

Exercise Improves High Fat-Induced Cognitive Impairment by Inhibiting Hippocampal Neuroinflammation *via* the Suppression of TLR4/MyD88/NF- κ B Signaling Pathway

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In the present study, the high fat diet was used to establish a murine model of obesity and treadmill exercise was applied to improve obesity. After exercise intervention, the novel object recognition test and three-chambered social test were performed to evaluate cognitive function. Enzyme-linked immunosorbent assay was employed to measure serum inflammatory factor levels and Western blot assay was performed to detect hippocampal protein levels in pro-inflammatory signaling pathway. We found that high fat diet mice spent less time on exploring novel objects and communication with unfamiliar mice in the novel object recognition and three-chambered social test, respectively. The cognitive decline in obese mice was significantly alleviated after treadmill exercise. Furthermore, serum levels of inflammatory cytokines, including interleukin-1 beta, tumor necrosis factor-alpha and interleukin-6 as well as hippocampal toll-like receptor 4/myeloid differentiation primary response 88/nuclear factor kappa B signaling pathway which can lead to the production of pro-inflammatory mediators were remarkably activated in obese mice. According to the random control experimental scheme and its big data analysis we found treadmill exercise inhibited the inflammatory response in the hippocampus. These results suggest that exercise may improve the high fat diet induced cognitive impairment by inhibiting hippocampal inflammation *via* the suppression of toll-like receptor 4/myeloid differentiation primary response 88/nuclear factor kappa B signaling pathway. Our work elucidates the mechanism underlying the improvement of exercise in obesity induced cognitive dysfunction and provides theoretical basis for the clinical application of exercise strategies for the treatment of obese patients with cognitive dysfunction.

Key words: Statistical analysis, treadmill exercise, high-fat diet, hippocampus, cognitive dysfunction

Big data statistics show that cognitive dysfunction causes learning and memory impairment and may lead to aphasia, apraxia, ignorance as well as disturbance in executive function, which severely affect the normal life quality of patients^[1]. It is noteworthy that obesity is often comorbid with cognitive dysfunction. As compared with normal-weight individuals, obese patients have a higher risk of neuropsychiatric disorders including cognitive^[2,3]. Hippocampal neuroinflammation is a significant contributing factor for the occurrence and development of cognitive dysfunction^[4]. Interestingly, previous research findings have shown that by the activation of microglia, obesity causes neuroinflammation and loss of hippocampal functional synapses^[5]. However, whether obesity affects

cognitive behaviors *via* neuroinflammatory pathway is still unclear.

Exercise forms an important part of life style intervention to control obesity without side effects. It is highly beneficial for human health such as bones and muscles growth, blood circulation, sleep quality and immunity^[6-10]. Additionally, the latest research and statistical analysis found that exercise suppressed intestinal barrier damage and neuroinflammatory response and improved depression-like behavior in mice^[11,12]. Recent studies reported that physical exercise increased the levels of growth factors in the brain and increased synaptic plasticity to promote cognitive formation^[13]. Furthermore, in clinical practice, exercise

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alleviates cognitive decline and dementia progression and dementia progression and reverses the symptoms of mild cognitive impairment in the elderly^[14]. However, the role of exercise in obesity-induced cognitive impairment and the underlying mechanism is not fully understood.

Our study is the first to evaluate the contribution of neuroinflammation in obese-induced cognitive dysfunction and explore the effect of exercise on cognition in obese mice and the underlying mechanism. In the present study, High Fat Diet (HFD) was used to establish an animal model of obesity and treadmill exercise was used as a therapeutic approach to treat obesity. Cognitive function of mice was evaluated by Novel Object Recognition (NOR) test and Three-Chambered Social (TCS) test followed by detection of serum levels of inflammatory cytokines, including Interleukin-1 Beta (IL-1 β), Tumor Necrosis Factor-Alpha (TNF- α) and Interleukin-6 (IL-6). Since Toll-Like Receptor 4 (TLR4)/Myeloid Differentiation primary response 88 (MyD88)/Nuclear Factor kappa B (NF- κ B) signaling pathway leads to the production of pro-inflammatory mediators^[15], the protein levels of TLR4, MyD88, p-p65NF- κ B, phosphorylated-I κ B kinase alpha (p-IKK α) and p-IKK β in the hippocampus were also measured in the present study.

Our results showed that obese produced significant hippocampal neuroinflammation *via* the activation of TLR4/MyD88/NF- κ B signaling pathway. Moderate-intensity aerobic exercise by treadmill suppressed the pro-inflammatory signaling pathway and significantly improved the cognitive impairment caused by obesity. These findings provide theoretical support for the further application of exercise training in the improvement of

obese induced cognitive decline.

MATERIALS AND METHODS

Animals:

4 w old male C57BL/6Cnc mice (n=60) were purchased from Zhejiang Vital River Laboratory Animal Technology Co. (Zhejiang, China) and housed with a 12 h light/dark cycle (8:00 am-8:00 pm), under controlled temperature (23 \pm 2 $^{\circ}$) and 50 %-60 % relative humidity. The mice were divided into three groups (n=20 per group): Control group (control mice); HFD group (HFD mice) and HFD with treadmill exercise group (HFD-M mice). All procedures on animals followed guidelines established by the Animal Ethical and Welfare Committee of Nanjing University and the China Council on Animal Care at Nanjing University.

HFD model:

The mice were fed with normal diet (Table 1) or HFD (Table 2) chow and were provided with sterilized water or 20 % fructose water^[16]. The obesity model was considered successful when the body weight was more than 20 % of the average body weight of normal mice.

Exercise protocol:

This experiment included 60 male C57BL/6Cnc mice aged 4 w, 40 of which were fed with a HFD for 6 w to establish an obesity model. After achieving HFD induced obesity, HFD-M mice were selected to acclimate to a treadmill undergo a 6 w training protocol. The HFD-M mice performed treadmill exercise at a speed of 12 m/min for 6 w, 5 d a week, once a day. For each exercise period, the mice were allowed 5 min of warm-up at 8 m/min and then continued to exercise at a speed of 12 m/min for 30 min/d at 2:00-4:00 pm^[17] (Table 3).

TABLE 1: COMPONENTS OF NORMAL DIET

	Components			Water
	Crude Fat (g)	Crude Protein (g)	Crude Fiber (g)	
Normal diet	40	180	50	Sterile water

TABLE 2: COMPONENTS OF HFD

	Components			Water
	Fat (%)	Protein (%)	Carbohydrates (%)	
HFD	60	20	20	20 % fructose water

TABLE 3: TREADMILL EXERCISE STARTED 6 W AFTER THE INTAKE OF HFD

Exercise period (w)	Warm-up (m/min)	Exercise speed (m/min)	Slope grade (%)	Time (min)
0-1	5		0	30
2-7	8	12	0	30

TCS test:

Social behavior or social memory was tested by the TCS test. The TCS test was conducted in a white plastic open box that was a 57 cm×45 cm×20 cm in size. There was a switchable channel at the bottom center of the two transparent partitions and metal cages at the corners of the left and right boxes. Before the initiation of the formal test, the test mice were placed in the middle box, in order to adapt for 5 min. During the first stage of the experiment, a stranger mouse of the same sex was placed in the metal cage at the corner of the left box. The test mice were placed in the middle box and then allowed to move freely in the three boxes for 10 min before being removed. In the second phase of the experiment, the second stranger mice of the same sex was placed in the metal cage at the corner of the right box and the test mice were placed in the middle box and then allowed to move freely in the three boxes for 10 min. During the experiment, a video camera was used to track the movement trajectory of the mice and the time and number of contacts between the test mouse and the metal cage were recorded^[18].

NOR test:

Cognitive abilities of mice were tested by the NOR test, which was conducted using a 30×30×20 cm box. First, the mice adapted to the environment in the experimental box for 5 min. Then, two identical items were placed into a symmetrical position in the box and the mice were allowed to freely explore the box. A video camera was used to track the movement trajectory of the mice in the box for 10 min. After 1 h, one item was replaced with another item of similar size but with completely different colors and shapes. The time and the number of times that the mice explored the two different objects within 10 min were recorded and a discrimination index was used to evaluate the animal's learning ability^[19].

Enzyme-Linked Immunosorbent Assay (ELISA):

ELISA was used to detect serum levels of IL-1 β , TNF- α and IL-6 inflammatory factors (n=10) according to the manufacturer's protocol. ELISA kits for detection of TNF- α (MTA00B), IL-6 (M6000B) and IL-1 β (MLB00C) were purchased from R&D Systems (Minneapolis, MN, USA).

Western blot assay:

The total protein in mice hippocampal tissue was extracted with tissue protein lysis buffer (P0013B, Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China) and a Pierce™ Bicinchoninic Acid (BCA)

assay protein analysis kit (23225, Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate protein extract concentration. The protein extract was directly mixed with the loading buffer and boiled. The samples were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and analyzed using the indicated primary antibody, which was followed by Horseradish Peroxidase (HRP)-coupled secondary antibody (anti-mouse IgG, 6805020 and anti-rabbit IgG, 671280; 1:5000 dilution; Biosharp, Hirono, Japan). Chemiluminescent HRP substrates (Miribo, Trikala, CA, USA) and Tanon-5200 chemiluminescence imager (Tanon Technology Co., Ltd., China Shanghai) were used for visualization. The antibodies used in this study were as follows: TLR4 (1:1000 dilution, 14358; CST, Danvers, MA, USA); MyD88 (1:1000 dilution, 4283; CST); p-p65NF- κ B (1:1000 dilution, 3039 ; CST); p65 NF- κ B (1:1000 dilution, 8242; CST); p-IKK α (1:1000 dilution, ab38515; Abcam, Cambridge, Massachusetts); IKK α (1:1000 dilution, 2682; CST); p-IKK β (1:1000 dilution, ab59195; MA, USA); IKK β (1:1000 dilution, 2684; CST); GAPDH (1:1000 dilution, 5174, CST).

Statistical analysis:

All data are expressed as the mean \pm standard deviation and statistical analysis was performed using GraphPad Prism 7.0 software. The Analysis of Variance (ANOVA) test and the Bonferroni post-test were conducted. Significance was established at the $p < 0.05$.

RESULTS AND DISCUSSION

The body weight, visceral adipose tissue and fat-to-body ratio of all animals were determined before the test and no significant difference was found among each group. After 13 w, the weight of the HFD mice was significantly higher than that of the control mice and the weight of the HFD-M mice was lower than that of the HFD mice ($p < 0.001$). In addition, we measured the visceral adipose tissue of the mice and calculate the fat-to-body ratio and we obtained the same results for both indicators. These results indicated that a HFD induced obesity, while treadmill exercise effectively improved the symptoms of obesity (fig. 1).

To examine the effect of treadmill exercise on cognitive impairment in HFD mice, the TCS and NOR tests were performed to evaluate learning and memory abilities. In the second phase of the TCS test, there was no difference in the exploration duration by HFD mice between the familiar mice (stranger 1) and stranger mice (stranger 2) ($p > 0.05$), indicating that the HFD induced obese mice

exhibited cognitive impairment and did not show any interest in the newly introduced stranger. However, the exploration time of the mice in the control and HFD-M groups were significantly higher than that in the first stage ($p < 0.001$). The exploration times of HFD-M mice in examining stranger 2 were also significantly increased ($p < 0.001$). For further confirmation, we subsequently carried out the NOR test.

In the second phase of the NOR test, there was no statistical difference in both time and frequency for HFD mice between the exploration of familiar and novel objects ($p > 0.05$). However, consistent with the TCS test results, the NOR test results showed that the time taken by HFD mice to explore a novel object

were significantly lower than those of control mice. And treadmill exercise obviously extended the time for obese mice to explore a novel object. All these results showed that treadmill exercise promoted short-term learning and memory abilities in obese mice (fig. 2).

Next, we attempted to explore the possible mechanisms of exercise in the improvement of cognitive dysfunction in HFD mice. We measured serum levels of inflammatory cytokines and the results indicated that the pro-inflammatory cytokines, including IL-1 β , TNF- α and IL-6 were significantly increased in HFD mice ($***p < 0.001$) and were significantly decreased after moderate exercise ($###p < 0.01$) (fig. 3).

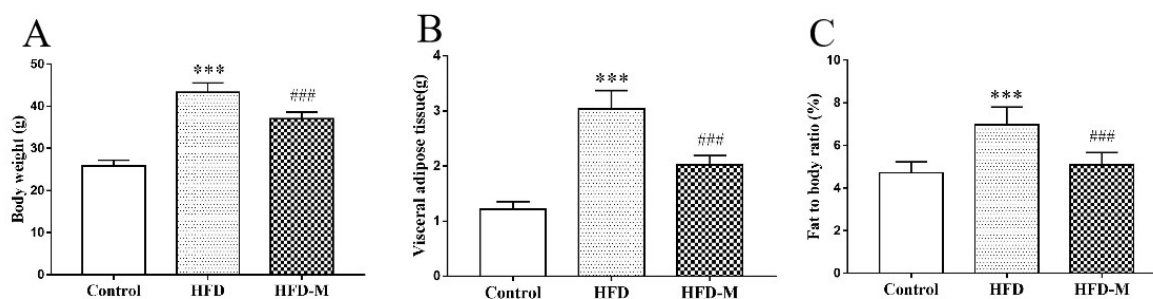


Fig. 1: Effects of treadmill exercise on body weight, visceral adipose tissue and fat-to-body ratio in obese model mice, (A) Body weight; (B) Visceral adipose tissue; (C) Fat to body ratio

Note: (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control mice; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. HFD mice)

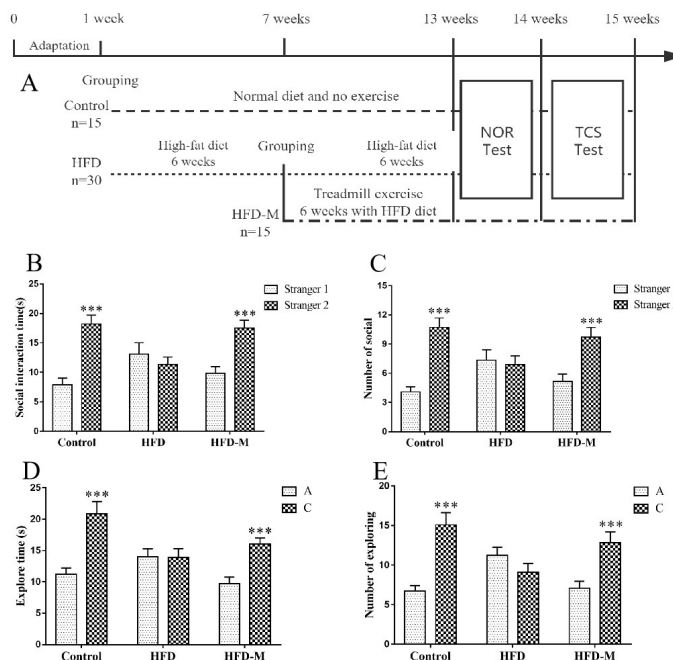


Fig. 2: Treadmill exercise decreased cognitive dysfunction in HFD-induced mice, (A) Outline of the experimental procedures; (B) Social interaction time (s) for the three-chambered social (TCS) test (* $p < 0.05$, ** $p < 0.01$, * $p < 0.001$ vs. stranger 1); (C) Number of social interactions; (D) Exploration time (s) for the novel object recognition (NOR) test; (E) Number of explorations (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control mice, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. A)**

Note: (□): Stranger 1; (▨): Stranger 1; (□): A; (▨): C

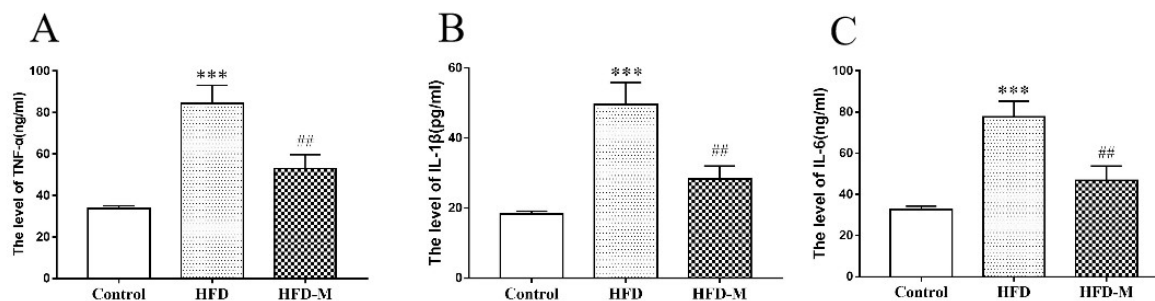


Fig. 3: Treadmill exercise increased the serum levels of inflammatory cytokines in HFD-induced mice. The hippocampal levels of (A) TNF- α ; (B) IL-1 β and (C) IL-6 by ELISA assay

Note: (* p <0.05, ** p <0.01, *** p <0.001 vs. control mice, # p <0.05, ## p <0.01, ### p <0.001 vs. HFD mice)

The TLR/MyD88/NF- κ B signaling pathway is an important pathway in the inflammatory response. It plays an important role in the occurrence and regulation of autoimmune diseases, inflammatory diseases and infectious diseases. Notably, it has been reported that obesity activates TLR4/MyD88/NF- κ B signaling pathway in adipose tissue^[20]. However, its role in hippocampal neuroinflammation has been poorly studied. The protein levels of the TLR4/MyD88/NF- κ B signaling pathway in the hippocampus were measured by Western blot assay. As shown in fig. 4, the results indicated that the protein levels of TLR4 (** p <0.01), MyD88 (* p <0.05), p-p65NF- κ B (* p <0.05), p-IKK α (** p <0.01) and p-IKK β (* p <0.05) were up-regulated in the HFD group compared with the control group. However, treadmill exercise down-regulated the levels of TLR4, MyD88, p-p65NF- κ B, p-IKK α and p-IKK β (# p <0.05) in HFD-M mice. Our results suggested that treadmill exercise may alleviate HFD induced cognitive impairment by inhibiting hippocampal inflammation through the suppression of TLR4/MyD88/NF- κ B pathway (fig. 4).

The global incidence of severe obesity in recent decades (Body Mass Index (BMI) ≥ 35 kg/m²) has been steadily increasing around the world^[21,22]. Obesity is associated with the occurrence and development of a variety of diseases, including cognitive dysfunction^[23-27]. In the present study, we reveal that the hippocampal neuroinflammation underlies the obese-induced cognitive decline and exercise rescues the cognitive impairment by suppressing of the pro-inflammatory TLR4/MyD88/NF- κ B signaling pathway in the hippocampus.

Diet high in fat and sugar is a major cause of obesity, which is significantly correlated with inflammation^[28,29]. In the present study, we have successfully developed a murine model of obesity through HFD. Animals of HFD model showed significant cognitive dysfunction, which manifested as significantly reduced communication time with strangers and reduced ability to recognize novel objects in NOR and TCS tests, respectively. Running at a speed of 12 m/min on a treadmill for rats is considered to be moderate intensity exercise, which can be interpreted as regular aerobic exercise^[30]. Notably,

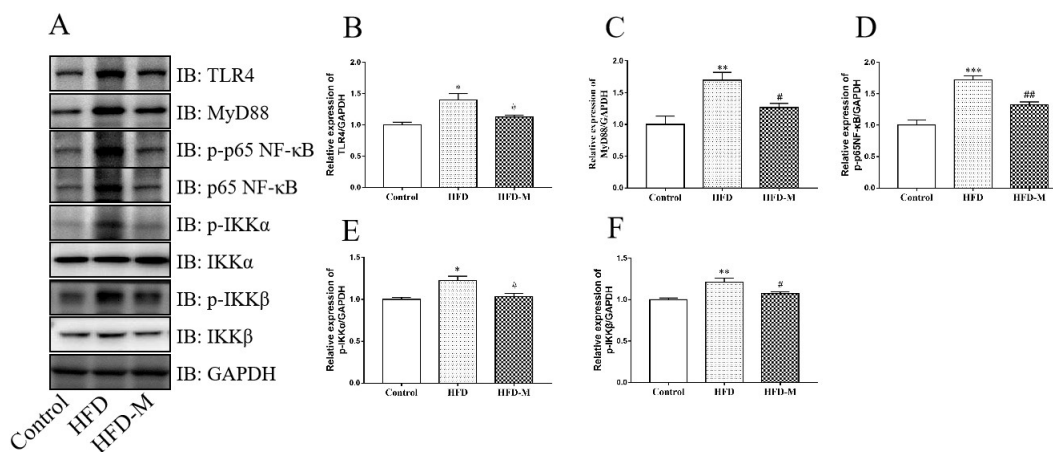


Fig. 4: Effects of treadmill exercise on the TLR4/MyD88/NF- κ B signaling pathway in the hippocampus, (A) Representative immunoreactive bands showing the protein levels of TLR4, MyD88, p-p65NF- κ B, p-IKK α and p-IKK β in the control, HFD and HFD-M mice; (B) TLR4; (C) MyD88; (D) p-p65NF- κ B; (E) p-IKK α and (F) p-IKK β

Note: (* p <0.05, ** p <0.01, *** p <0.001 vs. control mice, # p <0.05, ## p <0.01, ### p <0.001 vs. HFD mice)

we found that moderately intense exercise effectively reduced the weight gain and body fat mass of HFD mice. Furthermore, mice trained with moderate aerobic exercise showed significant improvement in cognitive dysfunction induced by obesity.

Currently, it has been found that inflammation is closely related to neurological diseases^[31-33]. Notably, exercise can reduce inflammation and counteract the symptoms of nervous system diseases and these effects are closely related to exercise intensity^[31]. Therefore, we examined the effects of obesity and moderate aerobic exercise on hippocampal inflammation. Serum levels of IL-1 β , TNF- α and IL-6 are important indicators of hippocampal inflammation^[28,34]. We found that serum levels of inflammatory factors significantly increased in HFD group and moderate intensity exercise inhibited the increase of inflammatory factors induced by obesity. In addition, TLR/MyD88/NF- κ B signaling pathway plays an important role in the occurrence and regulation of inflammatory response. Our results indicated that obesity activates the TLR/MyD88/NF- κ B signaling pathway and moderate intensity exercise significantly inhibits this pathway activation. These results are in support of ELISA data. Our findings suggest that moderate intensity exercise could rescue the cognitive dysfunction induced by obesity. The effect of moderate intensity exercise was mediated by reducing the inflammatory response in the hippocampus *via* inhibiting of the activation of TLR/MyD88/NF- κ B signaling pathway. However, the effects of different exercise intensity levels on hippocampal inflammation and cognitive dysfunction induced by obesity should be further studied.

In summary, the current study revealed that HFD-induced obesity led to the upregulation of inflammatory cytokines and produced cognitive dysfunction in mice. Notably, we observed that moderate exercise can suppress inflammatory responses and prevented cognitive damage caused by obesity. The activation and suppression of hippocampal TLR4/MyD88/NF- κ B signaling pathway underlies the obesity-induced cognitive dysfunction and the exercise-induced improvement of cognitive decline, respectively. These results may provide theoretical basis for the clinical application of exercise strategies for the treatment of obese patients with cognitive dysfunction.

Obesity is one of major health problems; the number of severely and morbidly obese people worldwide is increasing every year. Exercise is one of the most effective methods to control obesity without side effects.

Especially, treadmill exercise is associated with a lower BMI and lower body fat mass. In our study, we found that regular treadmill exercise rescue the cognitive dysfunction in HFD-fed obese mice. Furthermore, moderate aerobic exercise suppressed the hippocampus inflammatory response of the obesity-induced activation of TLR/MyD88/NF- κ B signaling pathway. Our work elucidates the mechanism by which exercise improves obesity-induced cognitive dysfunction and provides a novel exercise strategy for the clinical treatment of obese patients with cognitive dysfunction.

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Conflict of interests:

The authors declared no conflict of interest.

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