



Original Article

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Berberine Ameliorates Brain Inflammation in Poloxamer 407-Induced Hyperlipidemic Rats

Mia Kim¹, Tae-Woon Kim², Chang-Ju Kim², Mal-Soon Shin³, Minha Hong⁴, Hye-Sang Park⁵, Sang-Seo Park²

¹Department of Cardiovascular Neurologic Disease (Stroke Center), College of Korean Medicine, Kyung Hee University, Seoul, Korea

²Department of Physiology, College of Medicine, Kyung Hee University, Seoul, Korea

³College of Culture and Sports, Division of Global Sport Studies, Korea University, Sejong, Korea

⁴Department of Psychiatry, Myongji Hospital, College of Medicine, Hanyang University, Goyang, Korea

⁵Department of Kinesiology, College of Public Health and Cardiovascular Research Center, Lewis Kate School of Medicine, Temple University, Philadelphia, PA, USA

Purpose: Hyperlipidemia, which promotes the development of atherosclerosis, ischemic stroke, and other forms of brain injury, can be induced by poloxamer-407. Berberine is a primary pharmacological active component of *Coptidis Rhizoma* that has a number of therapeutic activities. This study investigated the effects of berberine on poloxamer-407-induced brain inflammation by evaluating its effects on short-term memory, cell proliferation, inflammation, and apoptosis in the hippocampus.

Methods: To induce hyperlipidemia in a rat model, 500 mg/kg of poloxamer-407 was injected intraperitoneally. Berberine was orally administered to the rats in the berberine-treated groups once a day for 4 weeks. The step-down task avoidance task was performed to measure short-term memory. An analysis of serum lipids, immunohistochemistry for 5-bromo-2'-deoxyuridine, glial fibrillary acidic protein (GFAP), and ionized calcium-binding adapter molecule 1 (Iba1) in the dentate gyrus, and western blot analysis for Bax, Bcl-2, and cytochrome c in the hippocampus were performed.

Results: In hyperlipidemic rats, berberine reduced the levels of triglycerides, total cholesterol, and low-density lipoprotein cholesterol and increased the level of high-density lipoprotein cholesterol in hyperlipidemic rats. Berberine also increased cell proliferation and short-term memory, as well as decreasing the expression of GFAP, Iba1, Bax, and cytochrome c and increasing Bcl-2 expression.

Conclusions: Berberine treatment improved short-term memory in hyperlipidemia by increasing neuronal proliferation and inhibiting neuronal apoptosis. Berberine treatment also improved lipid metabolism.

Keywords: Hyperlipidemia; Berberine; Poloxamer; Apoptosis; Glial fibrillary acidic protein; Iba1

- **Research Ethics:** The animal testing procedures were conducted in accordance with the regulations prescribed by the National Institutes of Health (NIH), and all guidelines of the Korean Medical Research Institute were followed during the experiment. Kyung Hee University Institutional Animal Care and Use Committee (Seoul, Korea) approved this experiment (KHUASP [SE]-18-047).
- **Conflict of Interest:** No potential conflict of interest relevant to this article was reported.

• HIGHLIGHTS

- Hyperlipidemia causes memory impairment.
- Berberine treatment enhances neuronal cell proliferation and suppresses apoptosis.
- Berberine treatment improves short-term memory in hyperlipidemic rats.

Corresponding author: Sang-Seo Park  <https://orcid.org/0000-0001-7033-9630>

Department of Physiology, College of Medicine Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, Korea

E-mail: s07@naver.com / Tel: +82-2-961-0282 / Fax: +82-2-964-2195

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INTRODUCTION

Hyperlipidemia refers to a state of abnormally elevated levels of 1 or more plasma lipids, including cholesterol, cholesterol esters, triglycerides (TG), phospholipids, and lipoproteins. The rise in plasma lipid levels is mainly due to genetic factors, and secondarily to diet, drugs, and disease. Increased triglyceride and cholesterol levels in the blood contribute to the loss of memory and learning ability [1]. The inflammatory responses caused by hyperlipidemia play an initiating role in the development of atherosclerosis, ischemic stroke, and many forms of brain injury [2].

Poloxamer-407 is a commonly used artificial surfactant that can cross the blood-brain barrier. Poloxamer-407 was observed to cause hyperlipidemia in experimental animals, as shown by increased blood levels of cholesterol and TG within 36 hours after an intraperitoneal injection of poloxamer-407 [3].

Increased protein expression or the condensation of proteins to form toxic condensates exacerbates the inflammatory response and worsens degenerative brain diseases [4]. Astrocyte-related etiologies lead to neurodegenerative diseases such as muscular dystrophy, Alzheimer disease, and Huntington disease. Glial fibrillary acidic protein (GFAP) is a well-known protein that acts as a biomarker in patients with brain damage caused by apoptosis or severe damage to the astrocytes in the brain [4]. The ionized calcium-binding adapter molecule 1 (Iba1) protein is an ionized calcium-binding adapter molecule, and Iba1 expression is known to be localized in macrophages and microglia [5]. Iba1 is an important molecule involved in membrane ruffling and phagocytosis in macrophages and microglia. Therefore, it is potentially relevant to neurodegenerative conditions, as activation of microglial cells has been proposed as an etiological mechanism for dopaminergic neuronal loss [6].

Berberine, which is a primary pharmacological active component of *Coptidis Rhizoma*, has a number of therapeutic activities, including anti-inflammatory, anticancer, antiviral, antibacterial, hypoglycemic, and lipid-regulating properties [7]. Berberine reduces blood lipid levels, thereby playing an important role in preventing hyperlipidemia [8]. Berberine reduces blood glucose levels, improves insulin sensitivity, and has been found to cause weight loss in animal and human experiments [9,10]. Berberine improves the condition of patients with diabetes mellitus to the point that it shows potential for diabetes treatment, and it also has anti-inflammatory effects [11]. Berberine exhibits an anti-apoptotic effect in cerebral ischemia by

decreasing the expression of caspase-3 and caspase-9 and increasing the Bcl-2/Bax ratio [12,13].

It is necessary to study the relationship between the inflammatory factors induced by hyperlipidemia and cognitive function. Therefore, this study aimed to investigate the effects of berberine on poloxamer-407-induced brain inflammation in a rat model. The effects of berberine on short-term memory, cell proliferation, inflammation, and apoptosis in the hippocampus were evaluated.

MATERIALS AND METHODS

Experimental Animals

The animal testing procedures were conducted in accordance with the regulations prescribed by the National Institutes of Health, and all guidelines of the Korean Medical Research Institute were followed during the experiment. Kyung Hee University Institutional Animal Care and Use Committee (Seoul, Korea) approved this experiment (KHUASP [SE]-18-047). We divided Sprague-Dawley male rats at 8 weeks of age (200 g) into 4 groups: a control group, a poloxamer-407 injection group, a poloxamer-407 injection and 50-mg/kg berberine treatment group, and a poloxamer-407 injection and 100-mg/kg berberine treatment group. Once a day for 5 consecutive days, the rats in all groups received an intraperitoneal injection of 100 mg/kg of 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO, USA) 30 minutes before berberine treatment. The rats in the berberine-treated groups received berberine orally (Sigma Chemical Co.) once a day for 4 weeks with the respective dose for each treatment group. The same amount of distilled water was administered to the rats in the control group.

Induction of Hyperlipidemia by Poloxamer-407

As described in a previous study [3], 500 mg/kg of poloxamer-407 was dissolved in a sterile cold isotonic NaCl solution, refrigerated overnight to facilitate its dissociation, and then intraperitoneally injected to induce hyperlipidemia. The same volume of sterile cold isotonic NaCl solution was applied to the rats in the control group.

Step-Down Avoidance Task

According to the method described earlier [14,15], the latency time of the step-down avoidance task was measured to evaluate short-term memory. At 28 days after the start of the experiment, the rats were trained to perform a step-down avoidance task.

During the training session, the rats were received a 0.5-mA scrambled foot shock for 2 seconds as soon as they descended. After the 2-hour training session, the latency time (seconds) in each group was determined. The rats were placed on a 42×25-cm grid of parallel stainless-steel bars at 1-cm intervals, with a 1-cm-high 5×25-cm platform with a diameter of 0.1 cm. The latency time was defined as the time interval at which rats descended and placed 4 feet on the grid. A latency time of more than 180 seconds was recorded as 180 seconds.

Analysis of the Serum Lipid Profile

Following a method that has been described elsewhere in more detail [16], rat blood was collected through the abdominal aorta. Blood samples without anticoagulant were collected in tubes, and after coagulation, the tubes were centrifuged for 20 minutes at 3,000 rpm at room temperature. The resulting serum samples were aliquoted into microtubes and stored on ice. Serum levels of total cholesterol (TC), TG, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were determined according to the manufacturer's instructions using commercially available kits.

Tissue Preparation

Following a previously described method, the rats were sacrificed immediately after the last behavioral test [17,18]. After being anesthetized with 40 mg/kg of Zoletil 50 (Vibac Laboratories, Carros, France), the rats received 50mM phosphate-buffered saline (PBS) through cardiovascular flow, and 4% paraformaldehyde in 100mM phosphate buffer (pH, 7.4) was infused. After dissecting the brain, the brain was fixed in the same fixative solution overnight and transferred to a 30% sucrose solution for protection against freezing. Frozen microtomes (Leica, Nussloch, Germany) were used to make 40- μ m-thick sagittal sections.

Immunohistochemistry for BrdU

Following a method that has been described elsewhere [14,19], the sections were first incubated with 0.5% Triton X-100 in PBS for 20 minutes and then pretreated with 50% formamide-2X standard saline citrate at 65°C for 2 hours. Each section was denatured with 2 N HCl for 30 minutes at 37°C, and washed 2 times with 100mM sodium borate (pH, 8.5). The sections were then incubated with BrdU-specific mouse monoclonal antibody (1:500; Roche, Mannheim, Germany) at 4°C during overnight. The sections were then washed 3 times with PBS and treated with biotinylated mouse secondary antibody (1:500;

Vector Laboratories, Burlingame, CA, USA) for 1 hour. The sections were then treated with ABC complex (1:100; Vector Laboratories) for 1 hour and 30 minutes. To visualize color development, the sections were treated for 5 minutes with 50mM Tris-HCl (pH, 7.6) containing 0.03% diaminobenzidine (DAB), 40-mg/mL nickel chloride, and 0.03% hydrogen peroxide. After BrdU labeling, mouse anti-neuronal nuclear antibody (1:1,000; Chemicon International, Temecula, CA, USA) was applied to the same section to stain the neurons. Then, the sections were washed with PBS 5 times and treated for 1 hour 30 minutes with biotinylated anti-mouse secondary antibody. For staining, the sections were treated for 3 minutes with a reaction mixture containing 0.03% DAB and 0.03% hydrogen peroxide. The sections were placed on gelatin-coated slides and the slides were air-dried overnight at room temperature and then mounted using Permunt (Thermo Fisher Scientific Inc., Waltham, MA, USA). The number of BrdU-positive cells in the dentate gyrus was calculated and the results were expressed as the number of BrdU-positive cells per square millimeter.

Immunohistochemistry for Iba1 and GFAP

Using techniques that have been described elsewhere [20], immunohistochemistry was conducted to detect GFAP and Iba1-positive cells in the dentate gyrus. The sections were treated for 5 minutes with 0.05M PBS and washed 3 times with 0.05M PBS. The free-floating sections were treated for 30 minutes with 3% H₂O₂, and then were treated for 2 hours with blocking solution (10% horse serum and 1% bovine serum albumin in 0.05M PBS) at laboratory temperature. Following overnight treatment with goat anti-GFAP antibody (1:500; Cell Signaling Technology, Danvers, MA, USA) and rabbit anti-Iba1 antibody (1:500; Cell signaling Technology), the sections were treated with biotinylated anti-goat and anti-rabbit IgG secondary antibodies (1:500; Vector Laboratories). The sections were treated with avidin-biotin-peroxidase complex (Vector Laboratories) for 1 hour at laboratory temperature. After treatment for 3 minutes with 0.03% H₂O₂ in 50mM Tris-HCl (pH, 7.6) containing 0.02% diaminobenzidine, the sections were treated with PBS. The sections were placed on gelatin-coated slides and the slides were air-dried overnight at room temperature and then mounted using Permunt (Thermo Fisher Scientific Inc., Waltham, MA, USA). GFAP-positive reactive astrocytes were assessed in a quantitative fashion based on optical density and the number of Iba1-positive microglia cells expressed as the number of Iba1-positive microglia cells per square millimeter.

Western Blotting for Bax, Bcl-2, and Cytochrome c

Following a previously described method [20,21], Bax, Bcl-2, and cytochrome c expression was measured by western blot analysis. Using protein lysis buffer, hippocampal samples were lysed and a colorimetric protein assay kit was used to detect protein concentrations (Bio-Rad, Hercules, CA, USA). After 30 μ g of protein was separated on sodium dodecyl sulfate-polyacrylamide gels, the separated protein was transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Anti-mouse β -actin antibody (1:1,000; Santa Cruz Biotechnology), anti-rabbit Bax antibody (Cell signaling Technology; 1:1,000), anti-mouse Bcl-2 antibody (1:1,000; Santa Cruz Biotechnology), and anti-rabbit cytochrome c antibody (1:1,000; Cell signaling Technology) were used as the primary antibodies. The secondary antibodies were horseradish peroxidase-conjugated anti-mouse antibodies (1:3,000; Vector Laboratories) for β -actin and Bcl-2, and horseradish peroxidase-conjugated anti-rabbit antibodies (1:5,000; Vector Laboratories)

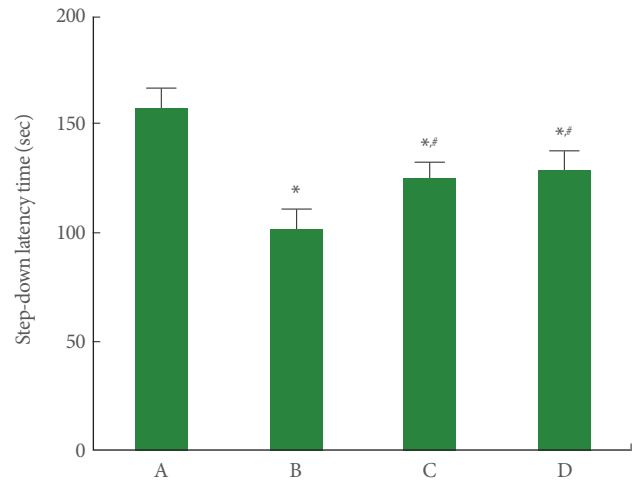


Fig. 1. Step-down avoidance task. A, control group; B, poloxamer-407 injection group; C, poloxamer-407 injection and 50-mg/kg berberine treatment group; D, poloxamer-407 injection and 100-mg/kg berberine treatment group. *P < 0.05 compared to the control group. #P < 0.05 compared to the poloxamer-407 injection group.

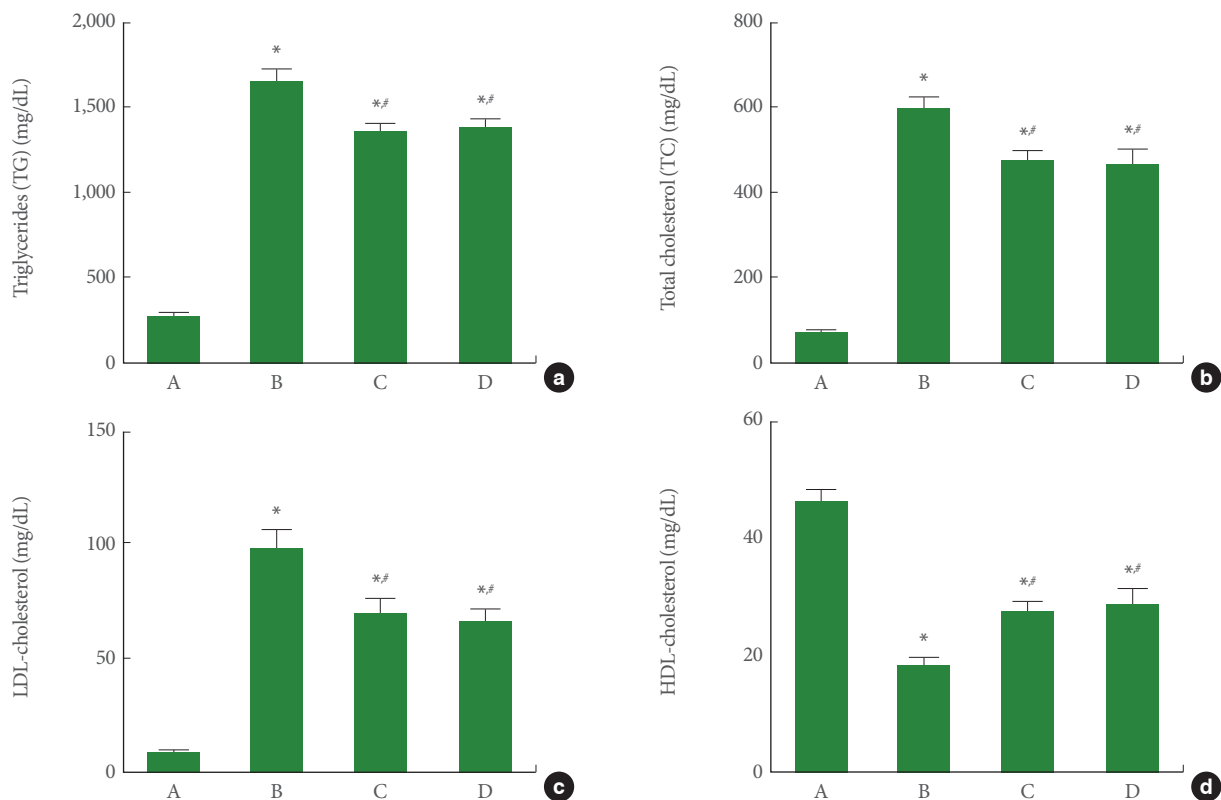


Fig. 2. Serum lipid levels. (a) Levels of triglycerides (TG). (b) Levels of total cholesterol (TC). (c) Levels of low-density lipoprotein (LDL) cholesterol. (d) Levels of high-density lipoprotein (HDL) cholesterol. A, control group; B, poloxamer-407 injection group; C, poloxamer-407 injection and 50-mg/kg berberine treatment group; D, poloxamer-407 injection and 100-mg/kg berberine treatment group. *P < 0.05 compared to the control group. #P < 0.05 compared to the poloxamer-407 injection group.

for Bax and cytochrome c. An enhanced chemiluminescence detection system (Amersham Pharmacia Biotech GmbH, Freiburg, Germany) was used for band detection.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics ver. 21.0 (IBM Co., Armonk, NY, USA). Groups were compared using 1-way analysis of variance with the Tukey *post hoc* test and the results were presented as the mean ± standard error of the mean. P-values of <0.05 were considered to indicate statistical significance.

RESULTS

Effect of Berberine on Short-term Memory

Fig. 1 shows the results of the step-down avoidance task. Short-term memory was reduced in the poloxamer-407 injection-induced hyperlipidemic rats ($P < 0.05$), but berberine treatment significantly improved their short-term memory ($P < 0.05$).

Effect of Berberine on Serum Lipids

Serum levels of TG, TC, LDL-C, and HDL-C are presented in Fig. 2. The levels of TG, TC, and LDL-C increased and the level of HDL-C decreased in the poloxamer-407 injection-induced

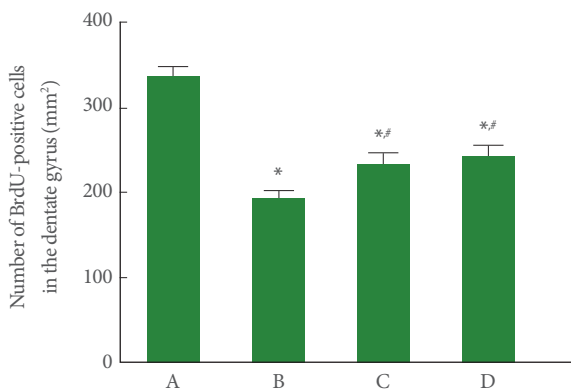
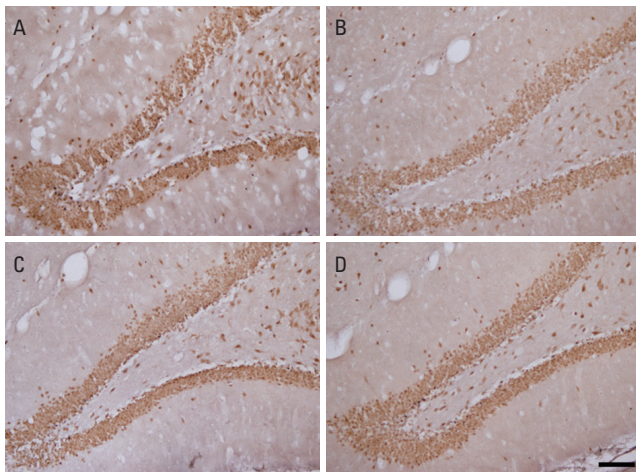


Fig. 3. Cell proliferation in the dentate gyrus. Upper panel: Photomicrograph of BrdU-positive cells. Lower panel: The number of BrdU-positive cells in each group. The scale bar represents 400 μm in each group. A, control group; B, poloxamer-407 injection group; C, poloxamer-407 injection and 50-mg/kg berberine treatment group; D, poloxamer-407 injection and 100-mg/kg berberine treatment group. * $P < 0.05$ compared to the control group. # $P < 0.05$ compared to the poloxamer-407 injection group.

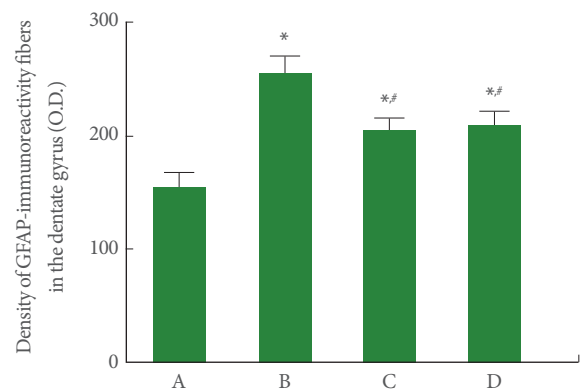
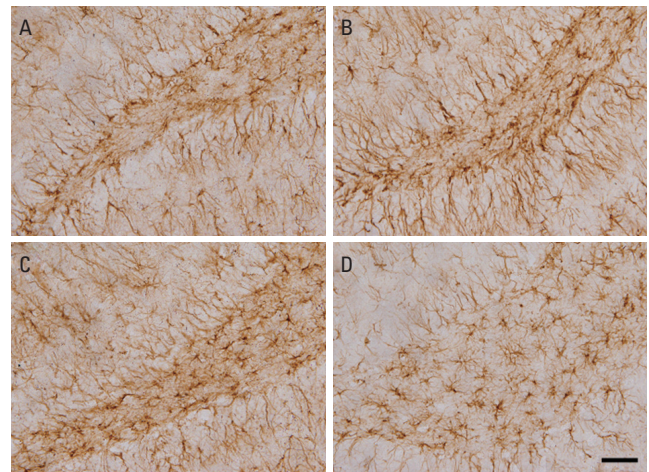


Fig. 4. Glial fibrillary acidic protein (GFAP) expression in the dentate gyrus. Upper panel: Photomicrographs of GFAP-positive fibers in the dentate gyrus. Lower panel: Optical density of GFAP-positive fibers in each group. The scale bar represents 400 μm in each group. A, control group; B, poloxamer-407 injection group; C, poloxamer-407 injection and 50-mg/kg berberine treatment group; D, poloxamer-407 injection and 100-mg/kg berberine treatment group. * $P < 0.05$ compared to the control group. # $P < 0.05$ compared to the poloxamer-407 injection group.

hyperlipidemic rats ($P < 0.05$). Berberine treatment reduced the levels of TG, TC, and LDL-C and increased the level of HDL-C in hyperlipidemic rats ($P < 0.05$).

Effect of Berberine on the Number of BrdU-Positive Cells

Fig. 3 shows the number of BrdU-positive cells in the dentate gyrus. The number of BrdU-positive cells decreased in the poloxamer-407 injection-induced hyperlipidemic rats ($P < 0.05$), but berberine treatment significantly increased the number of BrdU-positive cells ($P < 0.05$).

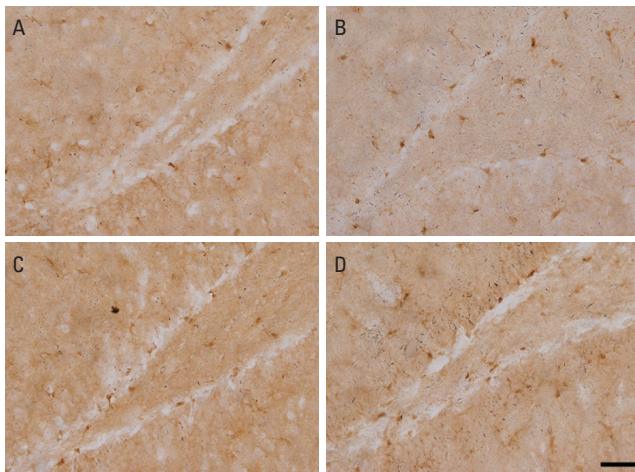


Fig. 5. Iba1 expression in the dentate gyrus. Upper panel: Photomicrographs of ionized calcium binding adaptor molecule 1 (Iba1) microglia cells in the dentate gyrus. Lower panel: Number of Iba1 microglia cells in each group. The scale bar represents 400 μm in each group. A, control group; B, poloxamer-407 injection group; C, poloxamer-407 injection and 50-mg/kg berberine treatment group; D, poloxamer-407 injection and 100-mg/kg berberine treatment group. * $P < 0.05$ compared to the control group. # $P < 0.05$ compared to the poloxamer-407 injection group.

Effect of Berberine on GFAP Expression

Fig. 4 presents the expression of GFAP in the dentate gyrus. The expression of GFAP increased in the poloxamer-407 injection-induced hyperlipidemic rats ($P < 0.05$), but berberine treatment decreased the expression of GFAP ($P < 0.05$).

Effect of Berberine on Iba1 Expression

Fig. 5 shows the expression of Iba1 in the dentate gyrus. The expression of Iba1 increased in the poloxamer-407 injection-induced hyperlipidemic rats ($P < 0.05$), but berberine treatment decreased the expression of Iba1 ($P < 0.05$).

Expression of Bax, Bcl-2, and Cytochrome c in the Hippocampus

Fig. 6 shows the expression of Bax, Bcl-2, and cytochrome c in the hippocampus. In the control group, the expression levels of Bax, Bcl-2, and cytochrome c were set at 1.00. The expression of Bax and cytochrome c increased and Bcl-2 expression decreased in the poloxamer-407 injection-induced hyperlipidemic rats ($P < 0.05$). Berberine treatment decreased the expression of Bax and cytochrome c and increased the expression of Bcl-2 in the hyperlipidemic rats ($P < 0.05$).

DISCUSSION

Hyperlipidemia is a major factor contributing to atherosclerosis and vascular diseases, including angina pectoris, myocardial infarction, and stroke [22]. Berberine, a compound derived from herbs, has been used as a therapeutic and prophylactic due to its numerous therapeutic activities, including diabetes improvement [9], blood lipid alterations [23], cell death suppression [12,13], anti-immune effects [11], and increased blood glucose uptake [9,10].

The step-down avoidance task is an experimental method used to evaluate short-term memory [14,24]. Treatment with berberine ameliorated the impairment of short-term memory caused by ischemia through activation of hippocampal phosphoinositide 3-kinase/protein kinase B pathway in gerbils [13]. In this study, berberine treatment led to an improvement in short-term memory, as shown by prolonged latency in the step-down avoidance task. No difference was found in the latency results according to the berberine dose.

Lee et al. [10] reported that berberine improved glucose metabolism, inhibited diabetes, and reduced lipid levels. In this study, berberine treatment reduced levels of TG, TC, and LDL-

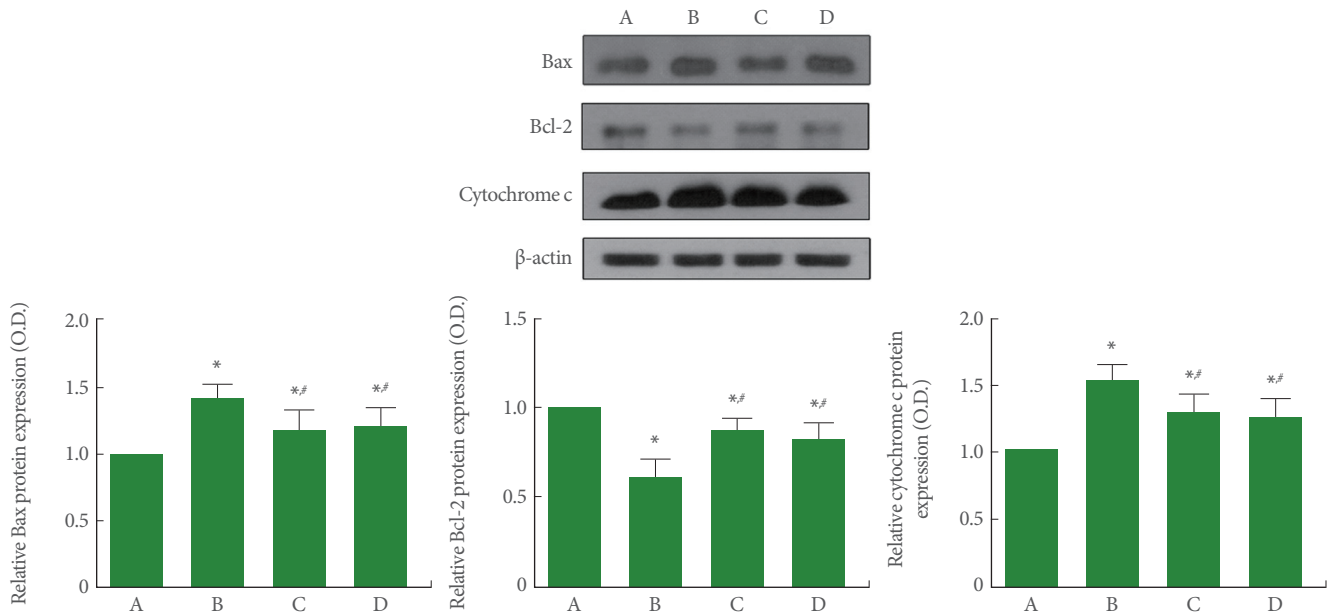


Fig. 6. Apoptosis in the hippocampus. Upper panel: Representative examples of Bax, Bcl-2, and cytochrome c expression. Lower panel: Relative optical density of Bax, Bcl-2, and cytochrome c in each group. The scale bar represents 400 μm in each group. A, control group; B, poloxamer-407 injection group; C, poloxamer-407 injection and 50-mg/kg berberine treatment group; D, poloxamer-407 injection and 100-mg/kg berberine treatment group. *P < 0.05 compared to the control group. #P < 0.05 compared to the poloxamer-407 injection group.

C and increased the levels of HDL-C in hyperlipidemic rats. No significant difference was found in serum lipid levels according to the berberine dose.

Reduced cell proliferation in the hippocampus causes memory impairment [14,15]. In this study, berberine treatment increased cell proliferation, which may have led to short-term memory improvements in the hyperlipidemic rats. No significant difference was found in cell proliferation according to the berberine dose.

Extensive oxidative stress, loss of antioxidant activity, and increased GFAP expression cause hippocampal neurodegeneration [25]. Stolmeier et al. [26] reported that an increase in factors associated with glial maturation, including GFAP and Iba1, antagonized hippocampal function. In this study, berberine treatment reduced the expression of GFAP and Iba1 in the hippocampus, indicating that berberine can act as a protective agent in hyperlipidemia. No significant difference was found in GFAP and Iba1 expression according to the berberine dose.

Bax, a pro-apoptotic protein, induces accelerated apoptotic cell death, while the anti-apoptotic protein Bcl-2 inhibits apoptosis [21,27]. Kim et al. [28] showed stress induced deterioration of the hippocampal function through apoptosis, with increased

expressions of caspase-3 and decrease of Bcl-2, and suggested it may lead to deterioration of lower urinary tract function. In this study, berberine treatment inhibited the expression of Bax and cytochrome c and enhanced the expression of Bcl-2, suggesting that apoptosis would be inhibited. The berberine dose showed no significant relationship with apoptosis.

A high-fat diet induces obesity and also causes a variety of health disorders, including cognitive decline [29]. Hippocampal function may be particularly vulnerable to the negative consequences of a high-fat diet. Chronic treatment with berberine for 30 days improved cognitive performance and lowered hyperglycemia, oxidative stress, and acetylcholinesterase activity in diabetic rats [30]. In conclusion, short-term memory was improved by berberine treatment in a hyperlipidemic rat model by increasing neuronal proliferation and inhibiting neuronal apoptosis. Berberine treatment also improved lipid metabolism.

AUTHOR CONTRIBUTION STATEMENT

• Full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis: SSP

- Study concept and design: *MK*
- Acquisition of data: *MK, TWK*
- Analysis and interpretation of data: *MK, TWK*
- Drafting of the manuscript: *SSP*
- Critical revision of the manuscript for important intellectual content: *CJK, SSP*
- Statistical analysis: *MSS*
- Administrative, technical, or material support: *MH, HSP*
- Study supervision: *CJK, SSP*

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