NEURAL REGENERATION RESEARCH
September 2014, Volume 9, Issue 17
www.nrronline.org

Damage of hippocampal neurons in rats with chronic alcoholism

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doi:*10.4103/1673-5374.141787*
http:*//www.nrronline.org/*

Accepted: *2014-05-10*

Abstract

Chronic alcoholism can damage the cytoskeleton and aggravate neurological deficits. However, the effect of chronic alcoholism on hippocampal neurons remains unclear. In this study, a model of chronic alcoholism was established in rats that were fed with 6% alcohol for 42 days. Endogenous hydrogen sulfide content and cystathionine-beta-synthase activity in the hippocampus of rats with chronic alcoholism were significantly increased, while F-actin expression was decreased. Hippocampal neurons in rats with chronic alcoholism appeared to have a fuzzy nuclear membrane, mitochondrial edema, and ruptured mitochondrial crista. These findings suggest that chronic alcoholism can cause learning and memory decline in rats, which may be associated with the hydrogen sulfide/cystathionine-beta-synthase system, mitochondrial damage and reduced expression of F-actin.

Key Words: *nerve regeneration; chronic alcoholism; hydrogen sulfide; cystathionine-beta-synthase; learning; memory; F-actin; mitochondria; hippocampus; NSFC grant; neural regeneration*

Funding: *This work was supported by the National Natural Science Foundation of China, No. 81471351; Key Project of the Science and Technology Foundation of Henan Provincial Education Department of China, No. 12A320013; the Funds of Henan Province Health Technology Talents Innovative Project, No. 3052; and the Open Fund of Henan Provincial Mental Hospital, Henan Key Laboratory of Biological Psychiatry.*

Du AL, Jiang HB, Xu L, An N, Liu H, Li YS, Zhang RL. Damage of hippocampal neurons in rats with chronic alcoholism. Neural Regen Res. 2014;9(17):1610-1615.

Introduction

Chronic alcoholism, commonly known as alcohol dependence or alcohol addiction, is one of the world's most important public health problems. In recent years, a large number of studies have shown that chronic alcoholism can affect whole brain energy metabolism, accelerate Ca^{2+} influx, increase oxygen free radicals, damage the cytoskeleton and increase the degree of neurological deficit (Yao et al., 2004; Hadjigeorgiou et al., 2012). The cytoskeleton is an important structure to maintain normal cell morphology and function. F-actin is an important part of the cytoskeleton. Complete F-actin is necessary for maintaining the normal function of endothelial cells and neurons (Chen et al., 2004; Tatavarty et al., 2012). F-actin is essential for mechanisms regulating microtubule entry into spinous processes and contribute importantly to our understanding of the role of microtubules in synaptic function and plasticity (Merriam et al., 2013). Endogenous hydrogen sulfide (H_2S) is mainly formed from cysteine by cystathionine-beta-synthase (CBS) in the brain and peripheral nervous system. Physiological concentrations of H_2S potentiate the activity of the N-methyl-D-aspartate receptor and enhance the induction of hippocampal long-term potentiation, as described by the synaptic model of learning and memory (Ren et al., 2010). Additionally, neurons in the hippocampus are more sensitive to alcohol, and are important areas of neuronal plasticity closely related to the formation of learning, memory and addiction (Liu et al., 2004; Burke et al., 2010). Therefore, this study investigated the effects of chronic alcoholism on rats and its mechanism of injury by observing learning and memory, the H_2S/CBS system and F-actin content in the hippocampus of a rat model of chronic alcoholism.

Materials and Methods

Establishing a model of chronic alcoholism

Forty clean, healthy, male Sprague-Dawley rats weighing 100–140 g were provided by the Experimental Animal Center of Henan Province, China (license No. SCXK 2010-0001). Rats were in the laboratory for 6–7 days to adapt to the environment (standard diet, free access to food and drinking water, natural light, air exhaustion cycle, at 22°C). Studies conformed to *the Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and the protocol

was approved by Institutional Animal Care Committee of Xinxiang Medical University in China. The rats were randomly divided into control group (n = 20) and model group (n = 20). Rats in the model group drank 6% (v/v) aqueous solution containing alcohol instead of water for 42 days. The alcohol solution was prepared and replaced at 9:00 everyday (Ren et al., 2007). After 42 days, the rat model was verified using the withdrawal score (Gray et al., 2010). Rats in the control group drank pure water.

Withdrawal score verification of the chronic alcoholism model

Withdrawal score (Gray et al., 2010) included assessment of stereotyped behaviors, agitation, tail stiffness and abnormal posture. There are 5 scores in each item. The maximum score is 20. A higher withdrawal score indicates poorer rat behavior.

Evaluation of learning and memory using a Y-maze test

Rats in each group underwent a Y-maze test after the withdrawal score test was completed (Jezek et al., 2002). The Y-maze discriminates learning, spatial reference memory and spatial working memory, which are related to the hippocampus and prefrontal brain regions in rodents (Xu et al., 2013). Inner and outer walls of the Y-maze (Shanghai Ruanlong Technology Development Co., Ltd., Shanghai, China) were made from PVC panels and labeled using black tape. The Y-maze has three arms, with the angle between the arms being 120°. Each arm size is 30 cm × 8 cm × 15 cm (length × width × height). A different geometrical pattern was created on each arm of the maze as a visual marker. The three arms of the Y-maze were randomized to I, II and III, and sawdust paved the way to the Y-maze. After the end of each training or test episode, the sawdust in each arm was changed to prevent interference from the animal's residual odor. A camera lens was placed 1.5 m above the Y-maze and was used during the whole process of recording. Training or testing began at 9:00 daily. The voltage was 40 V. The I arm was the starting area, with the safe area order alternating between I → II → III → I. A light at the top of each arm was lit for 5 minutes, indicating that this arm was the danger zone. Each rat was allowed to stand in the starting area to adapt for 3 minutes, and then electrically shocked, forcing the animal to flee to a safe area. The danger zone was lit for 15 minutes and the next study was started after lights were turned off and rested for 45 minutes.

(1) Learning capability: A correct response was interpreted when rats escaped directly from the starting area and fled to a safe area following stimulation by an electric shock. The number of shocks that were required before 9 out of 10 consecutive reactions were correct (the number of attempts) was recorded as the animal's learning ability. The number of attempts was maximized at 30 attempts. (2) Memory capability: Rats were rested for 24 hours after standard testing. Memory was determined using the same method described above. The number of correct responses in 10 shocks for each rat was recorded as memory performance.

Specimen collection

Ten rats from each group were anesthetized using intraperitoneal injection of 10% chloral hydrate (3 mL/kg), and brains were quickly removed. The hippocampus was isolated with a sharp blade on ice (Ren et al., 2010), frozen in liquid nitrogen, and stored at −80°C until the next day when they were prepared for use in electron microscopy. CBS activity and H_2S content were measured. The remaining rats in each group underwent thoracotomy under general anesthesia, exposing the heart. A small hole was cut at the right atrial appendage. A needle was inserted into the left ventricle to the ascending aorta. Saline (4°C) was injected into the ascending aorta. When the fluid from the right atrial appendage became clear, 250 mL of 4% paraformaldehyde was injected into the ascending aorta, and continued for an hour. Subsequently, the brains were removed. The brain tissue was fixed in 4% paraformaldehyde at 4°C for 6 hours, and embedded in paraffin after dehydration.

Determination of H_2S concentration in the rat hippocampus using the methylene blue method

H_2S level in the hippocampus was measured according to methods described by Geng et al. (2004). Briefly, 0.1 g hippocampal tissue was added to 0.5 mL of 1% zinc acetate, ground and homogenized, and then incubated with 0.5 mL N,N-dimethyl-p-phenylenediamine hydrochloride (20 mmol/L) and 0.5 mL ferric chloride (30 mmol/L) at room temperature for 10 minutes. 10% trichloroacetic acid (0.5 mL) and 2.5 mL distilled water were then mixed with this suspension and centrifuged for 10 minutes at 1,776 × g. The optical density values of the supernatant at 670 nm was read using a spectrophotometer (Shanghai Metash Instruments Co., Shanghai, China). H_2S concentration of each sample was calculated according to a standard curve of sodium hydrosulfide standard solution (7.81–250 μmol/L).

Indirect determination of CBS activity in the hippocampus using spectrophotometry

CBS is one of the main enzymes in the brain assisting in the production of H_2S. Therefore, the production of H_2S could be modulated with a change in CBS activity. CBS activity in the hippocampus was measured according to methods described by Ren et al. (2010). The reaction solution volume was 2 mL, containing 50 mmol/L potassium phosphate buffer (pH 8.0), 10 mmol/L L-cysteine, 2 mmol/L 5′-pyridoxal phosphate and 10% (w/v) hippocampal tissue homogenates. The mixture was evenly added in two 25 mL conical flasks, randomly labeled as A (CBS active group) and B (CBS inhibitory activity group). Distilled water (0.1 mL) was added to conical flask A, while 0.1 mL of CBS activity inhibitor, aminooxy titanium semi-hydrochloride (AOAA; Sigma-Aldrich, St. Louis, MO, USA), was added to conical flask B. Conical flasks were sealed with parafilm after being preserved in nitrogen for 30 seconds, and were then transferred to a 37°C water bath shaker to start the reaction. Ninety minutes later, the sediment was removed. Supernatant (0.1 mL) was harvested and mixed with 0.5 mL of 1% zinc acetate, and

then added to 0.5 mL N,N-dimethyl-p-phenylenediamine hydrochloride (20 mmol/L) and 0.5 mL of ferric chloride (30 mmol/L), and incubated at room temperature for 10 minutes. Following incubation, 0.5 mL of 10% trichloroacetic acid and 2.5 mL of distilled water was added to the mixture, and centrifuged for 10 minutes at 1,776 × *g*. The optical density value of the supernatant was measured using a spectrophotometer at 670 nm. H_2S concentration in the solution was calculated according to the H_2S standard curve. Optical density value of H_2S concentration in conical flask A and B represents the amount of H_2S generated by the CBS catalyst. The amount of H_2S of the unit weight of brain tissue is generated within a unit time, suggesting the amount of CBS activity (nmol/g per hour).

Transmission electron microscopy for the visualization of the ultrastructure morphology in the rat hippocampus

Hippocampal tissue was removed from the refrigerator (−80°C) and was quickly cut into 1-mm^3 tissue blocks. Tissue was fixed in 2.5% glutaraldehyde at 4°C for 2 hours, and stored at 4°C. Subsequently tissue was rinsed three times with phosphate buffer (0.2 mol/L, pH 7.2), fixed in osmium tetroxide for 1–2 hours, dehydrated using acetone (50%, 70%, 80%, 90%, and 100%) for 15 minutes, and embedded in entrapped liquid (Acetone: Epoxy = 1:1) at 37°C for 2 hours, ultimately forming ultra-thin slices. The obtained slices were detected using 3% uranyl acetate-lead citrate double staining. Images were captured using a transmission electron microscope (Hitachi, Tokyo, Japan).

Immunohistochemistry for F-actin expression in the rat hippocampus

Brain tissue was prepared into paraffin sections, which were dewaxed, rehydrated, and antigen retrieved with 0.01 mol/L citrate solution. Sections were then rinsed three times with PBS for 5 minutes. H_2O_2 solution (5%) was used to eliminate endogenous peroxidase at room temperature for 20 minutes. Sections were again rinsed three times with PBS for 5 minutes and blocked with serum at room temperature for 20 minutes. The sections were then incubated with rabbit anti-rat F-actin monoclonal antibody (1:100; Boaosen Company, Beijing, China) at −4°C overnight, washed 3 × 5 minutes with PBS, and incubated with goat anti-rabbit IgG (1:100; Boaosen Company) at 37°C for 60 minutes, and again washed for 5 minutes × 3 with PBS. Sections were then visualized with DAB at 37°C for 5 minutes and washed with distilled water to terminate the color reaction. After mounting using neutral gum, images were captured under a light microscope (Olympus, Tokyo, Japan). Expression of F-actin was analyzed using Image-Pro-plus 6.0 image analysis software (Media Cybernetics, Bethesda, Maryland, USA). The average optical density values were calculated and regarded as the F-actin expression (Ren et al., 2010).

Statistical analysis

All data are expressed as the mean ± SD and were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). The data were analyzed using independent Student's *t*-test for comparison between the two groups. The differences were considered to be statistically significant at $P < 0.05$.

Results

Successful establishment of a model of chronic alcoholism

Behavioral signs of alcohol withdrawal syndrome such as abnormal posture and gait, agitation, wet dog shaking, tail stiffness, tremor, and stereotyped behaviors appeared during the whole observation period of rats in the model group. The alcohol withdrawal score at 6 hours of alcohol withdrawal in the chronic alcoholism model was higher than the control group (13.82 ± 2.27 *vs*. 11.18 ± 1.40; $P < 0.01$).

Learning and memory decreased in rats with chronic alcoholism

The Y-maze test showed that the number of learning attempts of rats in the model group was increased ($P < 0.01$) compared with the control group. The incidence of memory retrieval was decreased in the model group ($P < 0.01$; **Figure 1A**), indicating that chronic alcoholism decreases the learning and memory ability of rats.

Change in H_2S content and CBS activity in the hippocampus of rats with chronic alcoholism

The methylene blue method showed that the H_2S content of hippocampal tissue in the model group was significantly higher than in the control group ($P < 0.01$; **Figure 1B**). Results of spectrophotometry showed that CBS activity in the hippocampus of the model group was significantly higher than that of the control group ($P < 0.01$; **Figure 1C**).

Changes in ultrastructure of hippocampal neurons in rats with chronic alcoholism

Transmission electron microscopy results suggested that the membrane structure of the neuronal nucleus in the rat hippocampus was clear, with no abnormal changes in the control group. The structure of the mitochondrial crista was visible, and the matrix was transparent (**Figure 2A**). Membrane structure of the neuronal nucleus in the hippocampus of model rats was not clear. Mitochondrial edema and some structural damage was observed. Mitochondrial cristae had dissolved and disappeared (**Figure 2B**).

Expression of F-actin increased in hippocampus of rats with chronic alcoholism

Immunohistochemistry showed that F-actin expression in hippocampal tissue of the model group was significantly lower than the control group ($P < 0.01$; **Figure 3**).

Discussion

Chronic alcoholism refers to a state of mind in the desire of drinking wine, which can continuously or periodically appear and experience the psychological effects of drinking, sometimes it indicates the discomfort caused by non-drinking, because the desire for drinking is often very strong. The choice of an appropriate and valuable animal model is an

important means for studying the pathogenesis and treatment of chronic alcoholism. In this study, we used alcohol withdrawal methods as previously described by Li et al. (2006). Rats were allowed to freely drink aqueous solution containing 6% (v/v) alcohol for 42 days, afterwards, rat behavior was assessed using the withdrawal score, to establish a rat model of chronic alcoholism (Li et al., 2011).

Increasing evidence has demonstrated that drinking alcohol can cause a decline in learning and memory ability, and produce behavioral and psychological dysfunction (Brown et al., 2009). Our experimental results showed that chronic alcoholism resulted in a decline in learning and memory capacities in rats. Growing evidence has shown that alcohol dependence increases the levels of ethanol, acetaldehyde and oxygen free radicals, causes cytosolic Ca^{2+} overload and production of high concentrations of H_2S (Carloni et al., 2004; Franco and Huttenlocher, 2005; Li et al., 2009). Ren et al. (2008) found that H_2S, which is higher in alcoholism compared with normal physiological concentrations, can reversibly and specifically inhibit the excitatory postsynaptic membrane, thereby blocking synaptic transmission in the hippocampus causing damage to tissues and cells. Gatto et al. (2003) have demonstrated that brain damage makes F-actin shift from specific sites to the cell surface, leading to the formation of filamentous and membranous pseudopodia. This occurrence ultimately results in abnormal connections between neurons and affects the function of the central nervous system. Therefore, we hypothesized that decreased ability of learning and memory in rats with chronic alcoholism may be related to changes in the H_2S/CBS system and F-actin.

H_2S is the latest discovered gaseous molecule. It is similar to NO and CO, has a small molecular weight and can freely pass through the cell membrane (Yan et al., 2004). After the sulfur-containing amino acid (methionine) is metabolized to cysteine *in vivo*, it can be catalyzed to generate H_2S by CBS and the cystathionine-gamma-cleavage enzyme (Ren et al., 2010). The cystathionine-gamma-cleavage enzyme is mainly distributed in the cardiovascular system, while CBS is mainly distributed in the central nervous system such as the hippocampus, cerebellum, cortex and brain stem. CBS is the major regulator enzyme to catalyze the generation of H_2S. In the brain of CBS knockout mice, there is almost no H_2S (Eto et al., 2002). Growing evidence has shown that endogenous H_2S is the third gaseous signaling molecule extensively involved in the body's physiological and pathological processes, and can reduce hippocampal pathological damage (Liu and Yan, 2007; Shao et al., 2007). H_2S at physiological concentrations can relax blood vessels and increase microcirculation (Zhao et al., 2001), promote the synthesis of the antioxidant glutathione in neurons (Kimura and Kimura, 2004), clear peroxynitrite and hydrogen peroxide, inhibit oxidative damage in the brain (Whiteman et al., 2004), as well as attenuate the inflammatory response (Wei et al., 2006). H_2S below physiological concentrations will lead to a series of pathological changes, and ultimately cause brain damage. H_2S beyond the physiological concentrations may enhance N-methyl-D-aspartate receptor-mediated Ca^{2+} overload, and trigger the swelling of mitochondria, energy metabolism disorder and progressive cellular necrosis (Nagai et al., 2004; Qu et al., 2006).

The results of this study showed that H_2S content and CBS activity in hippocampal tissue of the model rats with chronic alcoholism were significantly higher than that in the control group. Under transmission electron microscope, the nuclear membrane structure of neurons in the chronic alcoholism model was unclear and blurry, accompanied by chromatin condensation, mitochondrial edema, structural damage, mitochondrial cristae rupture, dissolution and disappearance. Therefore, we believe that chronic alcoholism increases the concentration of H_2S and oxidative damage, destroys mitochondrial function, thus leading to a decline in learning and memory capacity in rats.

Studies have found that mitochondrial damage and degeneration may result in energy metabolism disorders, and decreased ATP production. When ATP shortage occurs, G-actin cannot polymerize fully into F-actin. This will affect microtubule assembly, axonal transport, actin transport, and actin metabolism. Actin is one of the main skeletal components of eukaryotic cells. It exists in two forms: monomer (globular actin, G-actin) and fibrous (fibros actin, F-actin), and G-actin and F-actin can interact with each other. The link, elongation and depolymerization of F-actin are mediated by mitochondrial ATP (Belmont et al., 1999; Gomez and Spitzer, 1999; Mikule et al., 2002). Complete F-actin is necessary to maintain the normal function of cells (Chen et al., 2004). Ethanol (the main component of alcohol) and acetaldehyde (metabolites of ethanol) can inhibit GABA receptors causing a weakened inhibitory effect of GABA on glutamate neurotransmission, thus increasing excitatory amino acid neurotransmitter content. This increase in turn activates the NMDA receptor to make receptor-gated calcium channels open, and raise cytosolic Ca^{2+} concentration. Intracellular Ca^{2+} plays an important role in regulating cytoskeleton assembly and decomposition (Franco and Huttenlocher, 2005; Li et al., 2009). Several studies have shown that Ca^{2+} at high concentrations within the cells of the central nervous system can damage mitochondria, activate gelsolin and make it cut F-actin, degrading the neuronal cytoskeleton, membrane proteins, enzymes and substrates, thus damaging cell structure and function (Kinosian et al., 1998; Goll et al., 2003; Kiselar et al., 2003). The present study found that the structure of mitochondria in hippocampal cells of the model rats had abnormal changes and F-actin content was decreased, indicating a significant difference compared with the control group. This may be explained by a rise in ethanol, acetaldehyde and oxygen free radicals, intracytoplasmic Ca^{2+} overload and toxic effects of high concentrations of H_2S. These changes can make assembly energy insufficient and decomposition is exacerbated by lower content of F-actin in cells, leading to the dissolution, remodeling and collapse of the cytoskeleton and cell dysfunction.

In recent years, a large amount of evidence has shown that neurons of the hippocampus are sensitive to alcohol and are an important area of neuronal plasticity, which is closely

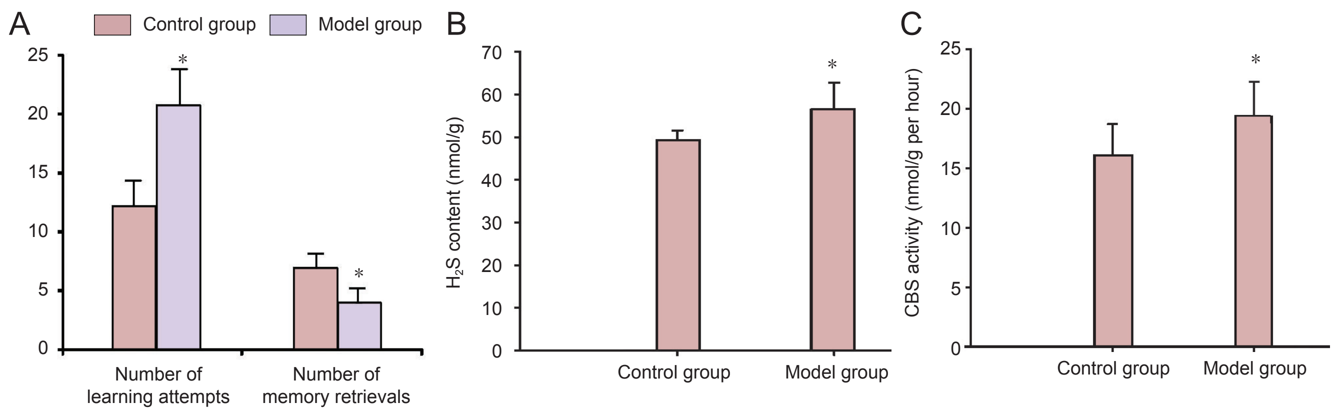

Figure 1 Change of learning and memory ability, hydrogen sulfide (H_2S) content and cystathionine-beta-synthase (CBS) activity in the hippocampus from rats with chronic alcoholism.
(A) Evaluation of learning and memory ability of rats with chronic alcoholism. (B) The changes of H_2S content in the hippocampus seen in both experimental groups. (C) The changes of CBS activity in the hippocampus of rats. Data were expressed as the mean ± SD, with 20 rats in each group. The data were analyzed using independent Student's *t*-test for comparison between the two groups. *$P < 0.01$, *vs*. control group.

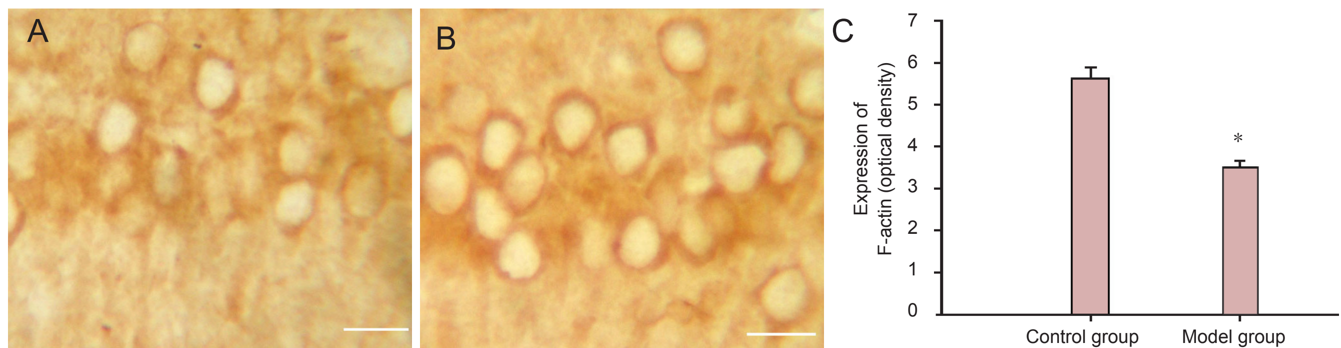

Figure 3 F-actin expression in hippocampus from rats with chronic alcoholism.
F-actin expression in neurons of the hippocampal CA1 region from the control group (A) and model group (B). Scale bars: 10 μm. (C) Average optical density values of F-actin in the hippocampus in both experimental groups. Data were expressed as the mean ± SD, with 20 rats in each group. Data were analyzed using independent Student's *t*-test for comparison between the two groups. *$P < 0.01$, *vs*. control group.

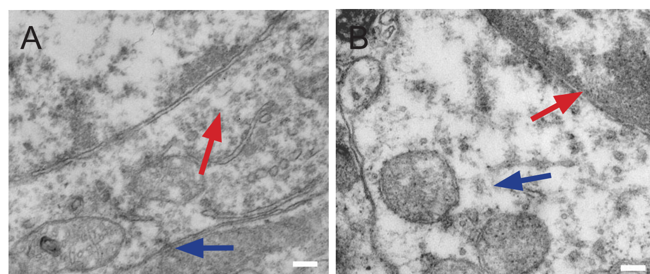

Figure 2 The ultrastructure of hippocampal neurons in rats with chronic alcoholism.
(A) The ultrastructure of the nucleus of hippocampal neurons was normal in the control group. (B) The ultrastructure of the nucleus of hippocampal neurons was changed significantly in the model group. Red arrows are nucleus, and blue arrows are mitochondria. Scale bars: 200 μm.

related to learning, memory and addiction formation (Liu et al., 2004; Burke et al., 2010). Our results demonstrated that chronic alcoholism may increase the activity of CBS and the amount of H_2S generation in hippocampal cells, thereby aggravating the toxic effect caused by H_2S, *i.e.*, increasing cytosolic Ca^{2+} overload and oxygen free radicals. This toxic effect may damage mitochondria, triggering the lack of ATP for energy, exacerbating decomposition and synthesis obstacles of F-actin, leading to the dissolution, remodeling and collapse of the cytoskeleton and cell dysfunction, and ultimately leading to a decline in learning and memory capacities in rats. However, there is no guarantee that every rat from the alcohol model group drank the same amount, which will cause individual differences. The content of H_2S, CBS and F-actin in the hippocampus of rats only be measured, which will provide a more scientific basis for the mechanism of injury and the corresponding treatment of chronic alcoholism.

Author contributions: *All authors were responsible for designing the experiment, implementing the experiments and evaluating the study. Du AL wrote the manuscript. All authors approved the final version of the manuscript.*
Conflicts of interest: *None declared.*

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Copyedited by Aprico K, Norman C, Yu J, Yang Y, Li CH, Song LP, Zhao M