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Effect of Formononetin on Lipopolysaccharide-Induced Depressive-Like Behaviors and Neuroinflammation in Mice

Mengyuan Li[1,2] Hui Liu[1,2] Shuaijun Peng[1,2] Pan Su[1] Erping Xu[1] Ming Bai[1] Yucheng Li[1]

[1] Henan Key Laboratory for Modern Research on Zhang Zhongjing's Prescriptions, Academy of Chinese Medical Sciences, Henan University of Chinese Medicine, Zhengzhou, Henan, China
[2] College of Pharmacy, Henan University of Chinese Medicine, Zhengzhou, Henan, China

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Address for correspondence Yucheng Li, PhD, Professor, Henan Key Laboratory for Modern Research on Zhang Zhongjing's Prescriptions, Academy of Chinese Medical Sciences, Henan University of Chinese Medicine, 156 Jinshui East Road, Zhengzhou, Henan 450046, China (e-mail: Liyucheng@hactcm.edu.cn).

Ming Bai, MD, Professor, Henan Key Laboratory for Modern Research on Zhang Zhongjing's Prescriptions, Academy of Chinese Medical Sciences, Henan University of Chinese Medicine, 156 Jinshui East Road, Zhengzhou, Henan 450046, China (e-mail: Baiming666@126.com).

Abstract

Objective The objective of this article is to explore the effect of formononetin (FMN) on depressive-like behaviors and neuroinflammation in lipopolysaccharide (LPS)-induced mice.

M**ethods** After acclimatization, male Institute of Cancer Research mice were randomly divided into normal group, LPS group, paroxetine group (20 mg/kg), FMN low-dose group (20 mg/kg, FMN20), and FMN high-dose group (40 mg/kg, FMN40), with eight mice in each group. The depressive-like behaviors were observed by sucrose preference test, tail suspension test (TST), and open field test. The protein and mRNA levels of interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) in the hippocampus were determined by enzyme-linked immunosorbent assay and real-time quantitative polymerase chain reaction. The expression level of ionized calcium-binding adapter molecule 1 (Iba-1) in the hippocampus was observed by immunofluorescence staining to evaluate the activation level of microglia.

Results Compared with the control group, the sucrose preference rate, the activity time of the central area, the distance of the central area, and the number of times of entering the central area were significantly decreased in the LPS group ($p < 0.01$), and the immobility time of TST was significantly prolonged ($p < 0.05$), the expression levels of IL-6, IL-1β, and TNF-α protein and mRNA in hippocampus were significantly increased ($p < 0.01$), and the fluorescence intensity of Iba-1 in CA1, CA3, and DG regions of hippocampus was significantly increased ($p < 0.01$). Compared with the LPS group, the sucrose preference rate, central area activity time, central area activity distance, and the number of times of entering the central area were significantly increased ($p < 0.05$ or $p < 0.01$) in the FMN group, and TST immobility time was significantly shortened ($p < 0.01$), the expression levels of IL-6, IL-1β, and TNF-α protein and mRNA in the hippocampus were significantly decreased ($p < 0.05$ or $p < 0.01$), and the fluorescence

Keywords
- formononetin
- depression
- LPS
- neuroinflammation
- proinflammatory cytokines
- activation of microglia

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intensity of Iba-1 in CA1, CA3, and DG regions of hippocampus was significantly decreased ($p < 0.01$).

Conclusion FMN could inhibit LPS-induced activation of microglia, reduce hippocampal neuroinflammation, and improve depressive-like behaviors in mice.

Introduction

Depression is a common mental disorder which is characterized by a persistent low mood or lack of pleasure. It can also be accompanied by varying degrees of cognitive and behavioral changes, even with suicidal tendencies. All over the world, there are approximately 350 million people suffering from depression.[1–3] However, clinical antidepressants have poor efficacy and side effects,[4] and there is an urgent need to develop new antidepressants.

Formononetin (FMN) is a natural isoflavone, which is widely present in plants such as Gancao (Glycyrrhizae Radix et Rhizoma) Jixueteng (Spatholobi Caulis) and Huangqi (Astragali Radix)[5] and has significant anti-inflammatory and neuroprotective activities.[6–8] We established a mouse model of depression by continuous injection of corticosterone and demonstrated the antidepressant effect of FMN for the first time in this model, but the mechanism remains to be elucidated.[9] Depression belongs to the category of "depression syndrome" in Chinese medicine, which is caused by stagnation of liver qi and emotional depression.[10] In Chinese medicine, "liver depression transforming into fire" is closely related to "inflammation" in Western medicine." Liver depression transforming into fire" results from prolonged liver depression, losing the function of conveyance and dispersion resulting in emotional depression and then developing into the inflammatory symptoms of the body. Modern medical studies have shown that neuroinflammation plays an important role in the pathogenesis of depression.[11–13] The levels of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) are significantly increased in the brains of patients with severe depression and depression model animals.[14] These cytokine-mediated neuroinflammatory responses not only inhibit neurogenesis but also lead to apoptosis of neurons. Inhibition of neuroinflammation can significantly improve depressive-like behaviors in animals,[15,16] which has become a hot content in antidepressant research. Lipopolysaccharide (LPS) is a bacterial endotoxin that can increase the release of proinflammatory cytokines and induce neuroinflammation and depressive-like behaviors in animals, which has been widely used in antidepressant research.[17–19] In order to reveal whether the antidepressant effect of FMN is related to the inhibition of neuroinflammation, LPS-induced neuroinflammation and depressive-like behaviors in the mice were examined in this study, and the antidepressant effect of FMN in this model was observed, which may provide a basis for further revealing the antidepressant mechanism of FMN.

Materials

Animal

Forty male specific pathogen-free-grade Institute of Cancer Research mice, weighing 18 to 20 g, 8 weeks old, were purchased from Beijing Chales River Experimental Animal Technology Co., Ltd.,(production license No.: SYXK [Beijing] 2021-0006). The mice were fed adaptively for 1 week, with an ambient temperature of 23 to 25 °C, a humidity of 40 to 60%, a 12-hours circadian rhythm, and free access to food and water. This experiment was approved by the Experimental Animal Ethics Committee of Henan University of Chinese Medicine with the approval number of DWLL201903018.

Drugs and Reagents

Drugs and reagents used were as follows: FMN (Dalian Meilun Biotechnology Co., Ltd., purity > 98%, No.:J0719C); LPS and Sucrose (Sigma-Aldrich Co., Ltd., No.:059M4031V, WXBD7917V); Paroxetine Hydrochloride Tablets (Sino-US Tianjin GSK Pharmaceutical Co., No.:8W4P); Ionized Calcium Binding Adapter Molecule 1(Iba-1) (Wuhan Servicebio Technology Co., Ltd.,No.:GB11105); RIPA lysate (ComWin Biotech Co., Ltd., No.: 01408/15322); BCA Protein Assay Kit (Solarbio Science&Technology Co., Ltd., No.:20221009); Total RNA Extraction Kit (Tiangen Biotech Co., Ltd., No.:R6816); Fluorescent Quantitative PCR Kit (Germany Qiagen, No.: 169047300); Enzyme-Linked Immunosorbent Assay (ELISA) Kits for IL-6, TNF-α, and IL-1β (US R&D systems, No.: P334473, P340966, and P334462).

Instrument

Smart 3.0 (Panlab, Spain), Gen5 multifunctional microplate reader (Bio Tek, United States), Nana drop One Micro nucleic acid protein analyzer (Thermo, United States), Q5 real-time fluorescence quantitative PCR instrument (Thermo, United States), and Nikon Eclipse E 10 fluorescence microscope (Nikon, Japan) were used for animal behavior video recording and analysis system.

Methods

Animal Model and Drug Treatment

After 1 week of adaptive feeding, the mice were randomly divided into five groups ($n = 8$): control group, LPS group, paroxetine group (20 mg/kg), FMN 20 group (20 mg/kg), and FMN 40 group (40 mg/kg). FMN and paroxetine were both prepared as suspensions in normal saline and orally administered once daily at the volume of 10 mL/kg for 14 days. The normal and LPS group received equal volumes of saline.

LPS was administered intraperitoneally on day 14. The behavioral tests were performed after 24 hours of LPS injection.

Behavioral Experiment

Sucrose Preference Test[20]

Before the sucrose preference test, the mice were trained to adapt to the sucrose solution. Each mouse was given a bottle of sucrose solution (1%, w/v) and a bottle of water to adapt for 48 hours, and the bottle position was changed every 12 hours during the adaptation period. After the adaptation, the mice were deprived of water and food for 24 hours and then given a bottle of sucrose solution (1%, w/v) and a bottle of water for 12 hours. The consumed sucrose and water were weighted, and the sucrose preference was calculated as follows: sucrose preference (%) = sucrose consumption/(sucrose consumption + water consumption) × 100%.

Tail Suspension Test[21]

The mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. The mice were suspended for 6 minutes, and the state of the mice within 6 minutes was recorded by animal behavior analysis system Smart 3.0, and the immobility time in the last 4 minutes was calculated.

Open Field Test[22]

The mice were individually placed in a box (50 × 50 × 50 cm), and the middle 9/25 area of the box was defined as the center. The movement of the mice within 5 minutes was tracked and recorded by Smart 3.0 (Panlab, Spain). The total distance, the center distance, the center time, and the number of entering center were autoanalyzed by Smart 3.0 software.

Real-Time Fluorescence Quantitative Polymerase Chain Reaction Detection of Proinflammatory Cytokine mRNA Expression Levels in Mouse Hippocampus

Total RNA was extracted from the hippocampus using the kit, and 30 μL RNase-free water was added to dissolve total RNA. The concentration of the extracted total RNA was determined using a micronucleic acid protein analyzer, and cDNA was synthesized by reverse transcription reaction. The amplification reaction was performed using a Q5 PCR instrument. The relative quantification of the mRNA expression levels of the target genes was performed using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the internal control, and the results were expressed as the fold of each group compared with the normal group. The primers were synthesized by Invitrogen Corporation (Shanghai, China), and the sequences are shown in ►**Table 1**.

Detection of Proinflammatory Cytokines in Mouse Hippocampus using the Enzyme-Linked Immunosorbent Assay Method

Hippocampus were homogenized with RIPA lysate and centrifuged at 12,000/min for 20 minutes. The supernatant was collected, and protein concentration was determined by the BCA kit. The levels of IL-1β, IL-6, and TNF-α were determined by ELISA kits according to the instruction of kits.

Observing the Activation Level of Microglia in Mouse Hippocampus by Immunofluorescence Staining

The whole brains of the mice were removed on ice and fixed with 4% paraformaldehyde. After paraffin-embedded sections (5 μm), the sections were placed in ethylenediaminetetraacetic acid antigen retrieval buffer (pH = 8.0), antigen retrieval was performed in a microwave oven and then blocked with 3% bovine serum albumin for 30 minutes. The blocked solution was gently shaken, and then, the sections with Iba-1 primary antibody (diluted ratio 1:1,000) were incubated at 4°C overnight. The sections were rinsed with phosphate buffer saline (pH = 7.4) and incubated with secondary antibody for 50 minutes. The nuclei were restrained with DAPI and incubated in the dark for 10 minutes. Finally, antifluorescence quenching blocking tablets were used for blocking, and the images were scanned with a fluorescence microscope, and the expression level of Iba-1 in hippocampus was observed.

Statistical Analysis

Statistical analysis was conducted using SPSS 25.0, and all data were represented as mean ± standard deviation ($\pm s$). The comparison between the normal group and the LPS group was conducted using *t*-test, while the other multiple-group comparisons were conducted using one-way

Table 1 Primer sequences

Primer	Sequence(5′-3′)	Product length/bp
IL-6	Forward primer CTGCAAGAGACTTCCATCCAG	131
	Reverse primer AGTGGTATAGACAGGTCTGTTGG	
TNF-α	Forward primer TAGCCCACGTCGTAGCAAAC	170
	Reverse primer GCAGCCTTGTCCCTTGAAGA	
IL-1β	Forward primer TGCCACCTTTTGACAGTGATG	136
	Reverse primer ATGTGCTGCTGCGAGATTTG	
GAPDH	Forward primer TCTCCTGCGACTTCAACA	117
	Reverse primer TGTAGCCGTATTCATTGTCA	

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6; IL-1β, interleukin-1β, TNF-α, tumor necrosis factor-α.

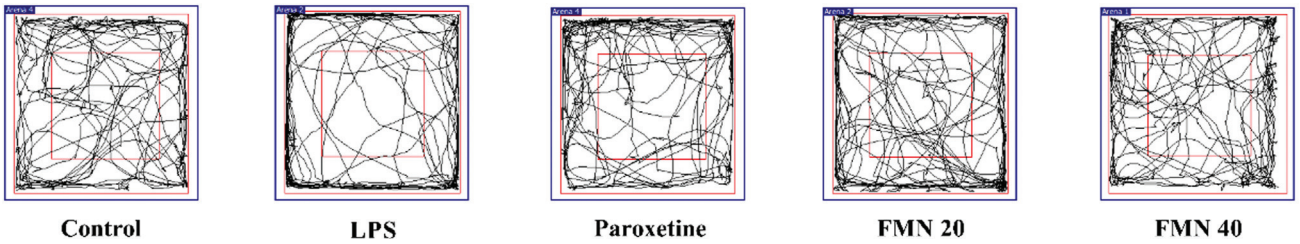

Fig. 1 The activity track of the mice in OFT.

Table 2 Effects of FMN on LPS-induced depressive-like behaviors in mice (Mean ± *s*, $n = 8$)

Group	Sucrose preference/%	Immobility time in TST(t/s)	OFT experiment			
			Total distance (s/cm)	Distance in center (s/cm)	The time in center (t/s)	The number of entering center/times
Control	88.29 ± 0.01	80.34 ± 45.66	2,525.05 ± 353.86	171.43 ± 45.88	11.93 ± 4.93	14.63 ± .66
LPS	62.83 ± 0.08[b]	127.7 ± 9.11[a]	2,206.31 ± 269.21	88.50 ± 46.41[a]	5.80 ± 4.00[a]	6.50 ± 5.04[b]
Paroxetine	73.43 ± 0.05[d]	57.11 ± 25.14[d]	2,166.03 ± 131.59	83.09 ± 22.73	6.91 ± 4.77	9.50 ± 5.90
FMN20	76.56 ± 0.07[d]	48.47 ± 13.86[d]	2,080.55 ± 301.75	159.75 ± 78.51	10.27 ± 6.45	11.75 ± 5.23
FMN40	80.22 ± 0.05[d]	40.98 ± 25.57[d]	2,375.67 ± 482.68	220.58 ± 97.38[d]	14.34 ± 7.46[c]	14.38 ± 1.57[c]

Abbreviations: FMN, formononetin; LPS, lipopolysaccharide; OFT, open field test; TST, tail suspension test.
Notes: Compared with the normal group
[a] $p < 0.05$
[b] $p < 0.01$, compared with the LPS group
[c] $p < 0.05$
[d] $p < 0.01$.

analysis of variance combined with Dunnett test. $p < 0.05$ indicates a statistically significant difference.

Results

Effect of Formononetin on Lipopolysaccharide-Induced Depressive-Like Behavior in Mice

Compared with the control group, sucrose preference was significantly reduced in the LPS group ($p < 0.01$) and increased in the paroxetine group and FMN group ($p < 0.01$) compared with the LPS group. In addition, the tail suspension test (TST) immobility time significantly increased in the LPS group ($p < 0.05$), while that decreased in the paroxetine group and the FMN group ($p < 0.01$). The results of open field test showed that there was no significant difference in the total distance, but the time in the center, activity distance in the center, and the number of entering the center were significantly reduced in the LPS group compared with the control group ($p < 0.05$ or $p < 0.01$), while those in FMN high-dose group significantly increased ($p < 0.05$ or $p < 0.01$), see ►**Fig. 1** and ►**Table 2**.

Effects of Formononetin on mRNA Expression Levels of Proinflammatory Cytokines in Hippocampus

As shown in ►**Table 3**, the mRNA levels of IL-6, IL-1β, and TNF-α in hippocampus were significantly increased in the LPS

Table 3 Effects of FMN on the mRNA levels of proinflammatory cytokines in the hippocampus (Mean ± *s*, $n = 6$)

Group	IL-6 mRNA	IL-1β mRNA	TNF-α mRNA
Control	1.00 ± 0.23	1.00 ± 0.50	1.00 ± 0.18
LPS	178.06 ± 52.00[a]	292.94 ± 112.99[a]	78.85 ± 32.63[a]
Paroxetine	103.25 ± 41.92[b]	158.59 ± 35.16[b]	47.82 ± 16.73[b]
FMN 20	90.40 ± 46.88[c]	160.99 ± 37.35[b]	28.87 ± 2.68[c]
FMN 40	86.73 ± 6.14[c]	142.92 ± 49.89[c]	44.80 ± 18.79[b]

Abbreviations: FMN, formononetin; IL-6, interleukin-6; IL-1β, interleukin-1β, TNF-α, tumor necrosis factor-α, LPS, lipopolysaccharide.
Notes: Compared with normal group
[a] $p < 0.01$, compared with LPS group
[b] $p < 0.05$
[c] $p < 0.01$.

Table 4 Effects of FMN on the levels of proinflammatory cytokines in the hippocampus (Mean ± *s*, *n* =6)

Group	IL-6(pg/mg)	IL-1*β*(pg/mg)	TNF-α(pg/mg)
Control	34.55 ± 5.62	4.86 ± 1.12	28.87 ± 3.27
LPS	62.02 ± 13.67[b]	30.60 ± 12.58[a]	30.55 ± 5.61[a]
Paroxetine	48.31 ± 8.50[c]	16.76 ± 5.46[c]	25.22 ± 5.46[c]
FMN 20	45.17 ± 9.49[c]	14.74 ± 5.47[c]	24.16 ± 5.01[c]
FMN 40	39.59 ± 4.75[c]	14.83 ± 3.85[c]	24.09 ± 1.14[c]

Abbreviations: FMN, formononetin; IL-6, interleukin-6; IL-1β, interleukin-1β, TNF-α, tumor necrosis factor-α, LPS, lipopolysaccharide.
Notes: Compared with the normal group
[a]$p < 0.05$
[b]$p < 0.01$, compared with the LPS group
[c]$p < 0.05$.

Table 5 Effects of FMN on the fluorescence intensity of Iba-1 in hippocampal microglia (Mean ± *s*, *n* = 2)

Group	CA1	CA3	DG
Control	25.16 ± 0.63	23.91 ± 1.11	27.20 ± 0.48
LPS	47.06 ± 0.58[a]	42.60 ± 0.92[a]	41.47 ± 0.63[a]
Paroxetine	34.08 ± 1.08[b]	33.91 ± 0.78[b]	36.12 ± 0.22[b]
FMN 20	28.07 ± 0.59[b]	31.86 ± 0.83[b]	34.30 ± 0.65[b]
FMN 40	26.16 ± 0.41[b]	28.36 ± 0.13[b]	27.83 ± 0.24[b]

Abbreviations: FMN, formononetin; LPS, lipopolysaccharide.
Notes: Compared with the normal group.
[a]$p < 0.01$, compared with the LPS group.
[b]$p < 0.01$.

group compared with the control group ($p < 0.01$). Compared with the LPS group, FMN significantly down-regulated the mRNA levels of IL-6, IL-1β, and TNF-α ($p < 0.01$or $p < 0.05$).

Effects of Formononetin on the Levels of Proinflammatory Cytokines in Hippocampus

►**Table 4** showed that the contents of IL-6, IL-1β, and TNF-α were also increased in the LPS group ($p < 0.05$ or $p < 0.01$). FMN significantly down-regulated the contents of IL-6, IL-1β, and TNF-α compared with the LPS group ($p < 0.05$).

Effect of Formononetin on the Activation Level of Hippocampal Microglia

To investigate the effects of FMN on microglia activation in hippocampus, a specific marker of microglia Iba-1 was determined by immunofluorescence. As shown in ►**Table 5** and ►**Fig. 2**, compared with control group, the fluorescence intensity of Iba-1 in the regions of CA1, CA3, and DG significantly increased in the LPS group ($p < 0.01$); compared with the LPS group, the fluorescence intensity of Iba-1 in the regions of CA1, CA3, and DG significantly decreased in paroxetine and FMN groups ($p < 0.01$).

Discussion

Depression is a complex mental disease, and its onset is affected by physiological, psychological, and social environment factors. In recent years, the neuroinflammation hypothesis has been paid attention to in the pathogenesis of depression, and the development of new antidepressant drugs from the perspective of inhibiting neuroinflammation has become a research hotspot. FMN is an isoflavone compound widely found in plants such as Gancao (Glycyrrhizae Radix et Rhizoma) and Huangqi (Astragali Radix), which can inhibit neuroinflammatory responses[23] and reduce the levels of inflammatory factors such as TNF-α and IL-6 in the blood. Our previous study confirmed the antidepressant effect of FMN in corticosterone-induced depression mouse model, but whether this effect is related to the inhibition of neuroinflammation is still unclear. In this study, depressive-like behaviors were induced by intraperitoneal injection of LPS in mice. FMN significantly increased the sucrose preference, increased immobility time in the TST, and increased the center distance, center time, and number of the center activities in OFT. These behavioral improvements indicate that FMN reverses LPS-induced depressive-like behaviors, further confirming the antidepressant activity of FMN.

The hippocampus is an important brain region that governs emotions, and it is also the most vulnerable region to damage under stress and inflammation.[24] Studies have shown that LPS-induced depressive-like behaviors are associated with the overexpression of proinflammatory cytokines (such as TNF-α, IL-1β, and IL-6) in the brain (especially

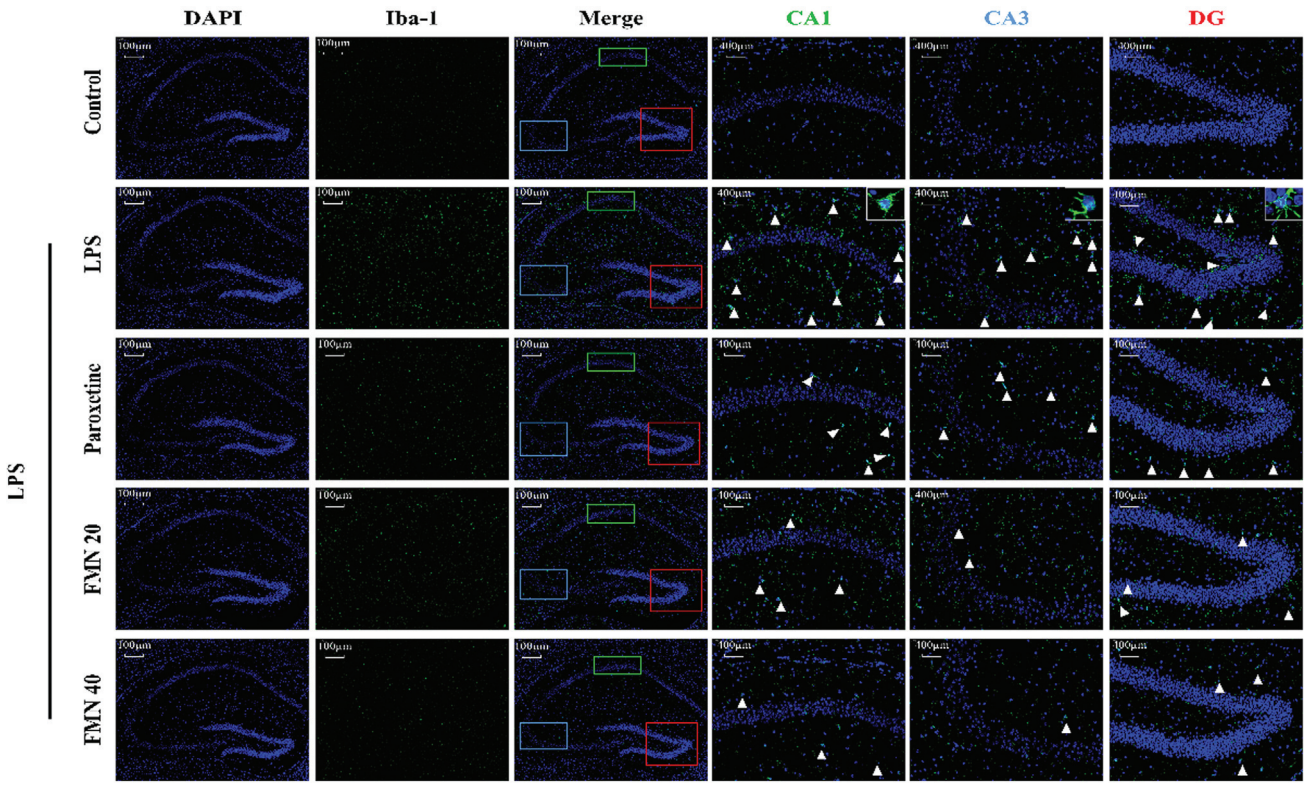

Fig. 2 Effect of FMN on the activation of microglia in the hippocampus.

in the hippocampal region).[25] To explore whether the antidepressant activity of FMN involves inhibition of hippocampal neuroinflammation, the contents of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the hippocampus were examined and it was found that LPS significantly increased the mRNA and protein levels of TNF-α, IL-1β, and IL-6 in the hippocampus. However, FMN decreased the mRNA and protein contents of these three proinflammatory cytokines in the hippocampus, indicating that FMN can inhibit LPS-induced neuroinflammation and reduce the release of inflammatory cytokines, which may be involved in its antidepressant mechanism.

Microglia are important immune cells in the central nervous system and play a key role in the occurrence of neuroinflammation.[26] Studies have shown that the activation of microglia is closely related to depression.[27] When microglia are overactivated, they will synthesize and release a large amount of inflammatory factors such as TNF-α, IL-1β, and IL-6, leading to neuronal damage and promoting apoptosis.[28] In the autopsy of patients with depression, the activation of microglia and the production of inflammation were found in the brain.[29]

In order to observe the effect of FMN on the activation of hippocampal microglia, the expression of hippocampal microglia-specific marker Iba-1 was detected. It discovered that LPS could activate microglia and promote the expression of Iba-1 in hippocampal CA1, CA3, and DG regions, while FMN could significantly reduce the expression of Iba-1 in these regions. These results indicate that FMN can reduce LPS-induced neuroinflammation by inhibiting the activation of hippocampal microglia and reducing the release of proinflammatory cytokines.

Conclusion

This study for the first time confirmed the antidepressant effect of FMN in LPS model and suggested that the antidepressant mechanism of FMN may be related to inhibiting the activation of hippocampal microglia and reducing neuroinflammation. However, the mechanism by which FMN inhibits the activation of microglia and whether the antidepressant effect is dependent on this pathway still need to be further studied.

CRediT Authorship Contribution Statement

M.L. was responsible for conceptualization, data curation, validation, and writing original draft. H.L. was responsible for methodology, and investigation. S.P. was responsible for methodology, and supervision. E.X. was responsible for project administration and funding acquisition. M.B. was responsible for validation. Y.L. was responsible for project administration, conceptualization, supervision, and funding acquisition.

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Conflict of Interest

The authors declare no conflict of interest.

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