BRAIN INJURY
https://doi.org/10.1080/02699052.2022.2037712

Taylor & Francis
Taylor & Francis Group

Study on the autophagy-related mechanism of puerarin in improving the cognitive impairment induced by alcohol in female mice

Huanhuan Bian[a*], Yi Wu[a*], Zhengguo Cui[b], Heyu Zheng[c], Yanyi Li[c], and Dan Zou[d]

[a]Department of Pathophysiology, Shenyang Medical College, Shenyang, Liaoning, PR China; [b]Department of Environmental Health, University of Fukui School of Medical Science, Fukui, Japan; [c]Clinical Medicine Major Grade 2018, Shenyang Medical College, Shenyang,Liaoning, PR China; [d]Department of Immunology, Shenyang Medical College, Shenyang, Liaoning, PR China

ABSTRACT

Primary Objective: This study aimed to investigate the effects of low-, medium-, and high-dose puerarin on cognitive impairment induced by 50% alcohol in mice and revealed the role of autophagy-related signaling pathways (mTOR and JNK pathways) in this process.

Research Design: The alcohol-induced brain injury model was treated with different concentrations of puerarin. The cognitive function of mice was evaluated by the behavioral test, and the changes of target proteins in hippocampus of each experimental group were detected.

Methods and Procedures: 40 female Kunming mice were randomly divided into 5 groups. The cognitive ability of mice was tested by Morris water maze, the morphological changes in the CA1 area of hippocampus were observed by HE staining, and the target proteins in hippocampus were measured by WB and IHC.

Main Outcomes and Results: Compared with the 50% alcohol group, the expression of p-mTOR/mTOR and p-4E-BP1/4E-BP1 in hippocampus was significantly decreased, while the expression of p-JNK/JNK, Beclin1, and LC3 was significantly increased in the medium- and high-dose puerarin groups.

Conclusions: Puerarin could improve the cognitive impairment induced by 50% alcohol. The mTOR and JNK pathways related to autophagy might be involved in this process.

ARTICLE HISTORY
Received 18 June 2021
Revised 20 December 2021
Accepted 31 December 2021

KEYWORDS
Puerarin; alcohol; learning and memory; autophagy; mTOR signaling pathway; JNK signaling pathway

Introduction

Alcoholism has become a very common medical and social problem. Studies have shown that one of the most serious effects of alcohol on brain function is the impairment of spatial learning and memory in hippocampus (1). The latest research in animal models suggested that compared with male, female is more likely to suffer from alcohol-induced neurotoxicity (2). Our previous study found that alcohol-induced cognitive dysfunction was related to the mTOR signal transduction pathway in the hippocampus of mice (3). The mTOR signaling pathway plays a central role in cellular growth, metabolism, and the negative regulation of autophagy (4). Autophagy not only isa normal physiological activity of cells but also can be activated when cells are subjected to various stimuli as a stress response to protect cells. Studies have shown that the c-Jun N end kinase (JNK) signaling pathway is also closely related to autophagy (5).

Puerarin is the major bioactive ingredient extracted from the root of the Pueraria lobata. Puerarin's pharmacological effects include promoting blood circulation, removing blood clots, improving microcirculation, expanding coronary arteries, attenuating insulin resistance, reducing cardiac oxygen consumption, etc. (6–8). It is widely used in the treatment of cardiovascular and cerebrovascular diseases, diabetes, cancer, Parkinson's disease, and Alzheimer's disease. In China, Pueraria lobata has been used to treat alcoholism since ancient times, but the specific mechanism remains unclear.

This study explores the effects of different doses of puerarin on the cognitive dysfunction induced by 50% alcohol and reveals the regulatory mechanism of mTOR/JNK signaling pathways to autophagy in the process. Our study provides new basic research data for puerarin to improve cognitive dysfunction caused by alcoholism.

Materials and methods

Animals and treatment

Experimental procedures were approved by the Animal Ethics Committee of Shenyang Medical College under guidelines from the National Medical Research Council of China. 40 female Kunming mice (8 weeks old, weighing 30 ± 5 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (SCXK2020-0001). Before the experiment, the mice were fed freely under a 12 hours light/dark cycle at the temperature of 21 ± 2°C for one week to adapt to the environment. Then, the mice were randomly divided into 5 groups, including the control group (normal saline), 50% alcohol group, 25 mg/kg puerarin+50% alcohol group, 50 mg/kg puerarin+50% alcohol

CONTACT Dan Zou zoudan@symc.edu.cn Department of Immunology, Shenyang Medical College, 146 Huang He North Road, Shenyang, Liaoning 110034, PR China

*These authors contributed equally to this work.

group, and 100 mg/kg puerarin+50% alcohol group. Normal saline and alcohol were administered by intragastric infusion at a dose of 10 ml/kg/d, and puerarin was administered by intraperitoneal injection at a dose of 20 ml/kg/d for 28 days. Puerarin was administered before alcohol, and the interval was 1 hour. Throughout the experiment, the mice were free to drink purified water and food.

After the spatial probe test of MWM, five mice in each group were anesthetized with ether and killed by cervical dislocation. The hippocampus was separated on ice and stored at −80 degrees before they were used in the Western blotting test. The remaining 3 mice in each group were anesthetized with ether, and then they were administered by intraperitoneal injection with 1% pentobarbital sodium (20160411; Merck, Germany) at a dose of 50 mg/kg. The mice were treated with 4% paraformaldehyde for cardiac perfusion and internal fixation, and then the brains were placed in 4% paraformaldehyde solution.

Morris water maze (MWM) test

MWM is a classic behavioral experiment on learning and memory. The test included 5 consecutive days of positioning navigation test and 1 day of spatial probe test (Figure.1). The test method of the experiment could be found in the previous reference (9).

Hematoxylin-eosin (HE) staining

The specific test method was carried out as previously described (3). The morphological changes in the CA1 area of hippocampus were observed in each group of female mice. Three mice of each group were used for HE staining.

Western blotting

The specific experimental method was described in the previous reference (3). The total protein (30 μg) was loaded on the 6% or 12% SDS-PAGE gel (KGP250; KeyGEN Biotech, Jiangsu, China). The primary antibodies were as follows: phospho-mTOR (phospho-S2448) antibody (1:8000, ab109268; Abcam, Cambridge, UK); anti-mTOR antibody (1:4000, ab32028; Abcam); phospho-4E-BP1 antibody (1:1000, #2855; Cell signaling Technology, Danfoss, Massachusetts, USA); anti-4E-BP1 antibody (1:1000, #9644; Cell signaling Technology); phospho-JNK1, JNK2, and JNK3 (phospho-T183+ T183+ T221) antibody (1:2000, ab124956; Abcam); anti-JNK antibody (0.2 μg/ml, AF1387-SP; Bio-Techne, Shanghai, China); anti-Beclin1 antibody (1:1000, ab210498; Abcam); and anti-LC3B antibody (1:1000, #3868; Cell Signaling Technology). The secondary antibody was goat antirabbit IgG (H-L) (peroxidase/HRP coupling) (1:4000, E-AB-1003; Elabscience, Wuhan, China). The β-actin antibody (1:10000, AC038; ABclonal, Wuhan, China) was an internal reference. Image J software was used to quantify the protein bands (Wayne Rasband, National Institutes of Health, Bethesda, Maryland). Five mice of each group were used for Western blotting.

Immunocytochemistry (IHC)

The specific test method was carried out as previously described (3). The primary antibodies were as follows: phospho-mTOR (phospho-S2448) antibody (1:200, YP0176; Immunoway); phospho-4E-BP1 antibody (1:400, #2855; Cell signaling Technology); phospho-JNK1, JNK2, and JNK3 (phospho-T183+ T183+ T221) antibody (1:200, ab124956; Abcam); anti-Beclin1 antibody (1:200, ab210498; Abcam); and anti-LC3B antibody (1:200, #3868; Cell Signaling Technology). The EnVision TM test kit (KIT-9901; Maxim, Fujian Province, China) was used for second antibody incubation. There were 3 mice in each group for IHC experiment (3 slices/protein/mouse). 2 fields of vision were selected from each slice for image acquisition by using a microscope digital camera system (Olympus Optical Co. Ltd, Japan), and Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, Maryland, USA) was used for semiquantitative analysis of protein distribution in the images.

Statistical analysis

All data were analyzed by using GraphPad Prism 9.0 software (GraphPad Software, La Jolla, California, USA). We repeatedly measured the differences in the escape latency in the same group on different days and different groups on the same day using ANOVA and Tukey's t-test. The indexes related to the spatial probe test were statistically analyzed in the same way. In the protein detection experiment (Western blotting and IHC), the comparison between different groups was performed using one-way ANOVA followed by Tukey's t-test and paired t-test. Data were expressed as the mean ± SEM, and statistically significance was observed at $P < .05$.

Figure 1. Experimental timeline.

Results

Spatial learning and memory function of mice were evaluated by the MWM test

Except for the 50% alcohol group, the escape latency showed a decreasing trend from the 1st day to the 5th day in each group, and the escape latency of the 5th day was significantly shorter than that of the 1st day in each group ($P < .05$) (Table 1). From the 1st day to the 2nd day, there was no significant difference in the escape latency between the groups [$F(4, 35) = 1.427$, $P > .05$ for the 1st day; $F(4, 35) = 2.640$, $P > .05$ for the 2nd day]. From the 3rd day to the 5th day, the escape latency of the 50% alcohol group was significantly longer than that of the control group [$F(4, 35) = 7.107$, $P < .01$ for the 3rd day; $F(4, 35) = 13.32$, $P < .01$ for the 4th day; and $F(4, 35) = 35.69$, $P < .01$ for the 5th day]. But compared with the 50% alcohol group, the escape latency of the medium- and high-dose puerarin groups was significantly decreased from the 4th day to the 5th day [$F(4, 35) = 13.32$, $P < .01$ for the 4th day; $F(4, 35) = 35.69$, $P < .01$ for the 5th day] [Figure.2(a)]. Data from the spatial probe test showed that the distance and time percentage in the target quadrant and the times of crossing the platform in the 50% alcohol group were significantly lower than those of the control group [$F(4,35) = 5.164$, $P < .01$ for distance; $F(4,35) = 7.324$, $P < .01$ for time; and $F(4, 35) = 6.490$, $P < .01$ for times of crossing the platform] [Figure. 2(b–d)]. But in the medium- and high-dose puerarin groups, the distance and time percentage in the target quadrant were significantly higher than those of the 50% alcohol group [$F(4,35) = 5.164$, $P < .01$ for distance; $F(4,35) = 7.324$, $P < .01$ for time] [Figure. 2(b,c)], and there was no significant difference between the medium- and high-dose puerarin groups ($P > .05$). These results were supported by representative images of traveling trace in the spatial probe test [Figure. 2(e)].

Effects of different doses of puerarin on the morphological structure of hippocampal CA1 neurons in mice treated with 50% alcohol

The results of HE staining showed that compared with the control group, the number of hippocampal neurons decreased and the arrangement was relatively loose in the 50% alcohol group. Compared with the 50% alcohol group, these pathological changes were obviously improved in the medium- and high-dose puerarin groups, it was visible that the number of pyramidal neurons was higher than that of the 50% alcohol group, and their arrangement was denser, while the improvement effect of the low-dose puerarin group was not so distinct [Figure. 3].

Effects of different doses of puerarin on the expression of p-mTOR/mTOR, p-4E-BP1/4E-BP1, p-JNK/JNK, Beclin1/β-actin, and LC3II/I in the hippocampus of mice treated with 50% alcohol

Compared with the control group, the ratio of p-mTOR/mTOR and p-4E-BP1/4E-BP1 in the hippocampus of mice increased significantly in the 50% alcohol group [$F(4,20) = 9.943$, $P < .01$ for p-mTOR/mTOR; $F(4,20) = 9.935$, $P < .01$ for p-4E-BP1/4E-BP1] [Figure. 4(b,c)], while the expression of p-JNK/JNK, Beclin1/β-actin, and LC3II/I decreased significantly [$F(4,20) = 14.10$, $P < .01$ for p-JNK/JNK; $F(4,20) = 23.26$, $P < .01$ for Beclin1/β-actin; and $F(4,20) = 7.217$, $P < .05$ for LC3II/I] [Figure. 4(d-f)]. Compared with the 50% alcohol group, the ratios of p-mTOR/mTOR and p-4E-BP1/4E-BP1 in the medium- and high-dose puerarin groups were significantly decreased, but the ratios of p-JNK/JNK, Beclin1/β-actin, and LC3II/I were significantly increased in the hippocampus of mice [$F(4,20) = 9.943$, $P < .05$ for p-mTOR/mTOR; $F(4,20) = 9.935$, $P < .01$ for p-4E-BP1/4E-BP1; $F(4,20) = 14.10$, $P < .05$ for p-JNK/JNK; $F(4,20) = 23.26$, $P < .01$ for Beclin1/β-actin; and $F(4,20) = 7.217$, $P < .01$ for LC3II/I] [Figure. 4(b-f)]. Furthermore, there is no significant difference between the medium and high doses of puerarin groups ($P > .05$). The expression of Beclin1/β-actin and LC3II/I was significantly higher in the low-dose puerarin group than those of the 50% alcohol group [$F(4,20) = 23.26$, $P < .05$ for Beclin1/β-actin; $F(4,20) = 7.217$, $P < .05$ for LC3 II/I] [Figure. 4(e,f)].

Effects of different doses of puerarin on the expression of p-mTOR, p-4E-BP1, p-JNK, Beclin1, and LC3 in the hippocampus of mice treated with 50% alcohol

To further confirm the Western blotting results, the protein expression of p-mTOR, p-4E-BP1, p-JNK, Beclin1, and LC3 was examined by IHC in the hippocampus of mice. Compared with the control group, the expression of p-mTOR and p-4E-BP1 increased significantly in the hippocampal CA1 area of the 50% alcohol group [$F(4,10) = 10.21$, $P < .01$ for p-mTOR; $F(4,10) = 46.15$, $P < .01$ for p-4E-BP1] [Figure. 5(b,c)], while the expression of Beclin1 and LC3 decreased significantly [$F(4,10) = 50.48$, $P < .01$ for Beclin1; $F(4,10) = 46.69$, $P < .01$ for

Table 1. Comparison of escape latency from the 1st day to the 5th day in each group.

Groups	1	2	3	4	5
Control	57.35±1.12	49.34±4.28	22.92±5.52**	11.25±1.86**	10.01±1.92**
50% A	59.13±0.46	54.93±3.06	51.03±3.26	47.28±3.92*	55.70±1.99
25mg/kg Pue+50% A	58.11±1.28	54.90±2.84	42.40±4.82*	34.66±5.08**	28.49±3.71**
50mg/kg Pue+50% A	59.33±0.19	46.62±2.75	19.99±5.02**	21.34±4.86**	15.93±3.40**
100mg/kg Pue+50% A	59.62±0.14	58.65±0.96	33.75±5.48**	16.20±3.56**	18.97±3.51**

The data were expressed as the mean ± SEM (n=8). Except for the 50% alcohol group, the escape latency decreased gradually from the 1st day to the 5th day and the escape latency on the 5th day was significantly shortened in each group compared to the 1st day. *$P < .05$ and **$P < 0.01$ versus the escape latency on the 1st day of each group.

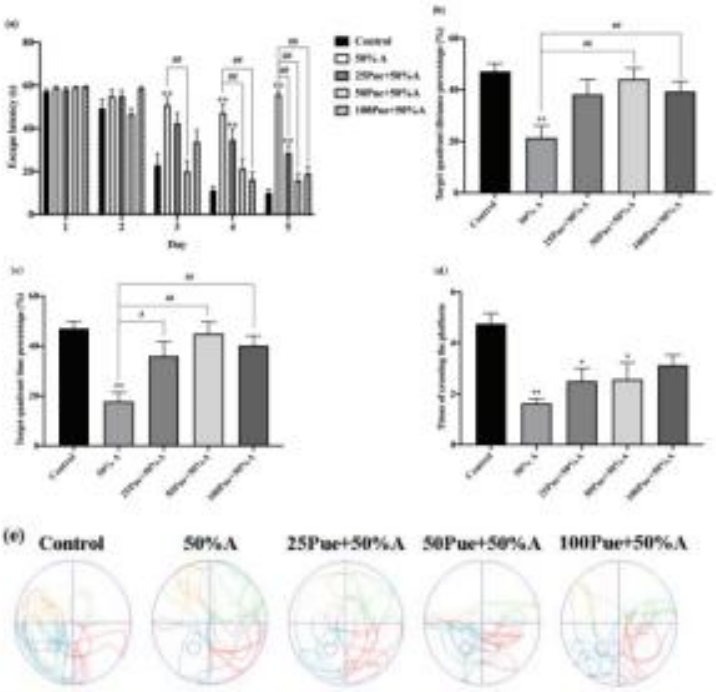

Figure 2. Cognitive function of mice was assessed by the MWM test. (a) The comparison of escape latency between groups. (b) The comparison of target quadrant distance percentage (%) between groups. (c)The comparison of target quadrant time percentage (%) between groups. (d) The comparison of times of crossing the platform between groups. (e) Representative path maps of each group. Data are expressed as the mean ± SEM (n=8). *P < 0.05 and **P < .01 versus control group. #P < .05 and ## P < .01 versus 50% alcohol group.

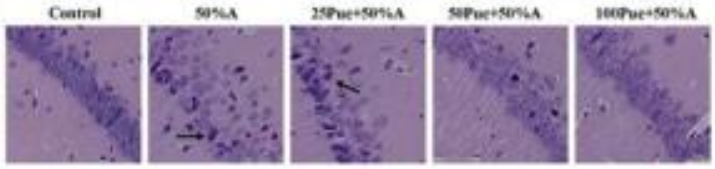

Figure 3. HE staining of the hippocampal CA1 area in each group of mice (original magnification 400×, n=3). Compared with the control group, the number of hippocampal neurons decreased and their arrangement was relatively loose in the 50% alcohol group. But in the medium- and high-dose puerarin groups, the number of pyramidal neurons was higher than that of the 50% alcohol group, and the arrangement was denser. The arrow indicates the the change in neuron.

LC3] [Figure. 5(e,f)]. Compared with the 50% alcohol group, the expression of p-mTOR and p-4E-BP1 in the hippocampal CA1 area of the medium- and high-dose puerarin groups was significantly decreased [F (4,10) = 10.21, P < .05 for p-mTOR; F (4,10) = 46.15, P < .01 for p-4E-BP1] [Figure. 5(b,c)] and the expression of Beclin1 and LC3 was significantly increased [F (4,10) = 50.48, P < .01 for Beclin1; F (4,10) = 46.69, P < .01 for LC3] [Figure. 5(e,f)]. In the hippocampal CA3 area of mice, the expression level of p-JNK decreased significantly in the 50% alcohol group and 3 doses of puerarin pretreatment groups compared with the control group [F (4,10) = 34.79, P < .01] [Figure. 5(d)]. However, the expression level of p-JNK in the medium-dose puerarin pretreatment group was higher than that of the 50% alcohol group [F (4,10) = 34.79, P < .05] [Figure. 5(d)]. Furthermore, there was no significant difference in the expression of the five proteins mentioned above between the medium- and high-dose puerarin groups (P > .05) [Figure. 5(b-f)].

Discussion

Alcohol is a neurotoxic compound. Excessive alcohol exposure can cause permanent damage to the central nervous system and lead to hippocampal-dependent learning and memory impairments (10). Studies have shown that puerarin has

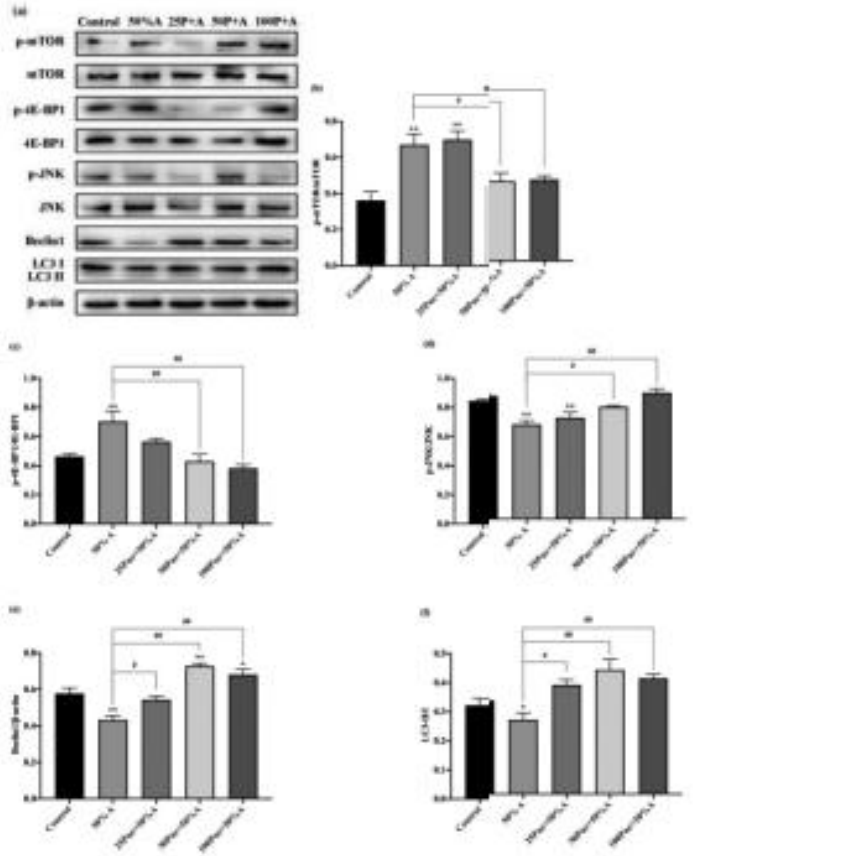

Figure 4. (a) Western blotting bands of p-mTOR, mTOR, p-4E-BP1, 4E-BP1, p-JNK, JNK, Beclin1, LC3 I, and LC3 II in each experimental group. (b-f) The comparison of p-mTOR/mTOR, p-4E-BP1/4E-BP1, p-JNK/JNK, Beclin1/β-actin, and LC3II/I of hippocampus in each group of female mice. Data are expressed as the mean ± SEM (n=5). *P < .05 and **P < .01 versus control group. #P < .05 and ## P < .01 versus 50% alcohol group.

properties of antioxidation, anti-inflammation, and antiapoptosis and can improve cognitive dysfunction caused by some [illegible] (11,18).

Puerarin can prevent the damage of spatial learning and memory caused by alcoholism in mice (13). Morris water maze results showed that on the 4th day and the 5th day of the positioning navigation test, compared with the control group, the escape latency in the 50% alcohol group was significantly prolonged, and in the medium- and high-dose puerarin pretreatment groups, it was significantly shorter than that of the 50% alcohol group. In the spatial probe test, compared with the control group, the residence distance and time in the target quadrant where the escape platform was located were significantly shortened in the 50% alcohol group. These two indexes of mice pretreated with medium- and high-dose puerarin were significantly longer than those of mice treated with 50% alcohol only, but there was no significant difference in these two indexes between the medium- and high-dose puerarin pretreatment groups. The [illegible]ies of crossing platform in the 50% alcohol group were significantly lower than those of the control group, while in the high-dose puerarin pretreatment group, they were not significantly different from those of the control group. The result of each group from the 1st day to the 5th day in the positioning navigation test showed that except for the 50% alcohol group, the escape latency decreased gradually from the 1st day to the 5th day and on the 5th day, it was significantly shortened in each group compared with the 1st day. The above results showed that 50% alcohol could damage the learning and memory function in mice, the pretreatment of medium- and high-dose puerarin could significantly improve the cognitive impairment induced by 50% alcohol in mice, and the effect was stable.

Long-term alcohol exposure can disrupt the generation and development of newborn neurons and induce changes in the morphology and molecular levels of neurons in the hippocampus, leading to cognitive dysfunction in mice (14–16). HE staining results showed the morphological characteristics of hippocampal pyramidal neurons in each group, respectively. Compared with the control group, 50% alcohol reduced the number of pyramidal neurons in the hippocampal CA1 area in female mice, with loose arrangement, unclear boundary, and severe cell damage. Compared with the 50% alcohol group, the number of pyramidal neurons in the hippocampal CA1 area was increased in medium- and high-dose puerarin pretreatment groups, the neuron arrangement was compact and relatively regular, and the degree of cell damage was significantly reduced. Our results showed that medium and high doses of puerarin have a protective effect on neuron damage induced by 50% alcohol in the hippocampal CA1 area of mice. Studies showed that enhanced autophagy could reduce the levels of apoptosis and necrosis in hypoxic-ischemic injury models (17).

Autophagy represents a homeostatic cellular mechanism for the turnover of organelles and proteins through a lysosome-dependent degradation pathway (18). As a dynamic recovery system, autophagy is involved in cellular renovation and internal environmental stability. It has been reported that the accumulation of ubiquitinated proteins caused by alcohol abuse might be correlated with impairment of the autophagy mechanism (19). Furthermore, the results of electron microscopy further proved that ethanol enhanced the neuropathological process by damaging autophagy (20).

Autophagy is an important physiological process downstream of mTOR. The mTOR signaling pathway is an important hub for regulating the cellular energy and nutritional metabolism and also a negative regulator for autophagy (21). Studies have shown that the PI3K/Akt signaling pathway can negatively regulate autophagy by regulating the phosphorylation of mTOR (22). 4EBP1 is a translation regulator, and p-4EBP1 can directly reflect the activity of mTOR (23). In mice myocardium, rapamycin can reduce the expression of p-mTOR and p-4EBP1 by inhibiting the mTOR-4EBP1 signaling pathway (24). Dephosphorylation of 4EBP1 inhibits protein synthesis and triggers autophagy (25).

Studies have shown that chronic ethanol exposure in adult mice can activate mTOR, impairing the autophagy-lysosome pathway in the brain, and further damages neurons in the central nervous system (CNS) (26). In our experiment, Western blotting results showed that p-mTOR /mTOR and p-4E-BP1 /4E-BP1 in hippocampal formation of mice in the 50% alcohol group were significantly higher than those of the control group and those of medium- and high-dose puerarin pretreatment groups. p-mTOR and p-4E-BP1 are activated forms of mTOR and 4E-BP1, respectively. Immunohistochemical results showed that the expression levels of p-mTOR and p-4E-BP1 in the 50% alcohol group were significantly higher than those of the control group and significantly higher than those of the medium- and high-dose puerarin pretreatment groups in the hippocampal CA1 area of mice. There was no significant difference in the expression of these proteins between the medium- and high-dose puerarin groups.

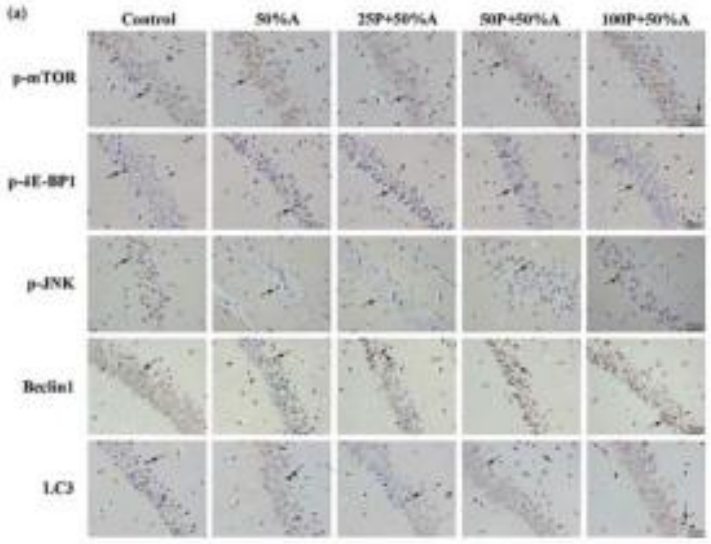

Figure 5. (a) Representative images of protein expression of p-mTOR, p-4E-BP1, Beclin1, and LC3 in the hippocampal CA1 area and protein expression of p-JNK in the hippocampal CA3 area in immunohistochemistry experiment (original magnification 400×). (b-f) The comparison of average optical density of p-mTOR, p-4E-BP1, p-JNK, Beclin1, and LC3 in the hippocampus. Data are expressed as the mean ± SEM (n=5). $^{*}P < .05$ and $^{**}P < .01$ versus control group. $^{\#}P < .05$ and $^{\#\#}P < .01$ versus 50% alcohol group. The arrow indicates the labeled protein.

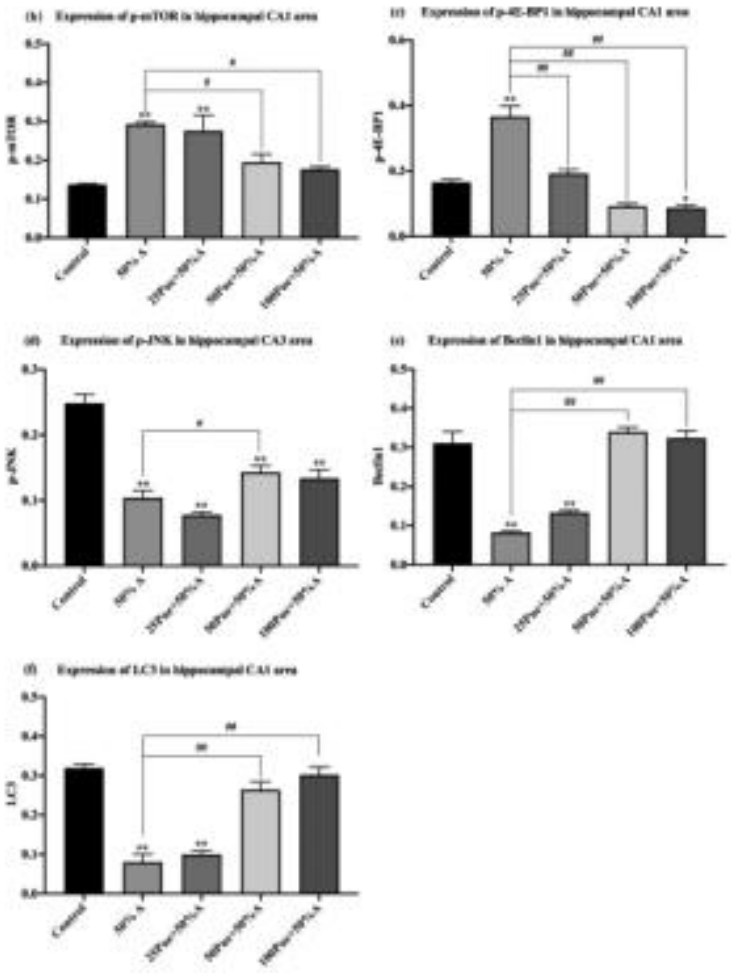

Figure 5. Continued

Studies have shown that the mTOR/BECN1 (S14) pathway is associated with alcohol-induced autophagy (27). Beclin1 and LC3 are important indicators to detect autophagy. Accumulation of LC3 II is considered as a marker of autophagy (28). Western blotting results showed that the expression levels of Beclin1 and LC3 II/I in hippocampal formation of mice in the 50% alcohol group were significantly lower than those of the control group and medium- and high-dose puerarin pretreatment groups. The expression levels of Beclin1 and LC3 II/I were not significantly different between the medium- and high-dose puerarin pretreatment groups. The immunohistochemical results of the hippocampal CA1 area in mice were consistent with the above results. It can be inferred from the above results that cognitive dysfunction induced by 50% alcohol in mice is related to the activation of the mTOR/4E-BP1 signaling pathway and the decrease of Beclin1 and LC3 II/I expression in the hippocampus of mice. Medium- and high-dose puerarin pretreatment improved cognitive impairment induced by 50% alcohol in mice. In the process, the activity of the mTOR/4E-BP1 signaling pathway in the hippocampus of mice was decreased and the expression of autophagy-related proteins Beclin1 and LC3 II/I was enhanced. Studies have shown that enhanced autophagy is a protective mechanism for alleviating ethanol-mediated oxidative stress and neuronal death (29).

The JNK signal transduction pathway is an important branch of the MAPK pathway, which is involved in the regulation of cell growth, proliferation, and apoptosis processes, and is closely related to autophagy. The JNK signaling pathway mainly functions through the phosphorylation of Bcl-2 family proteins and their disassociation with Beclin1 to regulate autophagy (30). Studies have shown that the learning and memory impairment induced by lanthanum may be associated with excessive autophagy in rats. Lanthanum activated the

JNK/c-Jun and JNK/FoxOs signaling pathways, inhibited the AKT/mTOR signaling pathway by inducing oxidative stress, and enhanced autophagy in the hippocampus (31). Curcumin can enhance autophagy by activating the AMPK-JNK signaling pathway. The enhancement of autophagy, in turn, promotes neurogenesis and synaptogenesis in the hippocampus of mice. Pretreatment with the autophagy inhibitor 3-methyladenine (3-MA) can eliminate the effect of curcumin on cognitive improvement (32). WB results of this study showed that p-JNK/JNK in hippocampal formation of mice in the 50% alcohol group was significantly lower than that of the control group and medium- and high-dose puerarin pretreatment groups. There was no statistically significant difference in p-JNK/JNK between the medium- and high-dose puerarin pretreatment groups. Immunohistochemical results showed that the expression level of p-JNK in the hippocampal CA3 area was significantly lower in the 50% alcohol group than that of the control group, and the expression level of p-JNK in the medium-dose puerarin pretreatment group was significantly higher than that of the 50% alcohol group. There was no significant difference in p-JNK expression between the groups pretreated with medium and high doses of puerarin. Therefore, we inferred that medium- and high-dose puerarin pretreatment could improve cognitive impairment induced by 50% alcohol, which may be related to activation of the JNK pathway and enhancement of autophagy.

Behavioral test results showed that low-dose puerarin pretreatment also could improve cognitive impairment induced by 50% alcohol in mice, but the effect was not as good as that of medium- and high-dose groups. Results showed that the expression of Beclin1 and LC3 II/I in hippocampus of the low-dose puerarin pretreatment group was significantly higher than that of the 50% alcohol group, but the expression of protein-related mTOR/JNK signaling pathways in the low-dose puerarin group was not completely synchronized with the medium- and high-dose groups. Learning and memory are complex neural activities in the brain, and the mechanism of low-dose puerarin to treat alcoholism needs further study.

Conclusion

Chronic and high-concentration alcohol exposure can damage cognitive function in female mice. Both 50 mg/kg and 100 mg/kg puerarin significantly improved cognitive impairment induced by 50% alcohol. Autophagy associated with activation of the JNK signaling pathway and inhibition of the mTOR/4E-BP1 signaling pathway may be involved in this process.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research was supported by the Scientific Research Project of Liaoning Province Education Administration [SYYX202002], the National Training Programs of Innovation and Entrepreneurship for Undergraduates (202010164003) and Science and Technology Innovation Fund for Master students of Shenyang Medical College (Y20210505).

References

1. Wang F, Li J, Li L, Gao Y, Wang F, Zhang Y, Fan Y, Wu C. Protective effect of apple polyphenols on chronic ethanol exposure-induced neural injury in rats. Chem Biol Interact. 2020 Aug 1;326:109113. doi:10.1016/j.cbi.2020.109113. Cited in PubMed; Cited in: PMID: 32360496.
2. Macht V, Eichert N, Crews F. Adolescent alcohol exposure produces protracted cognitive-behavioral impairments in adult male and female rats. Brain Sci. 2020 Oct 28;10(11):785. doi:10.3390/brainsci10110785. Cited in: PMID: 33126417.
3. Xing Y, Zou D. Ethanol-induced cognitive dysfunction is associated with alterations in the mammalian target of rapamycin signaling pathway in the hippocampus of male mice. Neuroreport. 2018 Sep 26;29(14):1230–37. doi:10.1097/WNR.0000000000001104. Cited in: PMID: 30063559.
4. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. Cell. 2017 Apr 6;169(2):361–71. doi:10.1016/j.cell.2017.03.035. Cited in: PMID: 28388417. Erratum for: Cell. 2017 Mar 9;168(6):960-76.
5. Zhang Y, Wu Q, Zhang L, Wang Q, Yang Z, Liu J, Feng L. Caffeic acid reduces A53T α-synuclein by activating JNK/Bcl-2-mediated autophagy in vitro and improves behaviour and protects dopaminergic neurons in a mouse model of Parkinson's disease. Pharmacol Res. 2019 Dec;150:104538. doi:10.1016/j.phrs.2019.104538. Cited in: PMID: 31707034.
6. Zhou YX, Zhang H, Peng C. Puerarin: a review of pharmacological effects. Phytother Res. 2014 Jul;28(7):961–75. doi:10.1002/ptr.5083. Cited in: PMID: 24339367.
7. Deng HF, Wang XL, Sun H, Xiao XZ. Puerarin inhibits expression of tissue factor induced by oxidative low-density lipoprotein through activating the PI3K/Akt/eNOS pathway and inhibiting activation of ERK1/2 and NF-κB. Life Sci. 2017 Dec 15;191:115–21. doi:10.1016/j.lfs.2017.10.018. Cited in PubMed; Cited in: PMID: 29037842.
8. Deng Y, Ng ES, Yeung JH, Kwan YW, Lau CB, Koon JC, Zhou L, Zuo Z, Leung PC, Fung KP, et al. Mechanisms of the cerebral vasodilator actions of isoflavonoids of gegen on rat isolated basilar artery. J Ethnopharmacol. 2012 Jan 6;139(1):294–304. doi:10.1016/j.jep.2011.11.021. Cited in: PMID: 22120017.
9. Cui SQ, Wang Q, Zheng Y, Xiao B, Sun HW, Gu XL, Zhang YC, Fu CH, Dong PX, Wang XM. Puerarin protects against damage to spatial learning and memory ability in mice with chronic alcohol poisoning. Braz J Med Biol Res. 2015 Jun;48(6):515–22. doi:10.1590/1414-431X20144250. Cited in: PMID: 25851201.
10. Qiao X, Sun M, Chen Y, Jin W, Zhao H, Zhang W, Lai J, Yan H. Ethanol-Induced neuronal and cognitive/emotional impairments are accompanied by down-regulated NT3-TrkC-ERK in hippocampus. Alcohol Alcohol. 2021 Feb 24;56(2):220–29. doi:10.1093/alcalc/agaa101. Cited in: PMID: 33103180.
11. Zhao SS, Yang WN, Jin H, Ma KG, Feng GF. Puerarin attenuates learning and memory impairments and inhibits oxidative stress in STZ-induced SAD mice. Neurotoxicology. 2015 Dec;51:166–71. doi:10.1016/j.neuro.2015.10.010. Cited in: PMID: 26511841.
12. Zhang J, Guo W, Tian B, Sun M, Li H, Zhou L, Liu X. Puerarin attenuates cognitive dysfunction and oxidative stress in vascular dementia rats induced by chronic ischemia. Int J Clin Exp Pathol. 2015 May 1;8(5):4695–704. Cited in: PMID: 26191159.
13. Li W, Zhang Y, Xue Y, Yu H, Zhang Y, Tao L, Yang Y, Qian Z. Effects of puerarin on spatial learning and memory function in mice with acute alcohol consumption: an evaluation based upon firing rate and oxygen saturation analysis. Adv Clin Exp Med. 2019 Feb;28(2):171–78. doi:10.17219/acem/81520. Cited in: PMID: 30503312.

14. Oliveira AC, Pereira MC, Santana LN, Fernandes RM, Teixeira FB, Oliveira GB, Fernandes LM, Fontes-Júnior EA, Prediger RD, Crespo-López ME, et al. Chronic ethanol exposure during adolescence through early adulthood in female rats induces emotional and memory deficits associated with morphological and molecular alterations in hippocampus. J Psychopharmacol. 2015 Jun;29(6):712–24. doi:10.1177/0269881115581960. Cited in: PMID: 25922423.
15. He J, Nixon K, Shetty AK, Crews FT. Chronic alcohol exposure reduces hippocampal neurogenesis and dendritic growth of newborn neurons. Eur J Neurosci. 2005 May;21(10):2711–20. doi:10.1111/j.1460-9568.2005.04120.x. Cited in: PMID: 15926919.
16. Guerri C. Neuroanatomical and neurophysiological mechanisms involved in central nervous system dysfunctions induced by prenatal alcohol exposure. Alcohol Clin Exp Res. 1998 Apr;22(2):304–12. doi:10.1111/j.1530-0277.1998.tb03653.x. Cited in: PMID: 9581633.
17. Loos B, Genade S, Ellis B, Lochner A, Engelbrecht AM. At the core of survival: autophagy delays the onset of both apoptotic and necrotic cell death in a model of ischemic cell injury. Exp Cell Res. 2011 Jun 10;317(10):1437–53. doi:10.1016/j.yexcr.2011.03.011. Cited in: PMID: 21420401.
18. Ryter SW, Mizumura K, Choi AM. The impact of autophagy on cell death modalities. Int J Cell Biol. 2014;2014:502676. doi:10.1155/2014/502676. Cited in: PMID: 24639873; PMCID: PMC3932252.
19. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, Johansen T. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem. 2007 Aug 17;282(33):24131–45. doi:10.1074/jbc.M702824200. Cited in: PMID: 17580304.
20. Pla A, Pascual M, Renau-Piqueras J, Guerri C. TLR4 mediates the impairment of ubiquitin-proteasome and autophagy-lysosome pathways induced by ethanol treatment in brain. Cell Death Dis. 2014 Feb 20;5(2):e1066. doi:10.1038/cddis.2014.46. Cited in: PMID: 24556681; PMCID: PMC3944260.
21. Rabanal-Ruiz Y, Otten EG, Korolchuk VI. mTORC1 as the main gateway to autophagy. Essays Biochem. 2017 Dec 12;61(6):565–84. doi:10.1042/EBC20170027. Cited in: PMID: 29233869; PMCID: PMC5869864.
22. Wang R, Zhang Q, Peng X, Zhou C, Zhong Y, Chen X, Qiu Y, Jin M, Gong M, Kong D. Stellettin B induces G1 Arrest, apoptosis and autophagy in human non-small cell lung cancer A549 cells via blocking PI3K/Akt/mTOR pathway. Sci Rep. 2016 May 31;6:27071. doi:10.1038/srep27071. Cited in PubMed; Cited in: PMID: 27245769; PMCID: PMC4886687.
23. Jin J, Hu K, Ye M, Wu D, He Q. Rapamycin Reduces Podocyte Apoptosis and is Involved in Autophagy and mTOR/ P70S6K/4EBP1 Signaling. Cell Physiol Biochem. 2018;48(2):765–72. doi:10.1159/000491905. Cited in: PMID: 30025409.
24. Jin B, Shi H, Zhu J, Wu B, Geshang Q. Up-regulating autophagy by targeting the mTOR-4EBP1 pathway: a possible mechanism for improving cardiac function in mice with experimental dilated cardiomyopathy. BMC Cardiovasc Disord. 2020 Feb 4;20(1):56. doi:10.1186/s12872-020-01365-9. Cited in: PMID: 32019530; PMCID: PMC6998347.
25. Chen Q, Zhou Y, Richards AM, Wang P. Up-regulation of miRNA-221 inhibits hypoxia/reoxygenation-induced autophagy through the DDIT4/mTORC1 and Tp53inp1/p62 pathways. Biochem Biophys Res Commun. 2016 May 20;474(1):168–74. doi:10.1016/j.bbrc.2016.04.090. Cited in: PMID: 27105917.
26. Luo J. Autophagy and ethanol neurotoxicity. Autophagy. 2014;10(12):2099–108. doi:10.4161/15548627.2014.981916. Cited in: PMID: 25484085; PMCID: PMC4502825.
27. Liu Y, Zhang Y, Peng J, Wang H, Li X, Li X, Rong X, Pan J, Peng Y. Autophagy alleviates ethanol-induced memory impairment in association with anti-apoptotic and anti-inflammatory pathways. Brain Behav Immun. 2019 Nov;82:63–75. doi:10.1016/j.bbi.2019.07.033. Cited in: PMID: 31376498.
28. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol. 2010 May;221(1):3–12. doi:10.1002/path.2697. Cited in: PMID: 20225336; PMCID: PMC2990190.
29. Maiuri G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol. 2014 Feb;15(2):81–94. doi:10.1038/nrm3735. Cited in: PMID: 24401948; PMCID: PMC3970201.
30. Zhou YY, Li Y, Jiang WQ, Zhou LF. MAPK/JNK signalling: a potential autophagy regulation pathway. Biosci Rep. 2015 Apr 22;35(3):e00199. doi:10.1042/BSR20140141. Cited in: PMID: 26182361.
31. Gao X, Yang J, Li Y, Yu M, Liu S, Han Y, Lu X, Jin C, Wu S, Cai Y. Lanthanum chloride induces autophagy in rat hippocampus through ROS-mediated JNK and AKT/mTOR signaling pathways. Metallomics. 2019 Feb 20;11(2):439–53. doi:10.1039/c8mt00295a. Cited in: PMID: 30617363.
32. Yi LT, Dong SQ, Wang SS, Chen M, Li CF, Geng D, Zhu JX, Liu Q, Cheng J. Curcumin attenuates cognitive impairment by enhancing autophagy in chemotherapy. Neurobiol Dis. 2020 Mar;136:104715. doi:10.1016/j.nbd.2019.104715. Cited in: PMID: 31843707.