Treadmill Exercise Ameliorates Short-term Memory Impairment by Suppressing Hippocampal Neuroinflammation in Poloxamer-407-Induced Hyperlipidemia Rats

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Purpose: Poloxamer-407 (P-407) is used to induce hyperlipidemia. Exercise is effective in improving arteriosclerosis and cognitive impairment. In this research, the effect of treadmill running on short-term memory in the P-407-treated hyperlipidemia rats was studied focusing on neuroinflammation.

Methods: Rats were classified in normal group, normal and treadmill exercise group, P-407-treated group, and P-407-treated and treadmill exercise group. Hyperlipidemia rats were made by single intraperitoneal injection with P-407 (500 mg/kg). Treadmill exercise was conducted for 30 minutes once a day, 5 days per week during 28 days. Step-down avoidance task was done to measure short-term memory. Glial fibrillary acidic protein and ionized calcium binding adaptor molecule 1 were assessed by immunohistochemistry. Expression of adhesion molecules and proinflammatory cytokines was determined by western blot analysis.

Results: Treadmill exercise alleviated lipid profiles in the P-407-induced hyperlipidemia rats. Treadmill exercise improved short-term memory, inhibited reactive astrogliosis and microglia activation, and suppressed expression of adhesion molecules and proinflammatory cytokines in the hyperlipidemic rats.

Conclusions: Treadmill exercise exerts alleviating effect on memory deficits by inhibiting hippocampal neuroinflammation in the hyperlipidemia. The current results suggest that treadmill running serves as the treatment strategy for the cognitive dysfunction caused by hyperlipidemia.

Keywords: Hyperlipidemia; Treadmill exercise; Glial fibrillary acidic protein; Ionized calcium binding adaptor molecules 1; Adhesion molecules; Proinflammatory cytokines

- *HIGHLIGHTS*
 - Injection of poloxamer-407 induced hyperlipidemia and impaired short-term memory.
 - Treadmill running improved short-term memory deficits in the hyperlipidemia rats.
 - Treadmill running inhibited hippocampal neuroinflammation in the hyperlipidemia rats.

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INTRODUCTION

Hyperlipidemia refers to a condition in which an excess of fat component is present in the blood and causes inflammation. Hyperlipidemia is diagnosed when fasting serum cholesterol level is greater than 220 mg/dL or triglyceride (TG) level is greater than 150 mg/dL [1]. Elevated blood lipids are known to be a major trigger for neurodegenerative diseases such as Alzheimer disease [2]. A single intraperitoneal injection of poloxamer-407 (P-407) induces a rapid onset of hyperlipidemia, which increases serum lipoprotein level by altering lipid metabolism and cholesterol biosynthesis by inhibiting lipoprotein lipase, which promotes hydrolysis of TG [3].

Astrocyte-related pathogenesis causes Alzheimer disease and Parkinson disease [4]. Glial fibrillary acidic protein (GFAP) is the main component of filaments of the cytoskeleton of astrocytes. GFAP was increased at the sites of forebrain ischemia areas with edema or neuronal damage [5]. Microglia in the central nervous system normally remain quiescent, but when activated, microglia can perform a variety of functions that may be beneficial or detrimental, depending on the situation [6]. The ionized calcium binding adapter molecule 1 (Iba-1) protein is a microglia and macrophage specific calcium binding protein. Activated microglia play an important role in immune-mediated neurodegenerative diseases [7]. Neutrophilia caused by hypercholesterolemia primarily invades arteries in the early stages of atherosclerosis [8]. Level of serum proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β, was elevated in hyperlipidemic patients with periodontitis [9].

Adhesion molecules in the vascular endothelium cause endothelial dysfunction and act as inducing factor of the atherosclerosis [10]. Vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 are expressed in the endothelial cell membrane and regulate adhesion of leucocytes and their migration to the endothelium of blood vessels [11]. VCAM-1 and ICAM-1 level was enhanced by increased expression of TNF-α, IL-6, IL-1β, and interferon (IFN)-γ [12]. Interestingly, enhanced expression of VCAM-1 and ICAM-1 in the Alzheimer dementia reflects morphologic change of white matter and impairment of short-term memory [13].

Exercise decreased serum level of TC and low-density lipoprotein cholesterol (LDL-C) and enhanced serum level of high-density lipoprotein cholesterol (HDL-C) [14]. The improving effect on short-term memory and the increasing effect on cell proliferation and synaptic plasticity of the treadmill running in mice with photothrombotic stroke are well documented [15]. Nevertheless, the effect of treadmill running on memory deficits in the hyperlipidemia has not yet been elucidated.

Therefore, the effect of treadmill exercise on short-term memory deficits in the hyperlipidemia-induced rats caused by P-407 injection was studied, focusing on neuroinflammation. Serum lipid profiles were analyzed by automated analyzer and short-term memory was measured by step-down avoidance task. Immunohistochemistry for GFAP and Iba-1 and Western blotting for VCAM-1, ICAM-1, TNF-α, IL-6, and IL-1β were done.

MATERIALS AND METHODS

Animals
The approval number of KHUASP (SE)-18-047 was obtained from the Institutional Animal Care and Use Committee of Kyung Hee University for this experiment. Sprague-Dawley male adult rats (180-200 g in weight and 8-week old) were used for this study. Rats were classified into 4 groups (n = 10 in each group): normal group, normal and treadmill exercise group, P-407-treated group, and P-407-treated and treadmill exercise group.

Treadmill Running Protocol
The amount of treadmill running was performed for 30 minutes a day, 5 times a week for 28 days. The exercise protocol consisted of 3 sessions. According to the method of Ko et al. [16], rats ran on the treadmill at 0 degree of inclination at a speed of 3 m/min for the first 5 minutes, 5 m/min for the next 5 minutes, and 8 m/min for the last 20 minutes.

Induction of Hyperlipidemia
After 28 days of treadmill exercise, for the induction of hyperlipidemia, 500-mg/kg P-407 (overnight in cold sterile NaCl 0.9% solution) was injected intraperitoneally one time, following the previously mentioned method [17]. The normal rats were injected same volume of 0.9% NaCl solution.

Measurement of Lipid Profile
Blood sample was collected immediately from abdominal aorta and centrifugation at 3,000 rpm for 15 minutes was done. Serum was then isolated and stored at -80°C until use. Serum level of TC, LDL-C, TG, and HDL-C was measured by the automatic analyzer (Hitachi, Tokyo, Japan).

Step-Down Avoidance Task

The step-down avoidance task was used for the determination of short-term memory at 29 days after experiment starting, following the previously mentioned method [18]. Rats were put on the platform and as soon as it came down, 0.5-mA paw shock applied for 2 seconds. After 2 hours of the training session, latency time was measured, which was defined as the time the rats spend on the platform. A latency time exceeding 300 seconds was regard as 300 seconds.

Tissue Preparation

After anesthetizing the rats by diethyl ether (Sigma Aldrich Co., St. Louis, MO, USA), 50mM phosphate-buffered saline (PBS) and 4% paraformaldehyde in 100mM phosphate buffer (pH, 7.4) were perfused through heart. After taking out the brain, the brain was treated with 4% paraformaldehyde at 4°C for overnight for fixation, and transferred in a 30% sucrose for cyroprotection. Freezing microtome (Leica, Nussloch, Germany) was applied to make 40-μm-thick coronal sections.

GFAP and Iba-1 Immunohistochemistry

Immunohistochemistry for GFAP and Iba-1 expression was used, following the previously mentioned method [19]. The sections were pretreated with 3% H_2O_2 for 30 minutes, treated with PBS, and incubated with 10% blocking solution at room temperature. The sections were incubated with goat anti-GFAP antibody (1:500; Cell Signaling Technology, Danvers, MA, USA) and rabbit anti-Iba1 antibody (1:500; Cell signaling Technology) at 4°C overnight. On the next day, the sections were treated with biotinylated secondary antibodies (1:200; Vector laboratories, Burlingame, CA, USA) against GFAP and Iba-1 for 2 hours, then the sections were treated with avidin-biotin peroxidase kit (1:100; Vector Laboratories) and 0.03% diaminobenzidine containing 0.03% H_2O_2 at room temperature. The GFAP immunoreactivity was expressed as the optical density and Iba-1 immunoreactivity was presented as the number of Iba-1-positive cells/mm².

Western Blot Analysis

Western blotting was done, following the previously mentioned method [18,19]. Hippocampal tissues were extracted by homogenization using lysis buffer (pH, 7.6) After separating 30 μg protein using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the protein was moved to a nitrocellulose membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The membrane was treated with 5% skimmed milk powder (BD Difco, Detroit, MI, USA) for blocking, and incubated with 0.1% Tween-20 (Sigma Aldrich Co.) for 1 hour at room temperature. The membrane was treated with the primary antibodies such as rabbit anti-VCAM-1 (1:1,000, Cell Signaling Technology), rabbit anti-ICAM-1 (1:1,000, Cell Signaling Technology), mouse anti-TNF-α (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-IL-6 (1:1,000; Santa Cruz Biotechnology), and mouse anti-IL-1β (1:1,000; Santa Cruz Biotechnology). Membranes were subsequently incubated for l hour with peroxidase-tagged secondary antibodies. Enhanced chemiluminescence detection kit (DoGen, Seoul, Korea) was used for visualization of protein, and quantitative analysis of bands was calculated using Image-ProPlus program (Media Cybernetics, Rockville, MD, USA).

Statistical Analysis

IBM SPSS Statistics ver. 26.0 (IBM Co., Armonk, NY, USA) software was used for the analysis of data statistically. One-way analysis of variance and Tukey *post hoc* test were applied for statistical processing of experimental results. The results were indicated as the mean ± standard error of the mean and $P < 0.05$ showed statistical significance.

RESULTS

Lipid Profiles

Fig. 1 shows lipid profiles. Serum TC, LDL-C, and TG level was increased and serum HDL-C level was decreased in the P-407-treated hyperlipidemia rats compared to the normal rats ($P < 0.05$). Treadmill running decreased serum TC, LDL-C, and TG level and increased serum HDL-C in the P-407-treated hyperlipidemia rats ($P < 0.05$). Statistical significance was not found between the normal rats and the normal and treadmill exercise rats.

Short-term Memory

Fig. 2 shows the result of step-down avoidance task. Latency time was decreased in the P-407-treated hyperlipidemia rats compared to the normal rats ($P < 0.05$). Treadmill running enhanced latency time in the P-407-treated hyperlipidemia rats ($P < 0.05$). Statistical significance was not found between the normal rats and the normal and treadmill exercise rats.
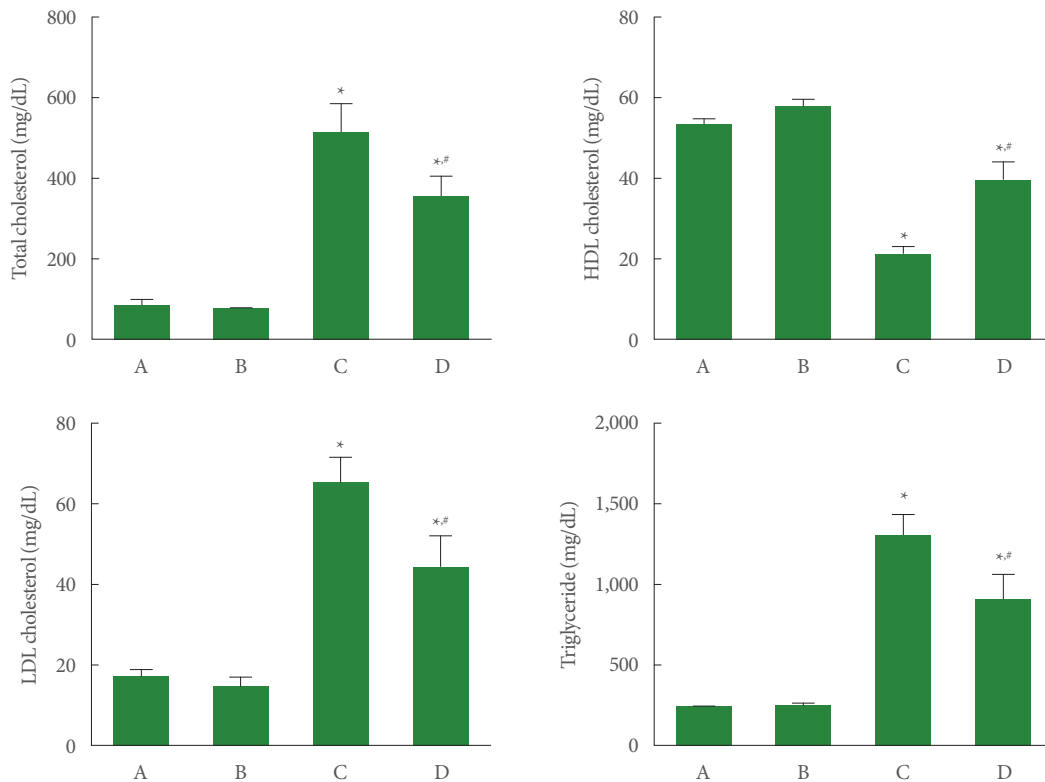
Fig. 1. Lipid profiles. Upper left panel: total cholesterol. Upper right panel: high-density lipoprotein (HDL) cholesterol. Lower left panel: low-density lipoprotein (LDL) cholesterol. Lower right panel: triglyceride. A, normal group; B, normal and treadmill exercise group; C, poloxamer-407-treated group; D, poloxamer-407-treated and treadmill exercise group. *P < 0.05 vs. normal rats. #P < 0.05 vs. poloxamer-407-treated rats.
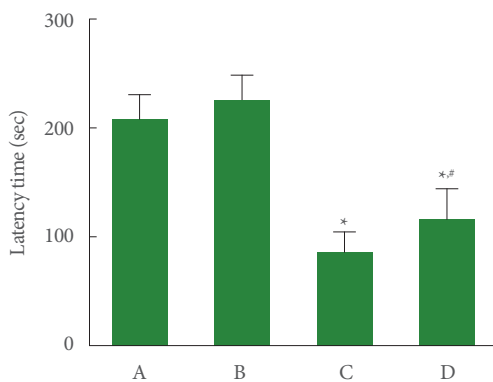

Fig. 2. Step-down avoidance task. A, normal group; B, normal and treadmill exercise group; C, poloxamer-407-treated group; D, poloxamer-407-treated and treadmill exercise group. *P < 0.05 vs. normal rats. #P < 0.05 vs. poloxamer-407-treated rats.

GFAP Expression in Hippocampal Dentate Gyrus

Fig. 3 shows GFAP expression in the hippocampal dentate gyrus. GFAP expression was increased in the P-407-treated hy-perlipidemia rats compared to the normal rats (P < 0.05). Tread-mill running decreased GFAP expression in the P-407-treated hyperlipidemia rats (P < 0.05). Statistical significance was not found between the normal rats and the normal and treadmill exercise rats.

Iba-1 Expression in Hippocampal Dentate Gyrus

Fig. 4 shows Iba-1 expression in the hippocampal dentate gy-rus. Iba-1 was increased in the P-407-treated hyperlipidemia rats compared to the normal rats (P < 0.05). Treadmill running decreased Iba-1 expression in the P-407-treated hyperlipidemia rats (P < 0.05). Statistical significance was not found between the normal rats and the normal and treadmill exercise rats.

Adhesion Molecules in Hippocampus

Fig. 5 shows expression of adhesion molecules in the hippo-campus. VCAM-1 and ICAM-1 expression was increased in the P-407-treated hyperlipidemia rats compared to the normal rats (P < 0.05). Treadmill running decreased VCAM-1 and
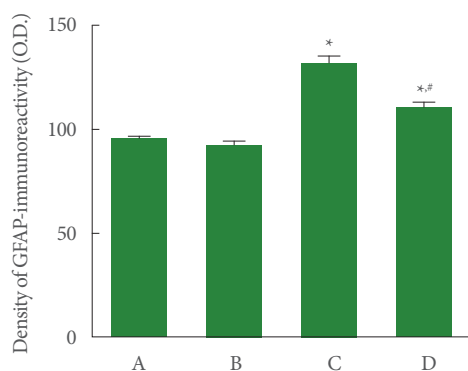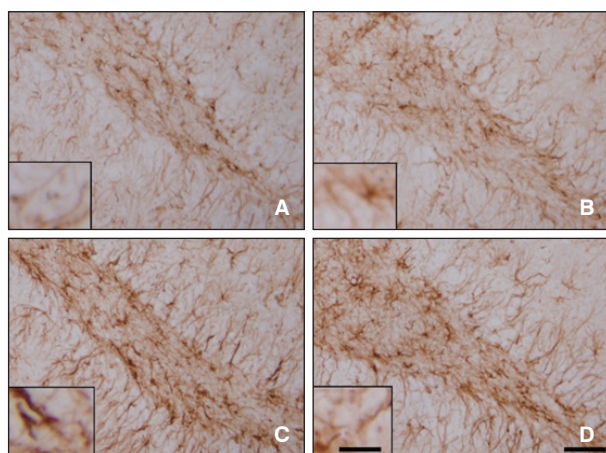
Fig. 3. Glial fibrillary acidic protein (GFAP) expression. Upper panel: photomicrographs of GFAP. Small square scale bar is 200 μm and large square scale bar is 50 μm. Lower panel: density of GFAP in each group A, normal group; B, normal and treadmill exercise group; C, poloxamer-407-treated group; D, poloxamer-407-treated and treadmill exercise group. *$P < 0.05$ vs. normal rats. #$P < 0.05$ vs. poloxamer-407-treated rats.

ICAM-1 expression in the P-407-treated hyperlipidemia rats ($P < 0.05$). Statistical significance was not found between the normal rats and the normal and treadmill exercise rats.

Proinflammatory Cytokines in Hippocampus

Fig. 6 shows expression of proinflammatory cytokines in the hippocampus. TNF-α, IL-6, and IL-1β expression was enhanced in the P-407-treated hyperlipidemia rats compared to the normal rats ($P < 0.05$). Treadmill running decreased TNF-α, IL-6, and IL-1β expression in the P-407-treated hyperlipidemia rats ($P < 0.05$). Statistical significance was not found between the normal rats and the normal and treadmill exercise rats.
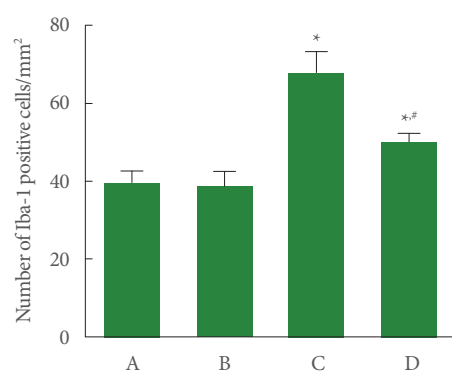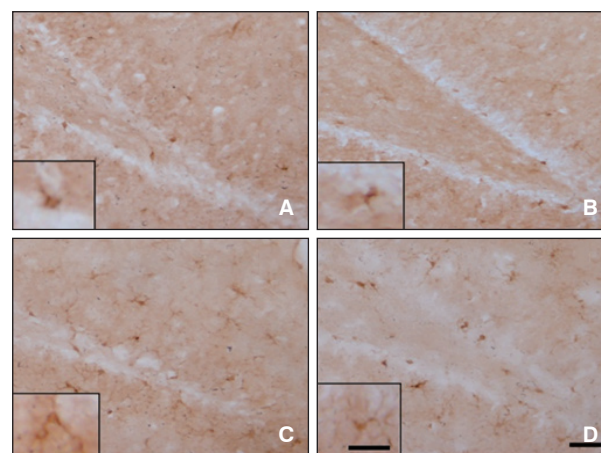

Fig. 4. Ionized calcium binding adaptor molecule 1 (Iba-1). Upper panel: photomicrographs of Iba-1. Small square scale bar is 200 μm and large square scale bar is 50 μm. Lower panel: number of Iba-1 in each group. A, normal group; B, normal and treadmill exercise group; C, poloxamer-407-treated group; D, poloxamer-407-treated and treadmill exercise group. *$P < 0.05$ vs. normal rats. #$P < 0.05$ vs. poloxamer-407-treated rats.

DISCUSSION

Elevated TC, LDL, and TG level and low HDL-C level are related with an increased risk of atherosclerosis and ischemic stroke [20,21]. P-407 is known to increase LDL-C and TG level and decrease HDL-C level [22]. In the current study, increment in serum TC, LDL-C, and TG level and decrement in serum HDL-C level were observed in the hyperlipidemia rats. Treadmill exercise suppressed serum TC, LDL, and TG level, and enhanced serum HDL-C level in the hyperlipidemia rats. The current results suggest that treadmill running has a preventive effect on hyperlipidemia.

Cholesterol is known to exerts significant effect on synaptic plasticity and brain function, including learning ability and
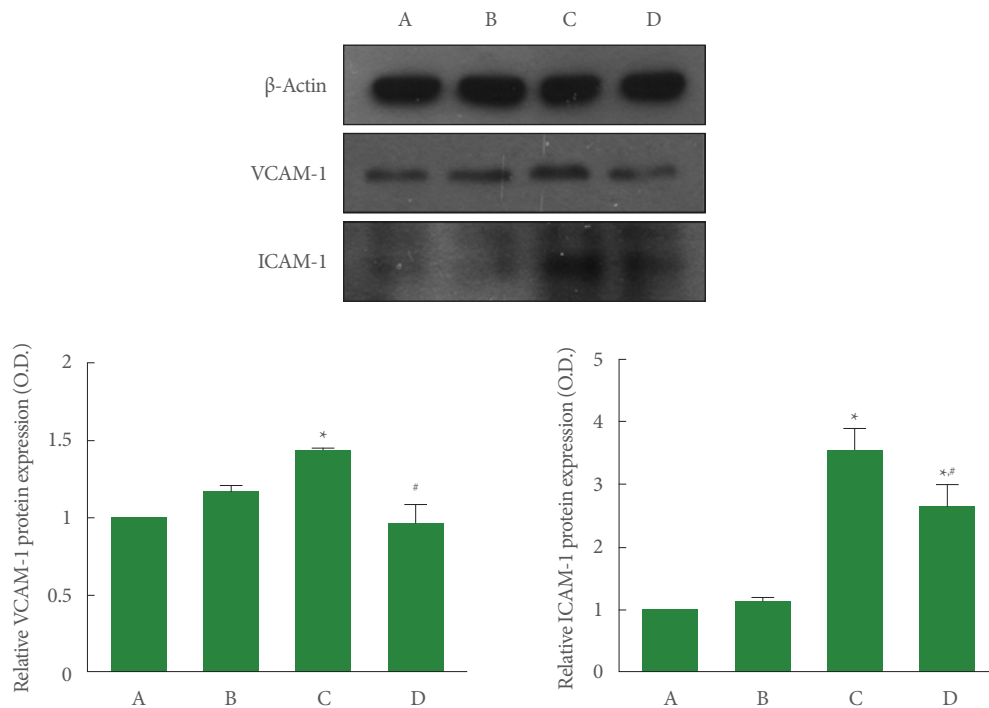
Fig. 5. Adhesion molecules expression. Upper panel: representative expression of vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1. Lower left panel: relative VCAM-1 expression in each group. Lower right panel: relative ICAM-1 expression in each group. A, normal group; B, normal and treadmill exercise group; C, poloxamer-407-treated group; D, poloxamer-407-treated and treadmill exercise group. *P < 0.05 vs. normal rats. #P < 0.05 vs. poloxamer-407-treated rats.

memory process [23]. Previous studies have demonstrated that hyperlipidemia is a inducing factor for the cognitive impairment of Alzheimer disease [24,25]. Hyperlipidemia induced by P-407 administration leads to cognitive impairment [17]. Exercise is one of the important treatments to improve cognitive function including memory ability [15]. In the current study, impairment of short-term memory was observed in the hyperlipidemic rats. Treadmill exercise significantly improved short-term memory in the hyperlipidemia rats. The current results suggest that treadmill running is effective in alleviating short-term memory deficits caused by hyperlipidemia.

Reactive astrogliosis and microglial activation has been considered as the hallmark of Alzheimer disease [26]. A high-cholesterol diet induced hippocampal morphological changes, including reactive astrogliosis, increased microglia activation, and decreased neurodevelopment [27,28]. In the current study, enhanced expression of GFAP and Iba-1 was found in the hyperlipidemia rats. Treadmill exercise significantly decreased the expression of GFAP and Iba-1 in the hyperlipidemic rats. The current results indicate treadmill running inhibits reactive astrogliosis and microglial activation in the hyperlipidemia rats.

The adhesion of vascular endothelium, regulated by many adhesion molecules, including VCAM-1 and ICAM-1, is one of the important mechanisms for the induction of inflammation in atherosclerosis [29]. VCAM-1 and ICAM-1 expression was upregulated by proinflammatory cytokines, such as TNF-α, IL-6, and IFN-γ [30]. Enhancement of VCAM-1 and ICAM-1 expression was strongly associated with increased incidence of atherosclerosis and Alzheimer's disease [31,32]. Exercise is considered to be one of the basic regulators of cell adhesion molecules [33], and aerobic exercise reduced endothelial activation makers, such as VCAM-1 and ICAM-1, and proinflammatory cytokines, such as TNF-α and IL-6, in old people. In the current study, the expression of VCAM-1 and ICAM-1 was increased with enhanced TNF-α, IL-6, and IL-1β expression in the hyperlipidemia rats. Treadmill exercise significantly decreased the expression of VCAM-1 and ICAM-1 with suppressed TNF-α, IL-6, and IL-1β expression in the hyperlipidemia rats. The current results indicate that treadmill running reduces the expression of adhesion molecules by suppressing proinflammatory cytokines in the hyperlipidemia rats.

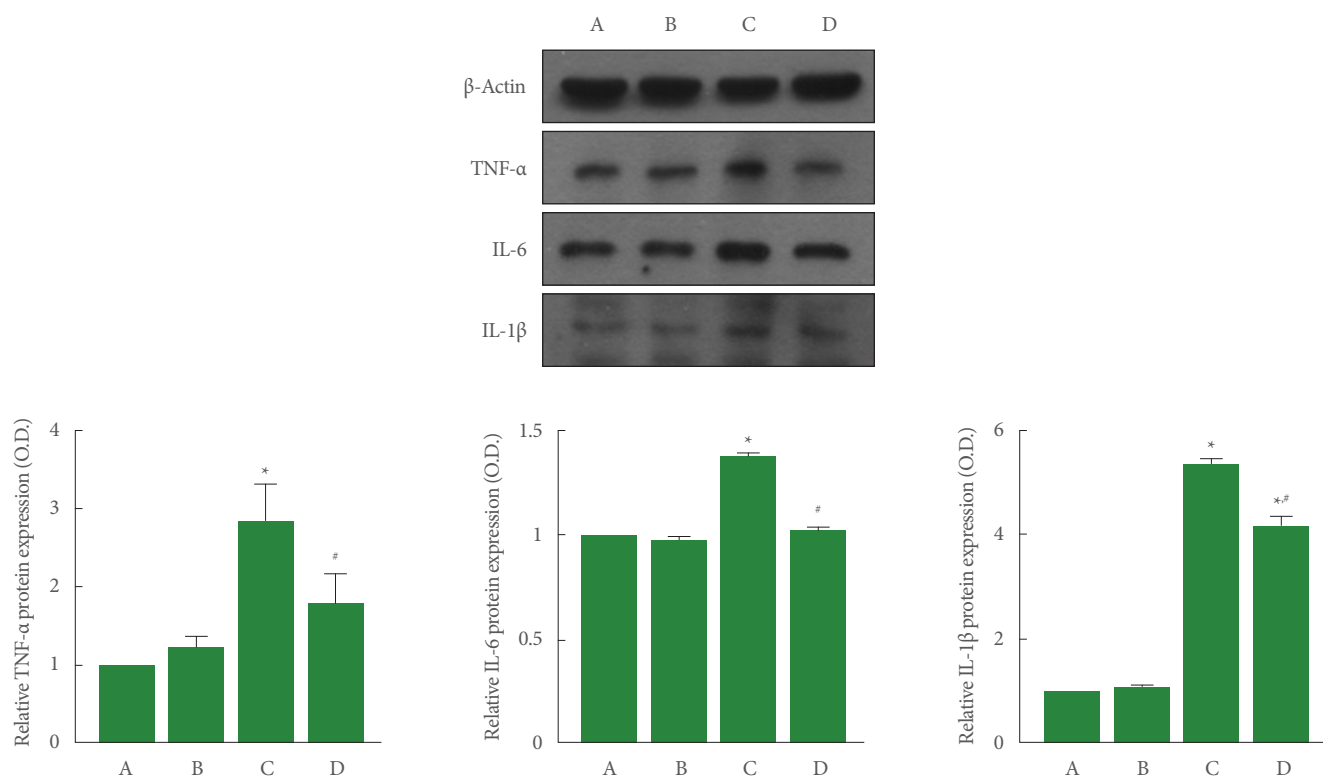In conclusion, treadmill running ameliorated memory defi-

Fig. 6. Proinflammatory cytokines expression. Upper panel: representative expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β. Lower left panel: relative TNF-α expression in each group. Lower middle panel: relative IL-6 expression in each group. Lower right panel: relative IL-1β expression in each group. A, normal group; B, normal and treadmill exercise group; C, poloxamer-407-treated group; D, poloxamer-407-treated and treadmill exercise group. *P < 0.05 vs. normal rats. #P < 0.05 vs. poloxamer-407-treated rats.

cits by inhibiting hippocampal neuroinflammation in the hyperlipidemia. The current study suggests that treadmill running serves as the treatment strategy for the cognitive dysfunction caused by hyperlipidemia.

AUTHOR CONTRIBUTION STATEMENT

· Conceptualization: *SSP*
· Data curation: *SSP*
· Formal analysis: *TWK, YHS, YJP*
· Funding acquisition: *MSS*
· Methodology: *MKK*
· Project administration: *MSS*
· Visualization: *TWK, YHS, YJP*
· Writing-original draft: *MSS*
· Writing-review & editing: *MSS*

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