide hydrochloride tablets (DBSJ, 250 mg/tablet) were purchased from Shanghai Roche Pharmaceutical Co. Ltd. (China).

Reagents and Apparatus

Rabbit anti-Fas and anti-FasL polyclonal antibodies and anti-Tamm-Horsfall (TH) polyclonal antibodies were purchased from Abcam (USA). Mouse anti-β-actin monoclonal antibodies, as well as horseradish peroxidase (HRP)-labelled goat anti-mouse and anti-rabbit secondary antibodies were bought from Wuhan Boster Biological Technology Co. Ltd. (China). Bicinchoninic acid protein assay kit and enzyme-linked immunosorbent assay kit were obtained from Shanghai Enzyme-Linked Biotechnology Co. Ltd. (China). Mouse anti-ionised calcium-binding adaptor molecule 1 (IBA-1, a microglia marker) antibodies, anti-CD86 (an M1 macrophage marker) antibodies and anti-CD206 (an M2 macrophage marker) antibodies were provided by Abcam (UK). Proteasome inhibitor (PSI) was purchased from Sigma (USA). In detail, 5 mg of PSI powders were added to 875 ìL of 100 percent dimethyl sulfoxide solution, stirred for dissolution, and then added to 375 ìL of distilled water into a solution, which was used immediately after preparation. The ZH-B brain stereotaxic apparatus was bought from Anhui Zhenghua Biologic Apparatus Facilities (China). The Histotome and Leica RM 2235 microplate reader were obtained from Beijing Perlong New Technology Co. Ltd. (China). The BX60 optical microscope was supplied by Olympus (Japan).

Grouping and Modelling

Forty rats were randomly assigned into a sham group (n=10) and a model group (n=30). Next, the model of chronic PD was established according to the method in a previous literature (El-Latif et al. 2023). In brief, the PSI solution was injected subcutaneously through the back at 75 ìL/100 g every Monday, Wednesday, and Friday for 2 weeks. The occurrence of PD-like symptoms such as hypoactivity, bradykinesia, decreased centre of gravity and limb stiffness 4 weeks after modelling and a decrease in the hanging score indicated successful modelling. For the sham group, an equal volume of 70 percent ethanol solution was subcutaneously injected from the back. Modelling failed in two rats, and three rats were eliminated to ensure that each group consisted of nine rats. Finally, 27 successfully modelled rats were allocated to three groups in a random manner, namely, PD group (n=9), BSZCF group (n=9), and DBSJ group (n=9).

According to a previous reference (Liu et al. 2024), 4 weeks after modelling, BSZCF (at 60 g/kg) and DBSJ Tablets (at 200 mg/kg) were administered intragastrically for BSZCF and DBSJ

groups respectively, once a day for three consecutive weeks. Normal saline was given to sham and PD groups in an equal volume by gavage for three consecutive weeks.

Behavioural Performance Test

The non-motor symptoms of rats were observed, including eating, hair, grooming, standing times, limb tremor, stiffness, etc.

Pole Test

A 50 cm long wooden pole was placed on a table at an angle of 45°, on top of which the rats were located. Then the time for rats climbing from the pole top to the bottom was recorded, and the average of three measurements was taken as the final pole-climbing time.

Wire Hanging Test

A 60 cm long wire was hung 30 cm above the ground. Next, the rats were hung on the wire with their two front paws, and their hanging time was recorded. The three measurement (with hanging conducted at an interval of 1 minute) results were averaged as the final hanging time. The scoring criteria were as follows: 0 point for 0-4 seconds, 1 point for 5-9 seconds, 2 points for 10-14 seconds, 3 points for 15-19 seconds, 4 points for 20-24 seconds, 5 points for 25-29 seconds, and 6 points for over 30 seconds.

Assessment of Neurological Impairment Score

At 24 hours following the last administration, the neurological function of rats was scored by the Zeo Longa 5-point assessment method, with the scoring criteria shown in Table 1 (Nim et al. 2023).

Table 1: Scoring criteria for neurological function

Score	*Condition*
0 point	No neurological impairment
1 point	Inability to fully extend the opposite front paw
2 points	Rotate to the opposite side when walking
3 points	Topple and fall to the opposite side when walking
4 points	Inability to walk independently, with clouding of consciousness

Sample Collection

After scoring of the neurological function, 3 percent pentobarbital sodium was intraperitoneally injected (dose: 50 mg/kg) for anesthetisation. Next, the rats were decapitated to harvest the brain on ice. Then the substantia nigra (starting from the ventral surface of the cross section of hippocampal junction and ending in the transverse fibers of pons) was collected and divided into three portions, with one portion rapidly preserved with liquid nitrogen in a -80°C refrigerator, one portion fixed in a 4 percent paraformaldehyde solution, and one portion stored in a -20°C refrigerator.

Hematoxylin-eosin Staining for Pathological Changes of the Substantia Nigra

The substantia nigra tissue fixed in the 4 percent paraformaldehyde solution was taken out, embedded in an embedding cassette, and flushed using running water to eliminate the fixative. Next, the tissue underwent dehydration with ethanol solutions at various concentrations and transparentization using xylene. Then the paraffin-embedded tissue was sliced into sections (5 μm) with the histotome. Afterwards, hematoxylin and eosin were utilised to stain the sections for 5 minutes and 1-2 minutes, respectively. After dehydration, the sections were blocked with neutral resin added dropwise, and observed under the optical microscope.

Enzyme-linked Immunosorbent Assay for Levels of Interleukin-4 (IL-4), IL-10, Tumour Necrosis Factor-α (TNF-α) and IL-6 in the Substantia Nigra

The substantia nigra tissue stored in the refrigerator at -20°C was taken out, added physiological saline at the ratio of 1:9, and ground on ice, with 13 minutes of 4°C centrifugation at 3000 rpm performed subsequently. Then EP tube was utilised to acquire the supernatant, in which IL-4, IL-10, TNF-α and IL-6 levels were measured according to the kit's instructions.

Detection of Cells Co-expressing Cluster of Differentiation 86 (CD86) or CD206 and Microglia Marker IBA1 Through Immunofluorescence Assay

The substantia nigra tissue taken from the -80°C refrigerator was cut into sections and left still for 30 minutes at room temperature. Then phosphate-buffered saline was added for washing the sections three times, and a blocking buffer was added dropwise, followed by 1 hour of 37°C culture in an incubator. After the excess blocking buffer was shaken off, corresponding primary antibodies against CD86 or CD206 and IBA1 were added dropwise to the sections, which were incubated at 4°C overnight. Afterwards, section washing in phosphate-buffered saline was conducted again, and fluorescent-labelled secondary antibodies were supplemented for 1 hour of room-temperature incubation prior to washing. Afterwards, the sections were added with a DAPI staining solution in drops for nuclear counterstaining. Ten minutes later, the sections were washed and blocked. A fluorescence microscope was utilised to observe and collect images, and an image analysis system was employed to count M1 or M2 microglia.

Western Blotting for Expressions of Fas, FasL and TH Proteins in the Substantia Nigra

The bicinchoninic acid method was employed to examine the substantia nigra for the concentration of total protein. The protein sample (a final concentration of 2 $\mu g \cdot mL^{-1}$) was boiled in hot water for 10 minutes, followed by storage in the -20°C refrigerator. Next, 50 μg of protein was loaded, added to the sample buffer, and boiled. Then SDS-PAGE separation and PVDF membrane transfer were performed for the denatured protein sample. Afterwards, the membrane was subjected to 1-hour blocking with skim milk (5%), rinsing in tris-buffered saline with Tween 20, and overnight incubation (4°C) using primary antibodies against Fas, FasL and TH (1:1000) in dark. Subsequently, secondary antibodies (1:5000) were added to incubate the membrane for 2 hours. At last, ECL solution was added for development and exposure, and protein bands were analysed using Image Lab software (Bio-Rad, USA) to obtain the gray value.

Statistical Analysis

Prism 8.0 software (GraphPad, USA) was utilised for statistical analysis. The format of mean ± standard deviation (åx ± s) was employed to describe all measurement data. The comparison of data between different groups was conducted using one-way analysis of variance, while pairwise comparison was executed through the LSD-*t* test. $P<0.05$ indicated a difference of statistical significance.

RESULTS

Behavioural Performance

In the sham group, no obvious abnormalities were observed in behaviours, activities, or diet. The rats in the PD group had obvious dry hairs, decreased food intake, limb tremor, markedly decreased voluntary activities, unstable gait, and dry stool. In BSZCF and DBSJ groups, the PD symptoms of rats were obviously relieved.

Behavioural Test Results

The pole-climbing time of rats was significantly prolonged, while the hanging score significantly decreased in the PD group in comparison to those in the sham group ($P<0.05$). The BSZCF group and the DBSJ group, compared with the PD group, had significantly shortened pole-climbing time and increased hanging score ($P<0.05$). The differences in pole-climbing time and hanging score were not significant between BSZCF and DBSJ groups ($P>0.05$) (Fig. 1).

Neurological Function Score

The PD group had a significantly higher neurological function score than that of the sham group [(3.26±0.47) points *vs.* 0 point] ($P<0.05$). The score significantly dropped in the BSZCF group [(1.32±0.28) points] and the DBSJ group [(1.18±0.20) points] compared with that in the PD group ($P<0.05$), while BSZCF and DBSJ groups had similar scores ($P>0.05$).

Pathological Changes in the Substantia Nigra

In the sham group, neurons in the substantia nigra were arranged neatly and closely, with-

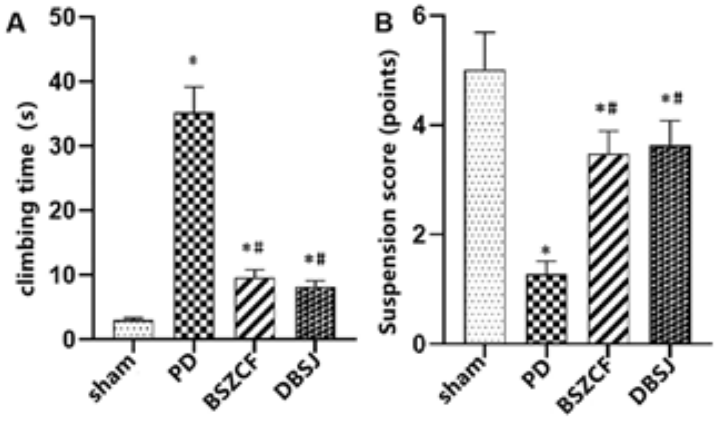

Fig. 1. Behavioral test results
A: Comparison of pole-climbing time
B: Comparison of hanging score
***P<0.05 vs. Sham Group, #P<0.05 vs. PD Group**
PD: Parkinson's disease

out obvious changes in cell morphology. In the PD group, evident pyknosis was found in the neuronal cell membrane from the substantia nigra, presenting reduced cytoplasm and incomplete nuclear membranes, as well as a prominently decreased number of nerve cells. Concerning BSZCF and DBSJ groups, the nerve cells in the substantia nigra had obviously alleviated morphology, with pyknosis found in only a few nuclei, and there were obviously more nerve cells (Fig. 2).

IL-4, IL-10, TNF-α and IL-6 Levels in the Substantia Nigra

The PD group had raised TNF-α and IL-6 levels together with reduced IL-4 and IL-10 levels in the substantia nigra compared with those of the sham group ($P<0.05$). Decreases in TNF-α and IL-6 levels in the substantia nigra as well as increases of IL-4 and IL-10 levels were observed in BSZCF and DBSJ groups in comparison to those in the PD group ($P<0.05$). The differences in TNF-α, IL-6, IL-4, and IL-10 levels in the substantia nigra between BSZCF and DBSJ groups were not significant ($P>0.05$) (Fig. 3).

Microglial Polarization in the Substantia Nigra

Compared to the sham group, the PD group had more $IBA1^+CD86^+$ cells, higher CD86/CD206 ratio, and fewer $IBA1^+CD206^+$ cells ($P<0.05$). The CD86/CD206 ratio and the $IBA1^+CD86^+$ cell counts were lower, whereas the $IBA1^+CD206^+$ cell count was higher in BSZCF and DBSJ groups

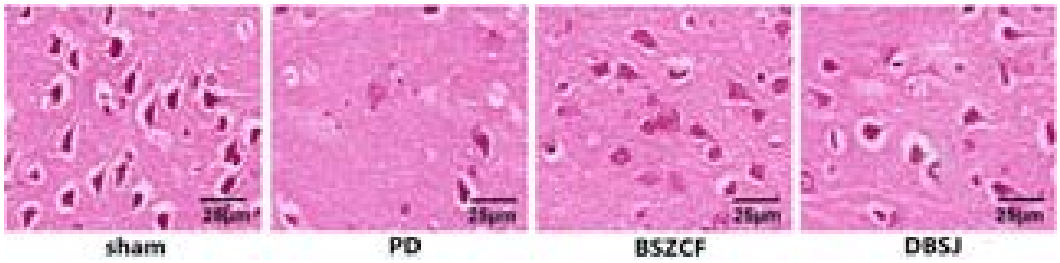

Fig. 2. Hematoxylin-eosin staining for the substantia nigra (magnification: ×400)

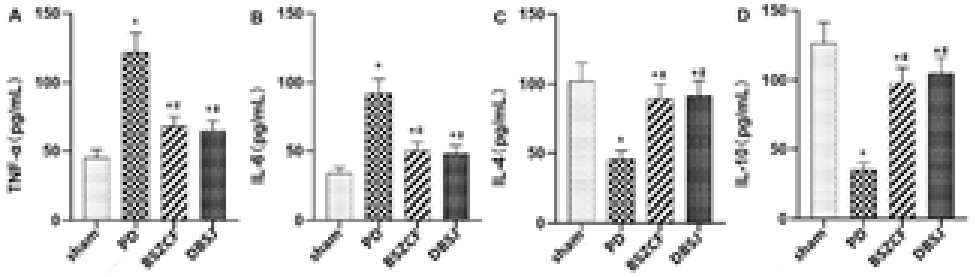

Fig. 3. IL-4, IL-10, TNF-α, and IL-6 levels in the substantia nigra
A: Comparison of TNF-α level
B: Comparison of IL-6 level
C: Comparison of IL-4 level
D: Comparison of IL-10 level
***P<0.05 vs. Sham Group, #P<0.05 vs. PD Group**
IL: Interleukin; PD: Parkinson's disease; TNF-α: tumor necrosis factor-α

than in the PD group (P<0.05). Moreover, the IBA1$^+$CD86$^+$ and IBA1$^+$CD206$^+$ cell counts together with the CD86/CD206 ratio presented no significant differences between BSZCF and DBSJ groups (P>0.05) (Fig. 4).

Expressions of Fas, FasL and TH Proteins in the Substantia Nigra

Significantly higher Fas and FasL protein expressions, as well as lower TH protein expres-

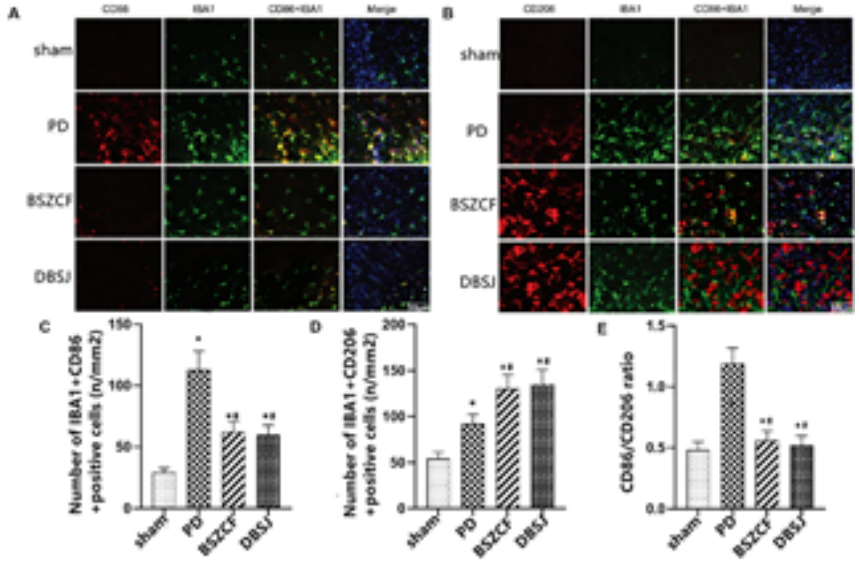

Fig. 4. Microglial polarization in the substantia nigra
A: CD86 expression determined using immunofluorescence assay
B: Expression of CD206 determined through immunofluorescence assay
C: Comparison of number of IBA1+CD86+ cells
D: Comparison of number of IBA1+CD206+ cells
E: Comparison of ratio of CD86/CD206
P<0.05 vs. Sham Group, #P<0.05 vs. PD Group
CD: Cluster of differentiation; IBA1: ionized calcium-binding adaptor molecule 1; PD: Parkinson's disease

sion, were detected in the substantia nigra from the PD group than those of the sham group ($P<0.05$). The BSZCF group and the DBSJ group, compared to the PD group, had significantly reduced Fas and FasL protein expressions and elevated TH protein expression in the substantia nigra ($P<0.05$). Besides, Fas, FasL and TH protein expressions from the substantia nigra had insignificant differences between BSZCF and DBSJ groups ($P>0.05$) (Fig. 5).

DISCUSSION

In the present study, BSZCF not only mitigated the symptoms of limb tremor, autonomic activity, tail and back stiffness, dry hairs, decreased food intake and dry stool in PD rats, but also increased the hanging score and shortened the pole-climbing time, suggesting that BSZCF can relieve the motor and non-motor symptoms of PD rats.

Dopaminergic neuron loss in the substantia nigra is the root reason for PD, and the state of dopaminergic neuronal survival in the brain can be reflected by TH (Tang et al. 2023). The researchers herein found that TH protein expression declined significantly in the substantia nigra of the PD group, proving the neuronal loss in PD rats. As one of the transmembrane proteins of the TNF receptor superfamily, Fas binds FasL to form an apoptosis-inducing complex. Fas can interact with FasL to induce the death of inflammatory cells, which is implicated in multiple sclerosis, cerebral infarction, and degenerative diseases (Krzyzowska et al. 2014; Malarkannan 2020; Lagunas-Rangel 2023). It was found in this study that BSZCF suppressed Fas and FasL but promoted TH in the substantia nigra at the level of protein expression, suggesting that BSZCF inhibited the Fas/FasL signalling pathway and raised the TH protein level to repress dopaminergic neuron loss in PD rats. Consistently, Li et al. (2020) confirmed that PD rats had high Fas and FasL protein expressions, activated Fas/FasL signalling pathway, and decreased number of dopaminergic neurons.

The main pathophysiological mechanism of PD is neuroinflammation mediated by microglial activation, as a crucial mechanism of dopaminergic neuron death (Lv et al. 2023). Under the action of many inflammatory factors, microglia, as one of the vital mediators of inflammatory responses, are activated, which can differentiate into typical pro-inflammatory M1 phenotype or protective anti-inflammatory M2 phenotype (Lind-Holm et al. 2023). M1 microglia secrete cytokines such as TNF-α and IL-6, aggravating the inflammatory reaction, while M2 microglia secrete IL-4, IL-10 and other anti-inflammatory factors and play a neuroprotective role by phagocytising misfolded proteins (Xu et al. 2023; Yu et al. 2023). Therefore, facilitating the M2 phenotype polarisation or suppressing the M1 phenotype polarisation of microglia while facili-

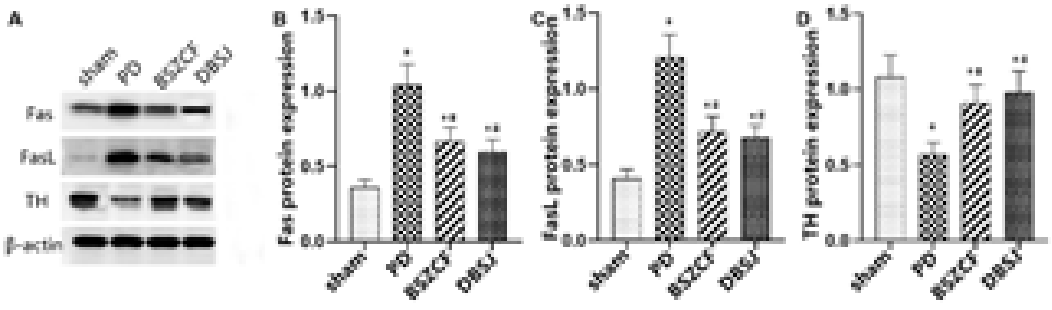

Fig. 5. Fas, FasL and TH protein expressions in the substantia nigra
A: Fas, FasL and TH protein bands in rat substantia nigra
B: Comparison of Fas protein expression in the substantia nigra
C: Comparison of FasL protein expression in the substantia nigra
D: Comparison of TH protein expression in the substantia nigra
***P<0.05 vs. Sham Group, #P<0.05 vs. PD Group**
FasL: Fas ligand; PD: Parkinson's disease; TH: Tamm-Horsfall

tating the microglia to polarise from M1 phenotype to M2 phenotype have potential treatment effects on PD (Guo et al. 2022). In this study, BSZCF applied to PD rats reduced M1-positive cell count, TNF-á and IL-6 levels in the substantia nigra, and neurological function score, but raised the M2-positive cell count and IL-4 and IL-10 levels, indicating that BSZCF facilitated the microglia transformation into M2 phenotype from M1 phenotype, mitigating central inflammatory responses, and inhibit the Fas/FasL signalling pathway-mediated dopaminergic neuron loss, relieving neurological injuries.

CONCLUSION

In conclusion, BSZCF can mitigate central inflammatory responses by promoting the microglia phenotype transformation from M1 to M2, and relieve neurological injuries by suppressing the Fas/FasL signalling pathway-mediated dopaminergic neuron loss.

RECOMMENDATIONS

The beneficial role of BSZCF in PD should be further tested by *in vitro* cell experiments and clinical studies.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

BSZCF: Bushen Zhichan Formula
CD: Cluster of differentiation
FasL: Fas ligand
IBA1: ionised calcium-binding adaptor molecule 1
IL: Interleukin
PD: Parkinson's disease
TH: Tamm-Horsfall
TNF-α: tumour necrosis factor-α

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