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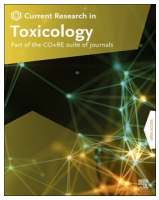

Research Paper

# L-3-n-butylphthalide alleviates intermittent alcohol exposure-induced hypothalamic cell apoptosis via inhibiting the IRE1α-ASK1-JNK pathway in adolescent rats

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ABSTRACT

Exposure to alcohol can induce different degrees of damage to various tissues and organs, and brain is the most vulnerable part affected by alcohol. However, there is no detailed report on whether intermittent alcohol exposure can result in pathological changes in the hypothalamus of adolescent rats and the detailed mechanism. This study investigated pathological changes in the hypothalamus, probed the levels of inflammatory factors, and detected the expression of proteins related to endoplasmic reticulum stress (ERS) to determine whether ERS is involved in the injury process of the hypothalamus and the protective mechanism of L-3-n-butylphthalide (L-NBP). The results showed that intermittent alcohol exposure induced hypothalamic nerve injury, including cell apoptosis, increased the levels of inflammatory factors, and upregulated the expression of glucose-regulated protein 78 (GRP78), p-Inositol Requiring Enzyme 1α (p-IRE1α), apoptosis signal-regulating kinase 1 (ASK1), and p-c-Jun N-terminal kinase (p-JNK)). Tauroursodeoxycholic acid (TUDCA), an ERS inhibitor, significantly reduced the pathological damage described above. The increases in the levels of inflammatory factors, pathological injury, and increased levels of proteins associated with the IRE1α-ASK1-JNK pathway were alleviated by L-NBP. The present study indicated that intermittent alcohol exposure could lead to hypothalamic cell apoptosis in adolescent rats and L-NBP could alleviate the above injury by inhibiting the IRE1α-ASK1-JNK pathway.

Abbreviations: Ang-2, Angiopoietin-2; ASK1, Apoptosis signal-regulating kinase 1; ER, Endoplasmic reticulum; ERS, Endoplasmic reticulum stress; ELISA, Enzyme-linked immunosorbent assay; GFAP, Glial fibrillary acidic protein; GRP78, Glucose-regulated protein 78; IBA1, Ionized calcium binding adapter molecule 1; i.p., Intraperitoneal; IRE1α, Inositol Requiring Enzyme 1α; JNK, c-Jun N-terminal kinase; L-NBP, L-3-n-butylphthalide; PND, Postnatal day; PVDF, Polyvinylidene difluoride; SDS-PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TRAF2, TNF-receptor associated factor 2; TUDCA, Tauroursodeoxycholic acid; VEGF, Vascular endothelial growth factor.

## Introduction

Alcohol consumption has emerged as a significant global issue with both medical and social implications (Nasui et al., 2021). The Global status report on alcohol and health 2018 released by the World Health Organization noted that in 2016, the number of current drinkers was approximately 2.4 billion and alcohol consumption resulted in 2.8 million deaths, accounting for 5.3 % of total deaths (World Health Organization, 2018a). Researchers have linked alcohol consumption to 60 diseases (Rehm et al., 2010). A study involving 28 million individuals indicated that the safest level of drinking was none (GBD 2016 Alcohol Collaborators, 2018). Alcohol is easily available for adolescents because of imperfect control measures (Peeters et al., 2016). 27 % of teenagers (aged 15–19) around the world are believed to have drunk alcohol in the past month, while the percentage is as high as 44 % in developed countries in Europe and America (Lees et al., 2020). The typical age of teenagers in the U.S. and Australia who drink alcohol is 15 (Aiken et al., 2018; Richmond-Rakerd et al., 2017). In Europe, the majority of teenagers begin consuming alcohol between the ages of 12 and 16 and 25 % of teenagers have their first drinking experience at age 13 (World Health

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Organization, 2018b). Alcohol consumption during adolescence generally occurs with a pattern of intermittent heavy drinking, which is more easily associated with dangerous consequences than in adults, including risky sexual behavior, interpersonal violence, and higher rates of auto accidents (Cservenka and Brumback, 2017; Hingson and Zha, 2018). As a crucial stage of physical development and maturity, adolescence is vulnerable to alcohol consumption-related problems (Aiken et al., 2018; Crews et al., 2007). However, most alcohol-related studies have looked at adults, and there has not been enough research on adolescent drinking problem.

Recent research has demonstrated that exposure to alcohol can induce different levels of damage to various tissues and organs (Patel et al., 2015), and the brain is the most vulnerable area affected by alcohol (Mira et al., 2020). Alcohol can easily cross the blood–brain barrier by binding to lipid components in cell membranes and then combining with lecithin in the brain to form conjugates, which can lead to a slowdown in alcohol metabolism (Vore and Deak, 2022; Siqueira and Stipursky, 2022). Moreover, alcohol can have serious neurotoxic effects on the brain by inducing the release of inflammatory and tumor necrosis factors from glial cells (Ehrlich et al., 2012). Substantial evidence indicates that alcohol can disrupt sleep rhythms and sleep disruption may contribute to the onset of alcohol use disorder, which may be associated with the homeostasis imbalance of the hypothalamic orexin system (Amodeo et al., 2020), however, the injury mechanism remains to be further studied. In view of the hypothalamus as an important center for regulating a variety of life activities, further research is needed to explore whether intermittent alcohol exposure induces pathological changes in the hypothalamus and the detailed mechanism.

Endoplasmic reticulum (ER) is the principal site of protein synthesis, folding, decoration, and secretion (Ron and Walter, 2007). Under normal physiological circumstances, the ability of the ER to fold proteins is balanced with the protein synthesis capacity of the cell (Yi et al., 2017). Homeostatic imbalance in cells can induce congestion of unfolded and misfolded proteins in the ER, which is termed ER stress (ERS) (Ren et al., 2021). Mild ERS can protect cells by promoting the production of molecular chaperone proteins, folding of unfolded proteins, and degradation of misfolded proteins (Qiao et al., 2018). On the other hand, excessive stress that exceeds the body's adjustment ability can induce injury and dysfunction (Yang et al., 2024). Current researches show that ERS plays a role in the pathological process of multiple diseases (Puri and Morris, 2018; Ren et al., 2021). However, it remains unclear whether ERS participates in the hypothalamic neuronal injury induced by alcohol exposure, and the underlying mechanisms remain to be explored.

L-NBP, a compound extracted from celery seeds, is commonly used to treat cerebral ischemia-related diseases (Zhao et al., 2014; Tu et al., 2020). L-NBP can reduce thrombus and vasoconstriction, increase local blood flow, and improve the circulatory function of cerebral arteries, thereby reducing ischemia/reperfusion injury (Qin et al., 2019). Current research has shown that L-NBP has multiple pharmacological effects, including improved microcirculation, protection of mitochondria, antioxidant activity, and inhibition of neuronal apoptosis (Chen et al., 2019). It is worth noting that L-NBP can relieve the extent of nerve injury induced by alcohol exposure (Diao et al., 2013), while further research is needed to fully understand the mechanism.

In this study, we successfully created a rat model of intermittent alcohol exposure that mimics the drinking habits of adolescents, probed the levels of inflammatory factors, investigated the pathological changes in the hypothalamus, and detected the expression of ERS-related proteins. The purpose was to determine whether ERS participates in the hypothalamic nerve injury process induced by intermittent alcohol exposure and the protective mechanism of L-NBP.

## Materials and methods

### *Animals*

Male *Sprague-Dawley rat* pups (Xinxiang Medical University, China) were placed in a room at a constant temperature of 22 °C with a light/dark cycle of 12/12 h. The week before the experiment was an adjustment period for the animals. The Animal Experimental Institution of Xinxiang Medical University approved all aspects of this study (Approval number: AE-2022-09/03). This study included the following groups: control group (control, n = 18), alcohol exposure group (alcohol, n = 18), alcohol + tauroursodeoxycholic acid group (alcohol + TUDCA, n = 13), alcohol + L-NBP group (alcohol + NBP, n = 18), TUDCA group (TUDCA, n = 13), and NBP group (NBP, n = 18).

### *Protocol of intermittent alcohol exposure*

A rat model of intermittent alcohol exposure was established as described previously (Gómez et al., 2018). Alcohol solution (3.0 g/kg, 25 % w/v mixed in isotonic saline) or isotonic saline was administered via gavage beginning on PND25. L-NBP (Sigma, 6066-49-5, New Jersey, USA; 2.5 mg/kg, 25 % w/v mixed in isotonic saline) was administered to the alcohol + NBP and NBP-treated rats 1 h before alcohol treatment via gavage. For the alcohol + TUDCA and TUDCA-treated rats, 1 h before alcohol treatment, TUDCA (Sigma, 35807-85-3, New Jersey, USA; 100 mg/kg, 30 % w/v mixed in isotonic saline) was administered by gavage. The second dose was administered on PND 26, followed by intermittent injections on PND 29, 30, 33, 34, 37, and 38. The injection dose depended on the weight of each rat. Food and water were provided to the rats ad libitum.

### *Tissue preparation*

Tissue preparation was performed as previously described (Yi et al., 2017). On PND39, all rats were anesthetized via an intraperitoneal injection of 1 % sodium pentobarbital (40 mg/kg) and sacrificed. The brains used for staining were immediately harvested and fixed in 10 % formalin for 48 h. The fixed brains were subsequently dehydrated in a graded ethanol series and embedded in paraffin. Coronal sections were obtained using a stereotaxic atlas (Paxinos and Watson, 2007) and rotary microtome (Leica RM2255, Shanghai, China). Consecutive 5-μm-thick coronal sections with the largest hypothalamus area that corresponded to −3.0 mm from the bregma were collected. The sections were prepared for thionine staining, hematoxylin and eosin (HE) staining, and immunohistochemical staining, and then examined under a light microscope (Olympus IX73; Olympus, Tokyo, Japan).

### *HE staining*

HE staining was performed as previously described (Liu et al., 2021). The sections were stained with hematoxylin for 1 min, transferred to 1 % hydrochloric acid alcohol differentiation solution for 5 s, stained with eosin for 3 s, and mounted with neutral gum.

### *Flow cytometry*

The rats were anesthetized by an intraperitoneal injection of pentobarbital sodium and sacrificed, after which the hypothalamus was removed. The samples were cut into pieces, and the tissue pieces were gently dissociated into single-cell suspensions with a Gentle MACS™ Dissociator (Miltenyi, NRW, Germany). The single-cell suspension was filtered through a cell strainer (with 40 μm pores) and then centrifuged for 5 min at 300 g. After discarding the supernatant, the cells were resuspended in 100 μL of staining buffer and then incubated with FITC-Annexin V and PI (BD Biosciences, CA, USA) at room temperature in the dark for 15 min. The final results were immediately analyzed by

acquiring 10,000 cells with a flow cytometer (Beckman, CA, USA).

*Thionine staining*

Thionine staining was performed as previously described (Yi et al., 2019). Deparaffinized sections were stained with 4 % thionine solution for 90 s at 60 °C, dehydrated with a graded series of alcohol, and mounted with neutral gum.

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA was carried out as previously mentioned (Yi et al., 2017). IL-1β, IL-2, IL-6, and TNF-α levels in the serum and hypothalamus were detected by ELISA Complete Kits (USCN Life Science Inc., Wuhan, China). Serum was then extracted after blood was drawn from the abdominal aorta of rats under anesthesia. Brain tissue was homogenized, and the supernatant was obtained. Subsequently, each well received the standard solution, serum, or supernatant before being incubated at 37 °C for 60 min. Horseradish peroxidase (HRP)-labeled anti-rat IL-1β, IL-2, IL-6, and TNF-α and melatonin secondary antibodies were added and incubated at 37 °C for 1.5 h. Tetramethylbenzidine (TMB) color development solution was used to create color responses after the liquid had been removed from the wells and after three rounds of washing. Sulfuric acid (2 mmol/10 ml) was added after 15 min at room temperature in the dark. ELISA reader was used to read the plates at 450 nm (Multiskan MS photometer type 352, Labsystems, Helsinki, Finland).

*Immunohistochemical staining*

Immunohistochemical staining was performed as previously described (Liu et al., 2021). After deparaffinization and antigen extraction using a microwave, the sections were incubated with 6 % $H_2O_2$ in chilled methanol for 15 min. The sections were incubated with goat serum for 30 min at 37 °C. The sections were then incubated with rabbit IBA1-specific monoclonal antibody (1:400, CST, #17198), rabbit GFAP-specific monoclonal antibody (1:100, CST, #80788), rabbit GRP78-specific monoclonal antibody (1:200, Abcam, ab108615), mouse ASK1-specific monoclonal antibody (1:200, Invitrogen, MA5-15861), and rabbit p-JNK-specific monoclonal antibody (1:100, CST, #4668) at 4 °C overnight. The next day, the sections were successively incubated with a biotinylated secondary antibody for 40 min and horseradish peroxidase (HRP)-conjugated biotin for 40 min (ZSGB-Bio, SP-9001, Beijing, China). Finally, 3,3′-diaminobenzidine (ZSGB-Bio, P0203, Beijing, China) was used as a chromogen to demonstrate protein localization. The sections were counterstained with hematoxylin and mounted with neutral gum. Image-Pro Plus 5.1 (Media Cybernetics, Houston, TX, USA) was used to detect the average fluorescence intensity (IOD), which was used to determine the expression levels of these proteins.

*Western blot analysis*

Three rats were randomly selected from each group for the western blot analysis. Protein extracts of the hypothalamus (30 μg of protein/lane) were loaded onto a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, separated by electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with rabbit IRE1α-specific monoclonal antibody (1: 1000, CST, #3294), rabbit p-IRE1α-specific monoclonal antibody (1: 1000, Invitrogen, MA5-35893), rabbit GRP78-specific monoclonal antibody (1: 1000, Abcam, ab108615), rabbit JNK-specific monoclonal antibody (1: 1000, Abcam, ab179461), rabbit p-JNK-specific monoclonal antibody (1: 1000, CST, #4668), mouse ASK1-specific monoclonal antibody (1: 1000, Invitrogen, MA5-15861), and mouse β-actin monoclonal antibody (1: 2000, Beyotime, AF0003) overnight at 4 °C. The next day, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (1:1000, Beyotime, A0208/ A0216). Finally, the membranes were visualized using an Odyssey gel imaging system (LI-COR, Inc., Lincoln, NE, USA).

*Statistical analysis*

The results are presented as the mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) followed by a post hoc least significant difference (LSD) *t*-test to determine specific group differences, and the effects of TUDCA and L-NBP were analyzed using two-way ANOVA. All statistical analyses were performed using SPSS 21.0. Significance was defined as $P < 0.05$ for all statistical tests.

**Results**

*HE staining showed the effect of TUDCA on pathological changes in the hypothalamus*

The hypothalamus of normal rats showed a complete and clear organizational structure, and the cell arrangement and shape were normal. Compared with the normal rats, the hypothalamus showed no difference in the TUDCA group, while tissue edema, cell pyknosis, and significant proliferation of microglia could be found in alcohol and alcohol + TUDCA groups. The injury degree of hypothalamus in alcohol + TUDCA group was significantly reduced than that in alcohol group (Fig. 1A).

*Flow cytometry showed the effect of TUDCA on cell apoptosis rate in the hypothalamus*

Compared with that of normal rats (3.99 ± 0.45 %), the apoptosis rate in the hypothalamus stayed at a low level in the TUDCA group (3.93 ± 0.42 %, $P > 0.05$) and was significantly increased in the alcohol group (11.06 ± 0.89 %, $P < 0.01$) and the alcohol + TUDCA group (7.54 ± 0.51 %, $P < 0.01$); compared with that in the alcohol group, the apoptosis rate was significantly decreased in the stress + TUDCA group ($P < 0.01$) (Fig. 1B).

*Western blot showed the effect of TUDCA on protein expression level of GRP78 in the hypothalamus*

Compared with that in the control group (0.59 ± 0.06), the expression of GRP78 remained at a low level in the TUDCA group (0.60 ± 0.13, $P > 0.05$) and was significantly upregulated in the alcohol group (1.64 ± 0.11, $P < 0.01$) and alcohol + TUDCA group (1.15 ± 0.11, $P < 0.05$). Compared with that in the alcohol group, the expression of GRP78 significantly decreased in the alcohol + TUDCA group ($P < 0.05$) (Fig. 1C).

*Thionine staining showed the effect of NBP on pathological changes in the hypothalamus*

In the control and NBP groups, the Nissl bodies were normal in shape and evenly distributed in the cytoplasm. Compared with those in the control group, disappearance of Nissl bodies and pyknosis of neurons were observed in the alcohol and alcohol + NBP groups. Compared with those in the alcohol group, pathological changes were significantly reduced in the alcohol + NBP group (Fig. 2A).

*Flow cytometry showed the effect of NBP on cell apoptosis rate in the hypothalamus*

Compared with that in the control group (3.43 ± 0.44 %), the apoptosis rate in the hypothalamus remained low in the NBP group (3.31 ± 0.33 %, $P > 0.05$) and was significantly increased in the alcohol group (9.95 ± 0.77 %, $P < 0.01$) and the alcohol + NBP group (5.41 ± 0.55 %, $P < 0.05$); compared with that in the alcohol group, the

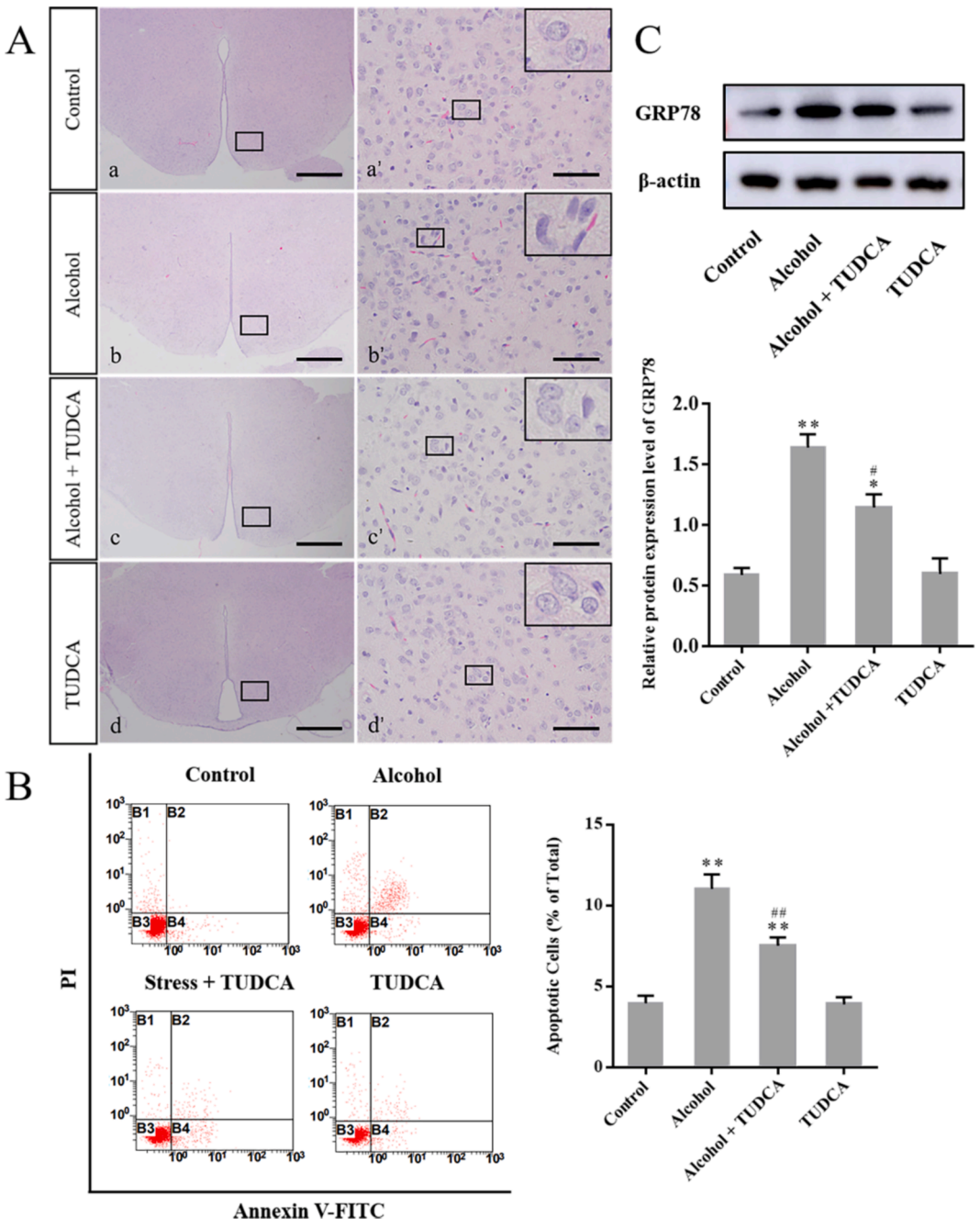


**Fig. 1.** (A) HE staining showed pathological changes in the hypothalamus. a′–d′ are magnified frames of a–d, respectively. Representative pathological changes are shown in the top right corners of a′–d′ (n = 5). (B) Flow cytometry showed the cell apoptosis rate in the hypothalamus (n = 5). (C) Western blot showed the expression level of GRP78 in the hypothalamus (n = 3). Bars = 500 μm in a–d; bars = 50 μm in a′–d′. The results are shown as the mean ± SEM, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. alcohol group. TUDCA: Tauroursodeoxycholic acid (color should be used).

apoptosis rate was significantly decreased in the stress + NBP group ($P < 0.01$) (Fig. 2B).

*Western blot showed the effect of NBP on protein expression levels of p-IRE1α, ASK1, and p-JNK in the hypothalamus*

Compared with that in the control group (0.44 ± 0.06), the expression of p-IRE1α remained at a low level in the NBP group (0.44 ± 0.04, $P > 0.05$) and was significantly upregulated in the alcohol group (0.82 ± 0.03, $P < 0.01$) and alcohol + NBP group (0.64 ± 0.04, $P < 0.05$). Compared with that in the alcohol group, the expression of p-IRE1α significantly decreased in the alcohol + NBP group ($P < 0.05$) (Fig. 2C and D).

Compared with that in the control group (0.31 ± 0.03), the expression of ASK1 remained at a low level in the NBP group (0.29 ± 0.02, $P > 0.05$) and was significantly upregulated in the alcohol group (1.16 ± 0.09, $P < 0.01$) and alcohol + NBP group (0.75 ± 0.06, $P < 0.01$). Compared with that in the alcohol group, the expression of ASK1 significantly decreased in the alcohol + NBP group ($P < 0.05$) (Fig. 2C and E).

Compared with that in the control group (0.36 ± 0.02), the expression of p-JNK remained at a low level in the NBP group (0.43 ± 0.03, $P > 0.05$) and was significantly upregulated in the alcohol group (0.84 ± 0.02, $P < 0.01$) and alcohol + NBP group (0.65 ± 0.04, $P < 0.01$).

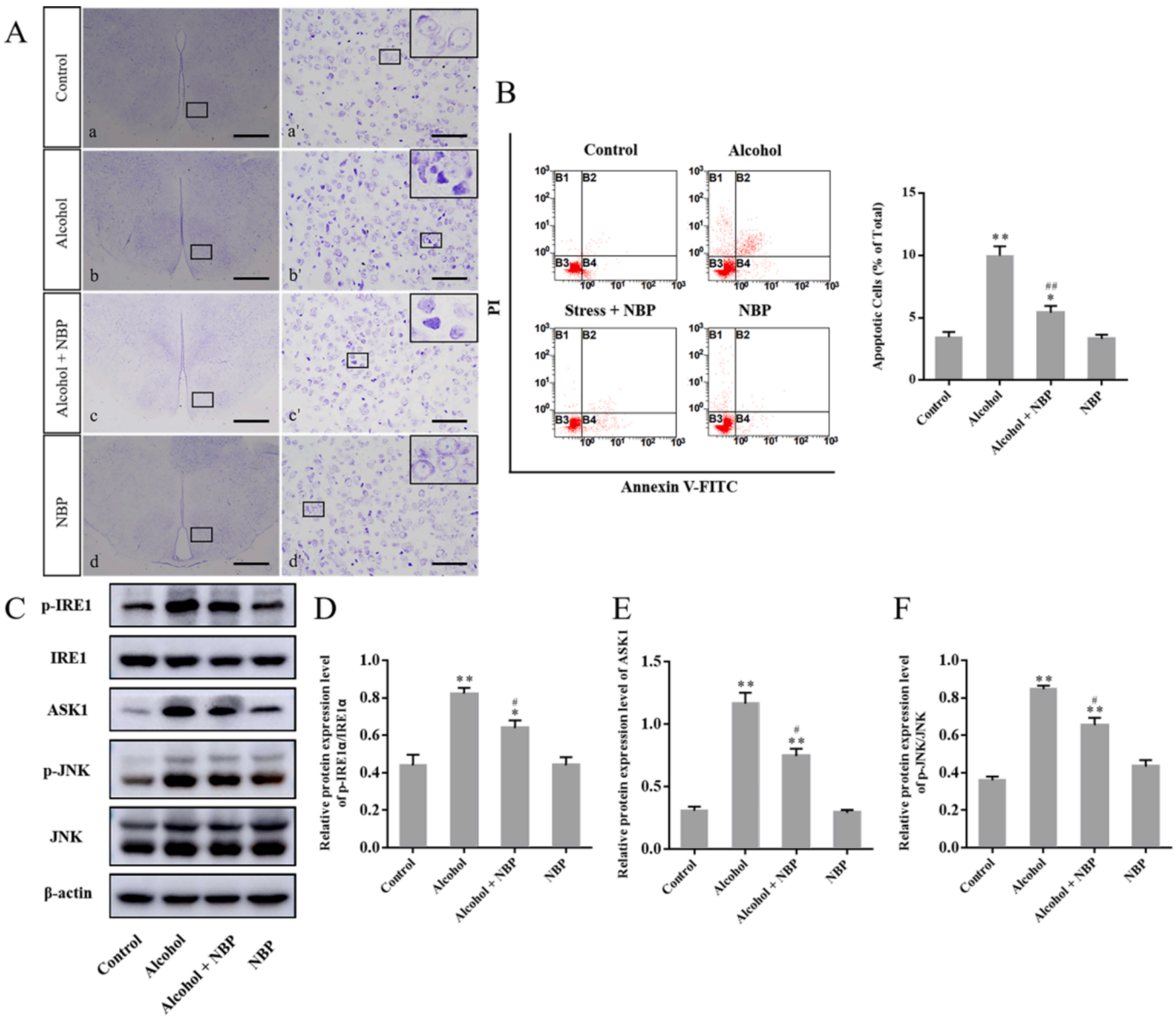


**Fig. 2.** (A) Thionine staining showed pathological changes in the hypothalamus. a′–d′ are magnified frames of a–d, respectively. Representative pathological changes are shown in the top right corners of a′–d′ (n = 5). (B) Flow cytometry showed the cell apoptosis rate in the hypothalamus (n = 5). (C) Western blot showed the expression levels of IRE1α-ASK1-JNK pathway-related proteins in the hypothalamus (n = 3). Bars = 500 μm in a–d; bars = 50 μm in a′–d′. The results are shown as the mean ± SEM, $^{**}$ $P < 0.01$, $^{*}$ $P < 0.05$ vs. control group; $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ vs. alcohol group. NBP: L-3-n-butylphthalide (color should be used).

Compared with that in the alcohol group, the expression of p-JNK significantly decreased in the alcohol + NBP group ($P < 0.05$) (Fig. 2C and F).

*Immunohistochemistry showed the effect of NBP on protein expression levels of p-IRE1α, ASK1, p-JNK, IBA1, and GFAP in the hypothalamus*

Compared with that in the control group (0.18 ± 0.03), the average IOD of p-IRE1α remained at a low level in the NBP group (0.18 ± 0.03, $P > 0.05$) and was significantly upregulated in the alcohol group (0.38 ± 0.02, $P < 0.01$) and alcohol + NBP group (0.30 ± 0.01, $P < 0.05$). Compared with that in the alcohol group, the average IOD of p-IRE1α significantly decreased in the alcohol + NBP group ($P < 0.05$) (Fig. 3).

Compared with that in the control group (0.57 ± 0.04), the average IOD of ASK1 remained at a low level in the NBP group (0.56 ± 0.04, $P > 0.05$) and was significantly upregulated in the alcohol group (1.09 ± 0.07, $P < 0.01$) and alcohol + NBP group (0.82 ± 0.05, $P < 0.05$). Compared with that in the alcohol group, the average IOD of ASK1 significantly decreased in the alcohol + NBP group ($P < 0.05$) (Fig. 4A and B).

Compared with that in the control group (0.35 ± 0.03), the average IOD of p-JNK remained at a low level in the NBP group (0.32 ± 0.04, $P > 0.05$) and was significantly upregulated in the alcohol group (0.87 ± 0.02, $P < 0.01$) and alcohol + NBP group (0.67 ± 0.05, $P < 0.01$). Compared with that in the alcohol group, the average IOD of p-JNK significantly decreased in the alcohol + NBP group ($P < 0.05$) (Fig. 4C and D).

Compared with that in the control group (0.38 ± 0.03), the average IOD of IBA1 remained at a low level in the NBP group (0.34 ± 0.04, $P > 0.05$) and was significantly upregulated in the alcohol group (0.84 ± 0.03, $P < 0.01$) and alcohol + NBP group (0.61 ± 0.05, $P < 0.05$). Compared with that in the alcohol group, the average IOD of IBA1 significantly decreased in the alcohol + NBP group ($P < 0.05$) (Fig. 5A and B).

Compared with that in the control group (0.55 ± 0.04), the average IOD of GFAP remained at a high level in the NBP group (0.58 ± 0.05, $P$

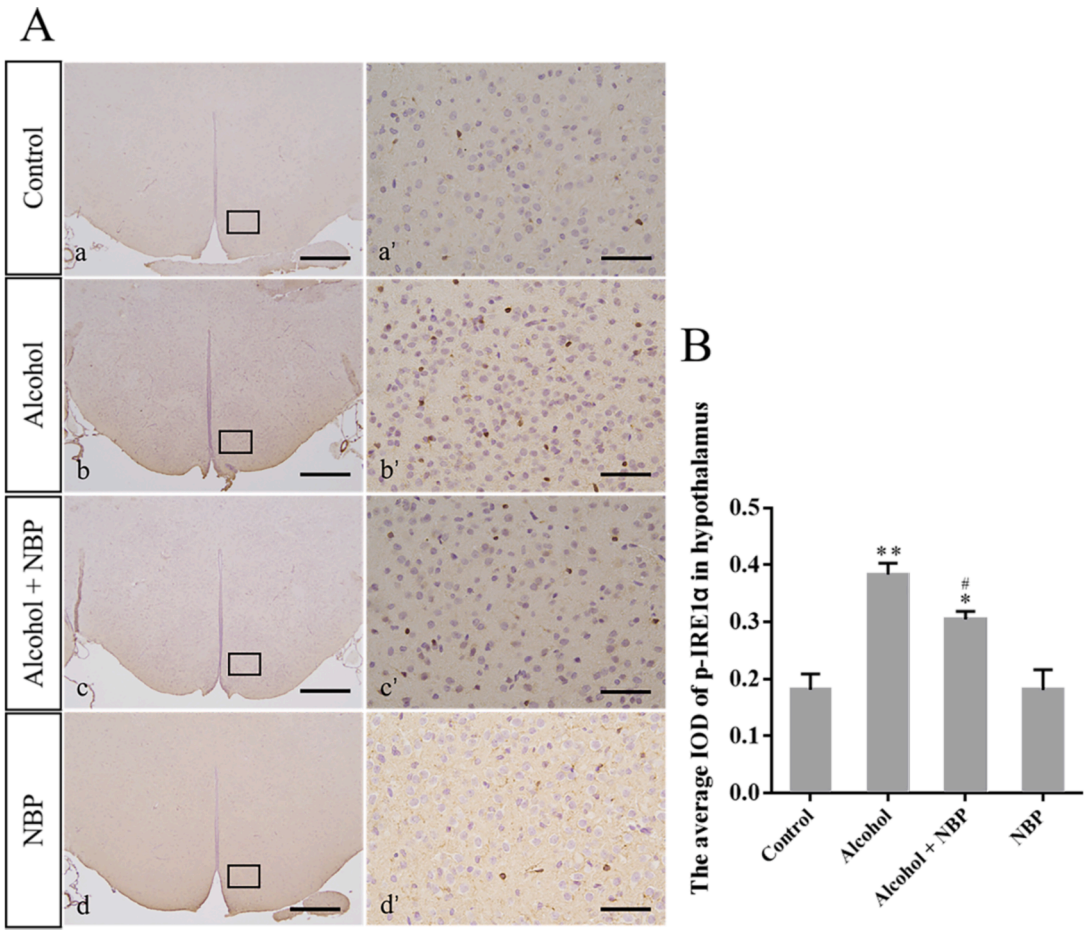


**Fig. 3.** Immunohistochemistry showed the expression level of p-IRE1α in the hypothalamus (n = 5). a′–d′ are magnified frames of a–d, respectively. Bars = 500 μm in a–d; bars = 50 μm in a′–d′. The results are shown as the mean ± SEM, ** $P < 0.01$, * $P < 0.05$ vs. control group; # $P < 0.05$ vs. alcohol group. NBP: L-3-n-butylphthalide (color should be used).

> 0.05) and was significantly downregulated in the alcohol group (0.22 ± 0.03, $P < 0.01$) and alcohol + NBP group (0.38 ± 0.03, $P < 0.05$). Compared with that in the alcohol group, the average IOD of GFAP significantly increased in the alcohol + NBP group ($P < 0.05$) (Fig. 5C and D).

*ELISA showed the effect of NBP on the levels of IL-1β, IL-2, IL-6, and TNF-α in the serum and hypothalamus*

In contrast to the control group (13.19 ± 1.17), the level of IL-1β in the serum remained at a low level in the NBP group (12.07 ± 2.02, $P >$ 0.05) and the alcohol + NBP group (20.07 ± 3.10, $P > 0.05$), and was markedly elevated in the alcohol group (31.00 ± 2.93, $P < 0.01$); in contrast to the alcohol group, the level of IL-1β was markedly reduced in the alcohol + NBP group ($P < 0.05$) (Fig. 6A).

In contrast to the control group (78.60 ± 11.09), the level of IL-2 in the serum remained at a low level in the NBP group (81.36 ± 7.08, $P >$ 0.05) and was markedly elevated in the alcohol group (361.2 ± 26.33, $P$ < 0.01) and the alcohol + NBP group (151.9 ± 19.76, $P < 0.05$); in contrast to the alcohol group, the level of IL-2 was markedly reduced in the alcohol + NBP group ($P < 0.01$) (Fig. 6B).

In contrast to the control group (84.70 ± 7.26), the level of IL-6 in the serum remained at a low level in the NBP group (80.38 ± 6.68, $P >$ 0.05) and was markedly elevated in the alcohol group (181.0 ± 20.47, $P$ < 0.01) and the alcohol + NBP group (120.3 ± 7.73, $P < 0.01$); in contrast to the alcohol group, the level of IL-6 was markedly reduced in the alcohol + NBP group ($P < 0.05$) (Fig. 6C).

In contrast to the control group (131.3 ± 14.81), the level of TNF-α in the serum remained at a low level in the NBP group (123.4 ± 14.88, $P$ > 0.05) and was markedly elevated in the alcohol group (441.1 ± 52.01, $P < 0.01$) and the alcohol + NBP group (205.2 ± 26.15, $P < 0.05$); in contrast to the alcohol group, the level of TNF-α was markedly reduced in the alcohol + NBP group ($P < 0.01$) (Fig. 6D).

In contrast to the control group (16.53 ± 2.43), the level of IL-1β in the hypothalamus remained at a low level in the NBP group (16.55 ± 2.91, $P > 0.05$) and was markedly elevated in the alcohol group (86.36 ± 9.95, $P < 0.01$) and the alcohol + NBP group (52.14 ± 4.17, $P < 0.01$); in contrast to the alcohol group, the level of IL-1β was markedly reduced in the alcohol + NBP group ($P < 0.05$) (Fig. 6E).

In contrast to the control group (47.33 ± 6.00), the level of IL-2 in the hypothalamus remained at a low level in the NBP group (49.85 ± 5.81, $P > 0.05$) and was markedly elevated in the alcohol group (182.6 ± 14.24, $P < 0.01$) and the alcohol + NBP group (89.70 ± 8.49, $P <$ 0.01); in contrast to the alcohol group, the level of IL-2 was markedly reduced in the alcohol + NBP group ($P < 0.01$) (Fig. 6F).

In contrast to the control group (50.05 ± 4.48), the level of IL-6 in the hypothalamus remained at a low level in the NBP group (45.16 ± 5.95, $P > 0.05$) and was markedly elevated in the alcohol group (149.0 ± 10.59, $P < 0.01$) and the alcohol + NBP group (85.63 ± 6.84, $P <$ 0.01); in contrast to the alcohol group, the level of IL-6 was markedly reduced in the alcohol + NBP group ($P < 0.01$) (Fig. 6G).

In contrast to the control group (96.29 ± 5.82), the level of TNF-α in the hypothalamus remained at a low level in the NBP group (98.51 ± 9.01, $P > 0.05$) and was markedly elevated in the alcohol group (283.7 ± 14.61, $P < 0.01$) and the alcohol + NBP group (189.9 ± 10.18, $P <$ 0.01); in contrast to the alcohol group, the level of TNF-α was markedly

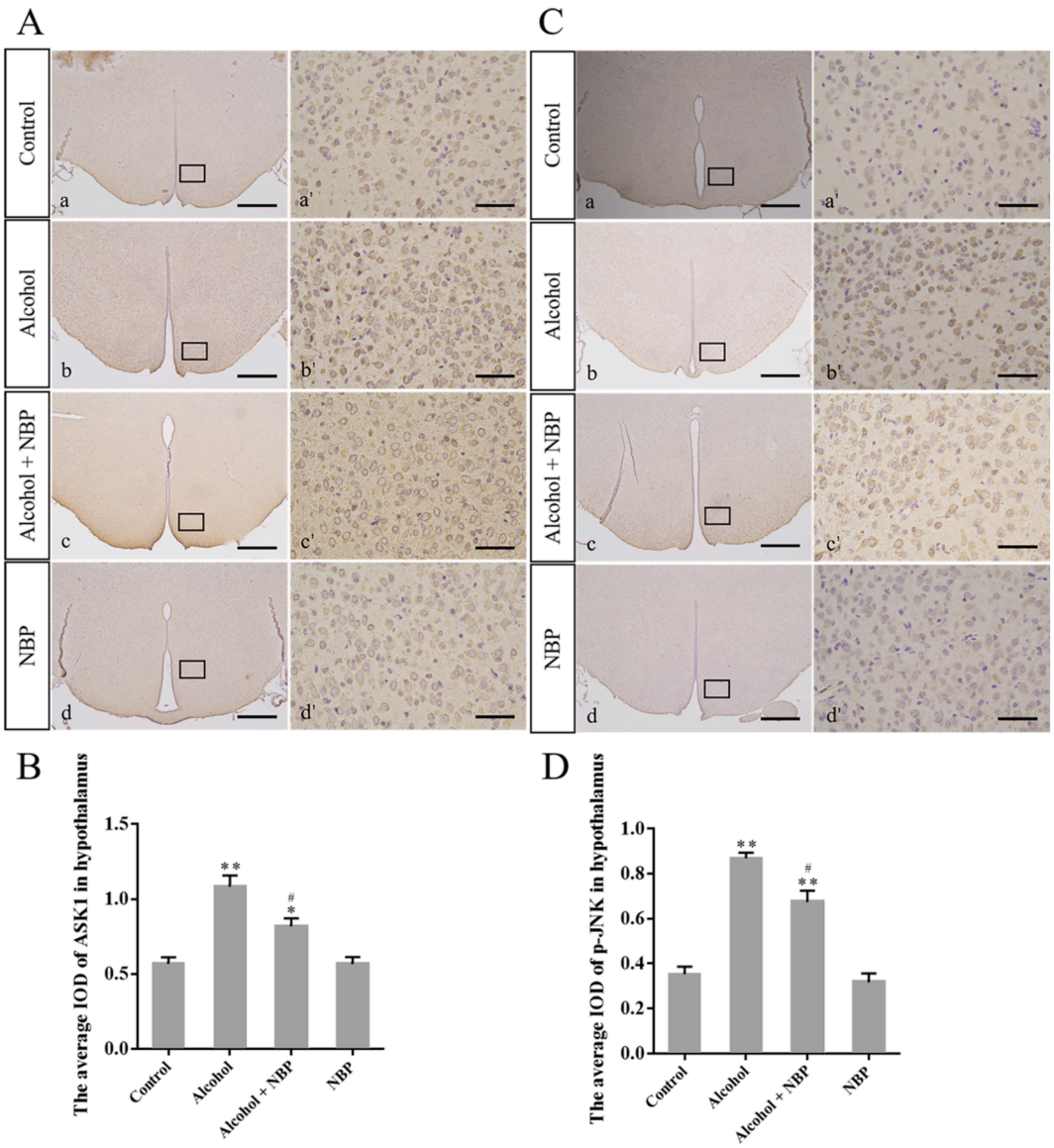


**Fig. 4.** (A and B) Immunohistochemistry showed the expression level of ASK1 in the hypothalamus (n = 5). (C and D) Immunohistochemistry showed the expression level of p-JNK in the hypothalamus (n = 5). a′–d′ are magnified frames of a–d, respectively. Bars = 500 μm in a–d; bars = 50 μm in a′–d′. The results are shown as the mean ± SEM, $^{**}$ $P < 0.01$, $^{*}$ $P < 0.05$ vs. control group; $^{\#}$ $P < 0.05$ vs. alcohol group. NBP: L-3-n-butylphthalide (color should be used).

reduced in the alcohol + NBP group ($P < 0.01$) (Fig. 6H).

## Discussion

The main objective of this study was to verify whether ERS plays a role in the hypothalamic nerve damage caused by intermittent alcohol exposure and explore the protective effect of L-NBP. In this study, we found that intermittent alcohol exposure induced hypothalamic nerve injury, including cell apoptosis, increased the levels of inflammatory factors (IL-1β, IL-2, IL-6, and TNF-α), and increased the expression of ERS-related proteins (GRP78, p-IRE1α, ASK1, and p-JNK), which indicated that intermittent alcohol exposure could trigger the activation of the IRE1α-ASK1-JNK pathway. TUDCA treatment significantly reduced pathological damage and restrained the increase in GRP78 expression. The increases in the levels of inflammatory factors, pathological injury, and the increases in the expression of IRE1α-ASK1-JNK pathway-related proteins were alleviated by L-NBP. These results showed that ERS was associated with the process of hypothalamic nerve injury induced by intermittent alcohol exposure, and L-NBP attenuated the above-mentioned injury and reduced the expression levels of proteins related to IRE1α-related pathway. Therefore, our study contributes to the understanding of hypothalamic nerve injury caused by intermittent alcohol exposure and the protective effect of L-NBP.

Alcohol can cause widespread damage to the central nervous system, resulting in motor incoordination, difficulty concentrating, memory impairment, and cognitive dysfunction (Loeber et al., 2009). In view of the hypothalamus as an important center for regulating a variety of life activities, there is no detailed report on whether intermittent alcohol exposure results in pathological change in the hypothalamus. Therefore, in this study, we focused on ascertaining pathological changes in the hypothalamus under intermittent alcohol exposure. Our results showed that alcohol exposure induced edema in neurons, scattered pyknotic neurons, and cell apoptosis. These findings demonstrate that intermittent alcohol exposure can severely damage the hypothalamus. Pathological changes in tissues and organs are accompanied by dysfunction. These results suggest that alcohol exposure can cause body dysfunction by damaging the hypothalamus.

As innate immune effector cells, microglia are quiescent in the

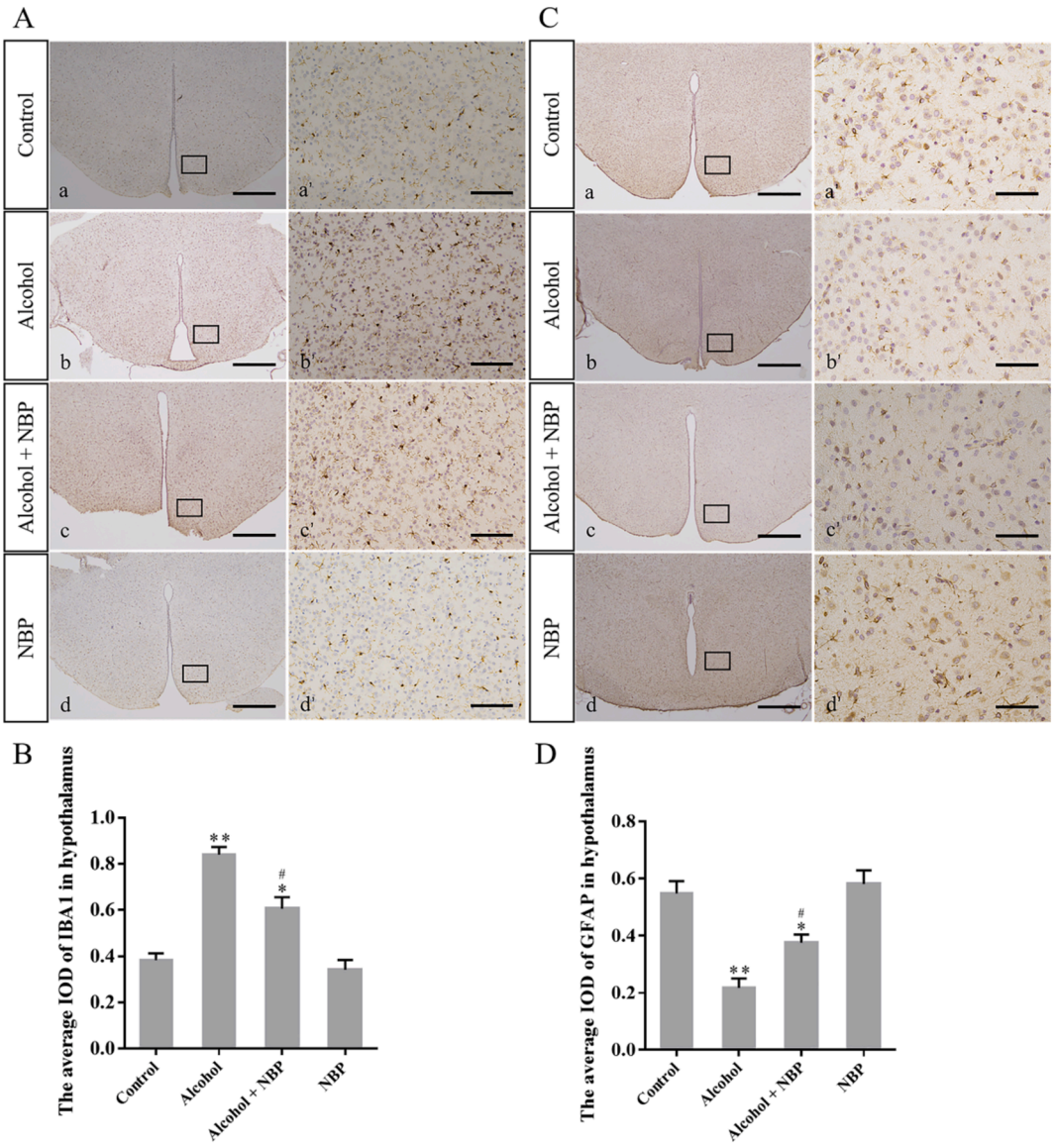


**Fig. 5.** (A and B) Immunohistochemistry showed the expression level of IBA1 in the hypothalamus (n = 5). (C and D) Immunohistochemistry showed the expression level of GFAP in the hypothalamus (n = 5). a′–d′ are magnified frames of a–d, respectively. Bars = 500 μm in a–d; bars = 50 μm in a′–d′. The results are shown as the mean ± SEM, $^{**}$ $P < 0.01$, $^{*}$ $P < 0.05$ vs. control group; $^{\#}$ $P < 0.05$ vs. alcohol group. NBP: L-3-n-butylphthalide (color should be used).

normal brain (Parajuli and Koizumi, 2023). Microglia move by branching to seek and devour neurotoxic proteins and damaged neurons (Kettenmann et al., 2011). When the body is damaged, microglia rapidly proliferate and can be activated, which can lead to enlarged cell bodies, retraction of processes, and increased release of inflammatory mediators, resulting in injury to glial cells and neurons (Borst et al., 2021; Prinz et al., 2021). Astrocytes are crucial for preserving nervous system homeostasis (Hasel and Liddelow, 2021). Damaged astrocytes can lead to the abovementioned dysfunctions, and some astrocytes can rapidly transform into reactive astrocytes, which can further exacerbate nerve injury (Ding et al., 2021). Therefore, quantity change of microglia and astrocytes can indicate that the body is in a state of injury. In the present study, immunohistochemical assay was used to identify changes in the quantity of microglia and astrocytes. Ionized calcium binding adapter molecule 1 (IBA1) is a specific marker of microglia, whereas glial fibrillary acidic protein (GFAP) is a specific marker of astrocytes. Thus, IBA1 and GFAP can be used to indicate changes in the quantity change of microglia and astrocytes in the hypothalamus. The expression of IBA1 was significantly upregulated after alcohol exposure. However, GFAP expression was significantly downregulated after exposure to alcohol. These findings demonstrate that intermittent alcohol exposure induce obvious changes in the number of microglia and astrocytes in the hypothalamus, which further verify that intermittent alcohol exposure can damage the hypothalamus.

Drastic changes in inflammatory factors can serve as early warning signs of body damage and play crucial roles in the development of several major diseases (Hu et al., 2022). Binge drinking can induce pathological changes in tissues, especially brain, and trigger an immune inflammatory response (Vore and Deak, 2022; Dukić et al., 2023). In this study, the levels of IL-1β, IL-2, IL-6 and TNF-α in serum and hypothalamus were robustly increased. These results declared that alcohol exposure brought about inflammatory responses in rats, which further suggests that intermittent alcohol exposure can lead to body damage.

When ERS persists, GRP78, as a specific marker for ERS activation, can promote cell apoptosis by activating downstream pathways (Yi et al., 2017; Xu et al., 2023; Sanderson et al., 2015). In the present study, compared with that in the control group, the expression level of GRP78 was significantly increased in the alcohol group; TUDCA, an ERS inhibitor, significantly reduced intermittent alcohol exposure-induced hypothalamic nerve injury, including cell apoptosis, and restrained the

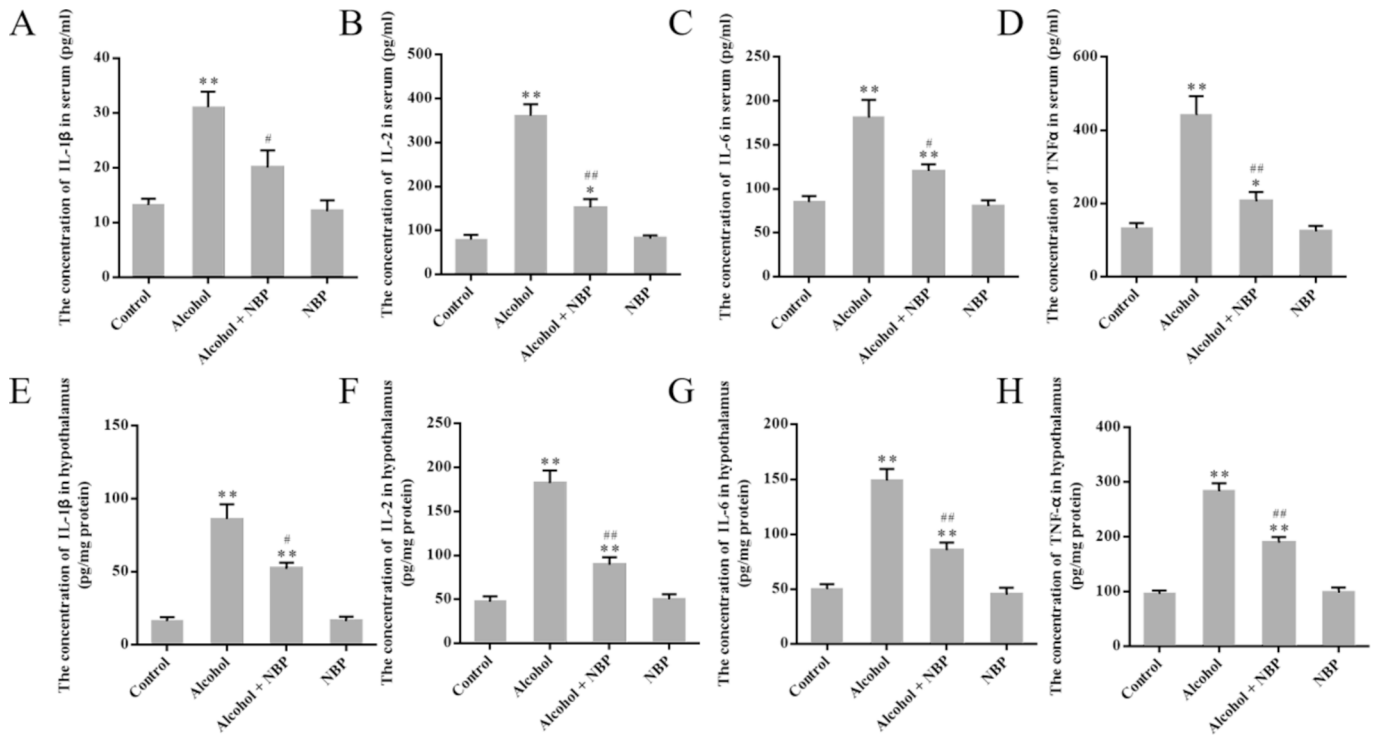


**Fig. 6.** (A) The concentration of IL-1β in serum (n = 5). (B) The concentration of IL-2 in serum (n = 5). (C) The concentration of IL-6 in serum (n = 5). (D) The concentration of TNF-α in serum (n = 5). (E) The concentration of IL-1β in hypothalamus (n = 5). (F) The concentration of IL-2 in hypothalamus (n = 5). (G) The concentration of IL-6 in hypothalamus (n = 5). (H) The concentration of TNF-α in hypothalamus (n = 5). The results are shown as the mean ± SEM, $^{*}$ $P < 0.05$, $^{**}$ $P < 0.01$ vs. the control group. $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.05$ vs. stress group. NBP: L-3-n-butylphthalide.

increase in GRP78 expression. These results suggested that ERS occurred in the hypothalamus of alcohol-exposed rats.

When ERS occurs, the IRE1α signaling pathway can be activated (Yang et al., 2024). Activated IRE1α can bind to TNF-receptor associated factor 2 (TRAF2) and ASK1 and form a protein complex, which can further activate JNK (Sozen et al., 2020). JNK, a key pro-apoptotic factor, can cause cell injury or death by blocking the cell cycle (Nagai et al., 2007). The results of this study indicated that alcohol exposure significantly upregulated the expression levels of p-IRE1α, ASK1 and p-JNK. After treatment with L-NBP, the expression levels of p-IRE1α, ASK1 and p-JNK were significantly decreased, the levels of IL-1β, IL-2, IL-6, and TNF-α and the degree of pathological damage, including cell apoptosis rate, were significantly reduced, suggesting that L-NBP can inhibit the activation of the IRE1α-ASK1-JNK pathway and alleviating the neurological injury caused by intermittent alcohol exposure. The above experimental results show that the IRE1α-ASK1-JNK pathway participates in intermittent alcohol exposure-induced hypothalamic nerve injury in adolescent rats and L-NBP can alleviate this damage by restraining the above pathway.

However, this study has several shortcomings. First, we studied the hypothalamus as a whole, the next step was to explore the key regions and cell populations of the hypothalamus involved in the process of nerve injury induced by alcohol exposure. In addition, the IRE1α-ASK1-JNK pathway may be only one of the pathways involved in the protective effect of L-NBP, and other potential mechanisms that need to be investigated.

## Conclusion

This study shows that intermittent alcohol exposure can lead to hypothalamic cell apoptosis in adolescent rats, and L-NBP can alleviate this injury via blocking the IRE1α-ASK1-JNK pathway of ERS. These new findings offer pathomorphological evidence to explain the mechanism behind hypothalamic nerve injury caused by intermittent alcohol exposure.

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## CRediT authorship contribution statement

**Shanyong Yi:** Conceptualization, Data curation, Investigation, Funding acquisition, Methodology, Writing – original draft. **Lai Wei:** Investigation, Formal analysis. **Bin Zhao:** Investigation, Formal analysis. **Zhijun Yao:** Validation, Visualization. **Bin Yang:** Investigation, Methodology, Writing – review & editing.

## Ethics approval

All attempts were approved by the Animal Experimental Institution of Xinxiang Medical University (Permission No: AE-2022-09/03).

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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