RESEARCH ARTICLE

Aminooxyacetic acid improves learning and memory in a rat model of chronic alcoholism

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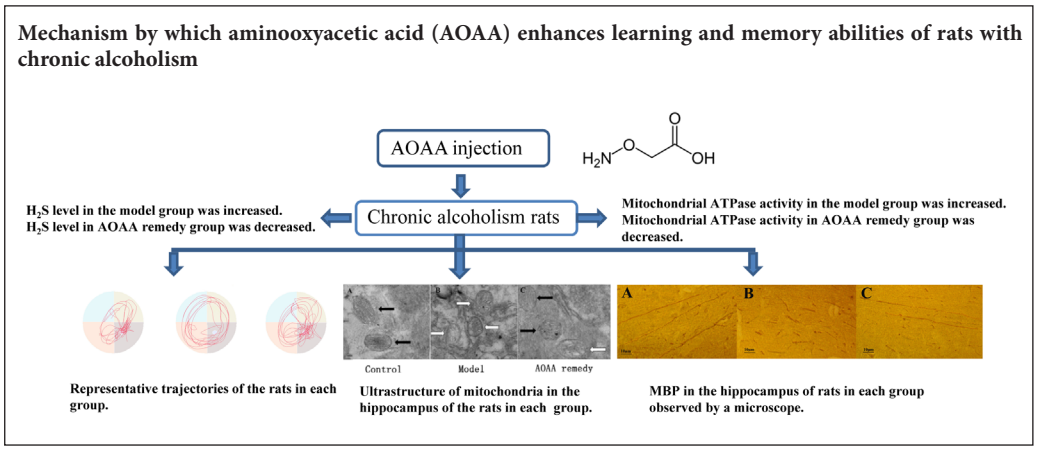
Graphical Abstract



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Abstract

Chronic alcoholism seriously damages the central nervous system and leads to impaired learning and memory. Cell damage in chronic alcoholism is strongly associated with elevated levels of hydrogen sulfide (H_2S) and calcium ion overload. Aminooxyacetic acid is a cystathionine-β-synthase activity inhibitor that can reduce H_2S formation in the brain. This study sought to observe the effect of aminooxyacetic acid on learning and memory in a chronic alcoholism rat model. Rats were randomly divided into three groups. Rats in the control group were given pure water for 28 days. Rats in the model group were given 6% alcohol for 28 days to establish an alcoholism rat model. Rats in the aminooxyacetic acid remedy group were also given 6% alcohol for 28 days and were also intraperitoneally injected daily with aminooxyacetic acid (5 mg/kg) from day 15 to day 28. Learning and memory was tested using the Morris water maze test. The ultrastructure of mitochondria in the hippocampus was observed by electron microscopy. H_2S levels in the hippocampus were measured indirectly by spectrophotometry, and ATPase activity was measured using a commercial kit. The expression of myelin basic protein was determined by immunohistochemistry and western blotting. Compared with the control group, latency and swimming distance were prolonged in the navigation test on days 2, 3, and 4 in the model group. In the spatial probe test on day 5, the number of platform crosses was reduced in the model group. Cristae cracks, swelling or deformation of mitochondria appeared in the hippocampus, the hippocampal H_2S level was increased, the mitochondrial ATPase activity was decreased, and the expression of myelin basic protein in the hippocampus was down-regulated in the model group compared with the control group. All the above indexes were ameliorated in the aminooxyacetic acid remedy group compared with the model group. These findings indicate that aminooxyacetic acid can improve learning and memory in a chronic alcoholism rat model, which may be associated with reduction of hippocampal H_2S level and mitochondrial ATPase activity, and up-regulation of myelin basic protein levels in the hippocampus.

Key Words: *nerve regeneration; aminooxyacetic acid; chronic alcoholism rat model; hydrogen sulfide; Ca^{2+} overload; brain injury; learning and memory abilities; Morris water maze; myelin basic protein; mitochondria; ATP enzyme activity; neural regeneration*

Introduction

Chronic alcoholism caused by prolonged overdrinking results in central nervous system damage. Chronic alcoholism is a progressive, potentially fatal disease, characterized by a strong desire for alcohol, an increase in tolerance, increased dependence, and non-control (Nam et al., 2012; Du et al., 2014). Long-term heavy drinking causes damage to the central nervous system and peripheral nerves (Li et al., 2005). The neurotoxicity of ethanol has been extensively studied, but its mechanism of action remains unclear. Decreased activity of cytochrome oxidase causes increased neuronal adenosine triphosphate (ATP) metabolism and calcium overload (Yang et al., 2011). Ethanol produces oxygen free radicals by the action of alcohol dehydrogenase and aldehyde dehydrogenase that damage neuronal DNA. N-methyl-D-aspartic acid (NMDA) and/or γ-aminobutyric acid

(GAGB) mediate alcohol-induced neuronal apoptosis (Sandra et al., 2001). Aminooxyacetic acid (AOAA) can inhibit the expression of cystathionine-beta-synthase, thereby reducing the formation of H_2S, inhibiting Ca^{2+} influx, and alleviating intracellular Ca^{2+} overload (Li et al., 2009). However, there is currently no study addressing AOAA treatment for chronic alcoholism. We speculate that AOAA can relieve nerve cell damage caused by chronic alcoholism and can improve central nervous system function by reducing the formation of H_2S and alleviating intracellular Ca^{2+} overload.

Myelin sheath is a single layer fat-protein tissue that envelops the axons of neurons. Myelination injury or shedding has an adverse effect on neuronal function (Sandell and Peters, 2003; Marina et al., 2008). Previous studies have shown cognitive and memory function defects related to changes in the myelination of nerve fibers (Luo et al., 2001; Long et al., 2005). In the central nervous system, myelin basic protein (MBP), accounts for 30–40% of myelin in the myelin sheath. MBP is an extrinsic protein localized exclusively at the cytoplasmic surface in the major dense line of the myelin sheath (Xie al., 2013). It is synthesized and secreted by oligodendrocytes (Li, 2007). MBP can promote axonal regeneration (Xu et al., 2005) and can also combine with calcium to inhibit calcium overload caused by free radicals during membrane lipid degradation (Li et al., 2006). A decrease in the level of MBP can inhibit myelin formation and affect the normal conduction function of the myelin sheath (Zhang et al., 2017). The hippocampus is strongly associated with advanced neural activities, such as learning and memory and emotions. There is a clear synaptic pathway within the hippocampus and the integrity of the hippocampal formation is important for learning and memory (Zao et al., 2015). In this study, we investigated a rat model of chronic alcoholism and observed the effect of AOAA treatment on changes in H_2S levels, mitochondrial structure and MBP levels in the hippocampus. We also tested the learning and memory capabilities of the model rats.

Materials and Methods

Animals

Sixty 2-month-old, male, Sprague-Dawley rats weighing 120–160 g were provided by the Animal Management Center of Zhengzhou University of China [SCXK (Yu) 2015-0004]. The experiment was approved by the Animal Ethics Committee of Xinxiang Medical College of China (No. 4107000081551).

The rats were housed at 22–25°C for 5–7 days to acclimatize to the laboratory environment. The rats were randomly divided into a control group, an AOAA remedy group, and a model group (chronic alcoholism model) (n = 20 per group).

Model establishment and drug administration

The control group was given free access to pure water every day, the water being changed every morning at 9:00 a.m. To establish a model of chronic alcoholism (Rong et al., 2008), the model and AOAA remedy groups were given free access to 6% (v/v) alcohol solution for 28 days, with the alcohol solution being changed at 9:00 a.m. every day. From day 15 to day 28, rats in the AOAA remedy group were intraperitoneally injected once a day with 5 mg/kg AOAA (Geel, Antwerp, Belgium) dissolved in 1 mL of saline (Tang et al., 2015). The control and model groups were intraperitoneally injected with 1 mL saline once a day from day 15 to day 28. After establishment of the model, the water maze test was performed to verify whether the model was successful. A significant difference in learning and memory abilities between the model and control groups indicates the model has been successfully established.

Evaluation of learning and memory abilities

On the 28th day after the start of model establishment, each group was tested in the Morris water maze test. This labyrinth system is widely used with rats in basic and applied neurobiology (Hu et al., 2000). The Morris water maze apparatus (Noldus, Wageningen, Gelderland, Netherlands) consisted of a cylindrical pool, a movable platform and a drainage system (Yu et al., 2015). The pool was circular with a black wall and bottom. The pool was surrounded by blue curtains to block visual references in the room. References of various shapes and colors can be fixed to the inner wall of the pool or other visible high points (Yan et al., 2004). During training, a platform was submerged 0.5 cm below the surface in a fixed position within a quadrant. The pool was divided into four quadrants: southwest, southeast, northwest and northeast. The submerged platform was placed in the center of the southeast quadrant. On the first day of the test, the platform was removed as a pre-experiment for the rats to become acclimatized to the environment and to identify and remove rats with poor swimming skills. Eight rats from each group were randomly selected for the navigation test and the spatial probe test. The positioning navigation test was performed on four consecutive days. The starting quadrant was changed each day in an anti-clockwise manner. Each rat was trained four times (in a different quadrant each time) every day. The swimming trajectory of rats was recorded with a camera and analyzed using behavioral record software, EthoVision XT 8 (Noldus). Each time the rat found the submerged platform, the rat was allowed to stay on the platform for 30 seconds to consolidate its memory. The maximum duration for each training was 60 seconds. If the submerged platform was not found within 60 seconds, the rat was directed to the platform and allowed to stay on the platform for 30 seconds. In the navigation test, the latency period to find the underwater platform and swimming distance of each rat were recorded. A shorter delay time and trajectory length indicated stronger learning ability. The spatial probe test was performed following the navigation test. The spatial probe test was conducted only once to measure the ability of rats to retain the memory of the platform position. The difference between this experiment and the previous one was that the platform was removed. The rats were then released from the center of the northwest quadrant, which was opposite the target quadrant, and the swimming time was 180 seconds. In the spatial probe test,

the more times the rat crossed the platform position and the longer the swimming distance within the platform quadrant, the stronger the learning ability. After the end of the Morris water maze test, the rats were euthanized. Four rats in each group were subjected to immunohistochemical staining and a total of eight hippocampi were analyzed. The left and right hippocampi of the remaining four rats were cryopreserved. H_2S levels, ATPase activity, and MBP protein levels in the eight hippocampi were measured, and the mitochondria were observed.

H_2S levels in the hippocampus measured by spectrophotometry

Twelve hours after euthanasia, H_2S absorbance in hippocampal tissue was measured using an ELx800 automatic microplate reader (Bio-Tek, Venuschi, VT, USA) at the wavelength of 670 nm. H_2S levels in the hippocampus were measured according to an H_2S standard curve and expressed in terms of H_2S level per unit weight of tissue (nmol/g) (Ou et al., 2007). The levels of H_2S in the eight hippocampi were measured.

Morphology of mitochondria observed by electron microscopy

Twelve hours after euthanasia, 1 cubic millimeter pieces of hippocampus were prepared. The pieces were fixed with 4% glutaraldehyde for 1 minute, and then stored at 4°C for 3 hours. The samples were then washed with PBS and immersed in 1% citric acid for approximately 1 hour. After washes with PBS, these samples were dehydrated with acetone and embedded in epoxy resin. The samples were then sliced into ultrathin sections. The sections were stained with citrate and uranyl acetate and observed by transmission electron microscopy (Hitachi, Tokyo, Japan). The morphology of mitochondria in neurons of the hippocampus was compared among groups (with assistance from the Electron Microscopy Department of Xinxiang Medical College of China).

Determination of mitochondrial ATPase activity

Twelve hours after euthanasia, according to the instructions of the Ultramicro Total ATP Enzyme Assay Kit (Nanjing Institute of Bioengineering, Nanjing, China), samples were pretreated, then tissues were accurately weighed, and physiological saline added to give a weight (g):volume (mL) ratio of 1:9. Samples were mechanically homogenized under ice-water bath conditions and centrifuged at 2500 × *g* for 10 minutes. The supernatant was taken to measure mitochondrial ATPase activity. The supernatant was taken for enzymatic reaction, and centrifuged at 3500 × *g* for 10 minutes, and then treated with the fixed phosphorus reagent. The optical density (OD) values were measured using a spectrophotometer at 636 nm. The ATPase activity was calculated by (measured OD value − control OD value)/(standard OD value − blank OD value) × standard concentration × 6 × 7.8 ÷ sample protein concentration.

MBP immunohistochemistry

The brains of four rats from each group were fixed with 4% paraformaldehyde for 12 hours, paraffin embedded and sectioned. Using an SP Kit for immunohistochemistry (Bioss, Beijing, China), sections were dewaxed and rehydrated, and 0.01 mol/L citrate solution was used for antigen retrieval. The slices were washed in PBS for 5 minutes three times, incubated in 5% H_2O_2 solution at 37°C for 20 minutes (SP Kit; Bioss Inc.), washed in PBS for 5 minutes three times, and blocked with normal goat serum working fluid at 37°C for 20 minutes. A rabbit anti-MBP polyclonal antibody (Bioss Inc.) was added, incubated at 37°C for 20 minutes and then rinsed with PBS for 5 minutes three times. Biotin-labeled secondary antibody working fluid was added and incubated at 37°C for 60 minutes. Sections were then washed with PBS for 5 minutes three times. Horseradish peroxidase-labeled streptavidin working fluid was added and incubated at 37°C for 50 minutes. Staining was developed with diaminobenzidine at 37°C for 5 minutes and observed and photographed under a light microscope. Image-pro-plus 6.0 image processing software was used to analyze the positive expression of MBP. The value of the integrated OD (mean density) of the effective target protein divided by the effective target distribution area is the mean density value.

MBP western blot assay

Three days after euthanasia, the hippocampus was added in tissue lysate (1 mg: 10 μL,Beyotime Biotechnology Co., Ltd., Shanghai, China) containing protease inhibitor (50:1; Beyotime Biotechnology Co., Ltd.) and incubated for 30 minutes and then centrifuged to extract whole protein. Protein concentration was measured using a bicinchoninic acid protein quantification kit (Beyotime Biotechnology Co., Shanghai, China). The extracted protein was diluted to give the same concentration in all samples and incubated at 100°C in a water bath for 10 minutes, followed by storage at −80°C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed on 40 μg of protein at 120 V for 90 minutes (BioRad, Hercules, CA, USA). Proteins were then transferred onto polyvinylidene fluoride membrane at 100 V for 90 minutes (BioRad). The membrane was then incubated with a rabbit anti-MBP polyclonal antibody (1:500; Bioss) at 4°C overnight. The blot was then rinsed with Tris-buffered saline containing Tween 20 (1:1000), incubated with goat anti-rabbit IgG (1:1000; Beyotime Biotechnology Co.) and then rinsed again in Tris-buffered saline with Tween 20. Strip analysis was then performed using an imaging system (UVP, Upland, CA, USA). The value of the integrated optical density of the target protein divided by the integrated optical density of the internal reference protein gave the relative value of the target protein.

Statistical analysis

Data are expressed as the mean ± SEM and were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). The comparison between groups was performed by one-way analysis of variance followed by the least significant difference *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

Results

Learning and memory in each group

As shown in **Figure 1**, the learning and memory of the the model group was significantly worse than that of the control group, showing longer latency periods and swimming distances. There was a significant difference between the groups on day 2 and day 4 ($P < 0.01$). The latency periods and swimming distances in the AOAA remedy group were significantly decreased compared with the model group, especially on day 2 and day 4 ($P < 0.01$).

Figure 2 shows the representative exploratory trajectories of the rats on day 5. Compared with the control group, the trajectories of the rats in the model group were remarkably more evenly distributed in each quadrant with a decreased number of platform crossings. The trajectories of the rats in the AOAA remedy group were significantly concentrated in the quadrant of the platform with an increased number of platform crossings compared with the model group.

H_2S levels in the hippocampus

As shown in **Figure 3**, compared with the control group, H_2S levels in the hippocampus of rats of the model group were significantly increased ($P < 0.01$). H_2S levels were significantly decreased in the hippocampus in the AOAA remedy group compared with the model group ($P < 0.01$).

Morphology of mitochondria in the hippocampus observed by electron microscopy

The morphology of mitochondria in the hippocampus of each group was observed by electron microscopy. As shown in **Figure 4**, mitochondria indicated by black arrows were intact. There was no cavitation, cristae breakage, or swelling. The mitochondria indicated with white arrows show vacuolization, and broken and deformed cristae. The mitochondria in the control group were intact and there were no vacuoles, cristae breaks, or swelling. The mitochondria in the model group showed cristae breakage, swelling and deformation. Mitochondrial morphology was improved after AOAA treatment.

Mitochondrial ATPase activity

As shown in **Figure 5**, mitochondrial ATPase activity in the hippocampus of the model group was significantly decreased compared with that of the control group ($P < 0.01$). The activity of mitochondrial ATPase in the AOAA remedy group was significantly higher compared with that in the model group ($P < 0.01$).

MBP expression in the hippocampus

MBP expression was observed by immunohistochemistry in the hippocampal CA1 region in each group (**Figure 6A–C**). The average OD shows the level of MBP in the hippocampal CA1 region. Compared with the control group, the MBP level was significantly decreased in the model group ($P < 0.01$). The MBP level was significantly increased in the AOAA remedy group compared with the model group ($P < 0.01$) (**Figure 6D**).

Western blot assay of MBP is shown in **Figure 7**. MBP levels were significantly decreased in the hippocampus of the model group compared with the control group ($P < 0.01$). MBP levels were significantly increased in the AOAA remedy group compared with the model group ($P < 0.01$).

Discussion

Chronic alcoholism seriously damages the central nervous system and leads to impaired learning, memory, and cognitive judgment (Li et al., 2016; Le et al., 2017). The mechanism of nerve damage by alcohol has been investigated by different studies. Decreased activity of cytochrome oxidase causes reduced neuronal ATP metabolism and calcium overload. Ethanol produces oxygen free radicals under the action of alcohol dehydrogenase and aldehyde dehydrogenase, which injure neuronal DNA. Ethanol-induced neuronal apoptosis is mediated by NMDA and/or GAGB, and serotonin receptors (Sandra et al., 2001). AOAA can inhibit the expression of cystathionine-beta-synthase, thereby reducing the formation of H_2S, inhibiting Ca^{2+} influx and alleviating intracellular Ca^{2+} overload, which relieves cell damage (Li et al., 2009). However, no study has addressed AOAA treatment for chronic alcoholism. The results of this study indicate that AOAA can alleviate the impaired learning and memory caused by chronic alcoholism.

Along with CO and NO, H_2S is a gaseous signaling molecule (Li et al., 2016; Le et al., 2017). In the brain, cystathionine-beta-synthase catalyzes the formation of H_2S (Zhao et al., 2001; Cui et al., 2016) from cysteine. Physiological concentrations of H2S can help blood vessel dilation and the anti-oxidative response (Jung et al., 2014; Huang et al., 2017) to maintain learning and memory abilities (Li et al., 2015; Tian et al., 2016). High concentrations of H_2S can, however, impair organs (Jiang et al., 2016). It inhibits central nervous system function, and specifically inhibits the excitatory postsynaptic membrane potential and blocks synaptic transmission. It also enhances NMDA receptor-mediated calcium overload, which leads to increased intracellular calcium concentration and damage to mitochondrial morphology and energy metabolism, eventually resulting in cell necrosis. Chronic alcoholism can activate cystathionine-beta-synthase, and catalyze the formation of H_2S (Zao et al., 2001). AOAA is a cystathionine-beta-synthase inhibitor (Sandell and Peters, 2003; Niu et al., 2018), and can reduce H_2S formation in the brain by inhibiting cystathionine-beta-synthase activity (Donovan et al., 2017).

ATPase can catalyze the oxidation of ADP to ATP, which plays an important role in material transport and information transfer (King et al., 2016). The activity of ATPase can change with the onset of diseases (Li et al., 2000). By detecting the activity of mitochondrial ATPase in the hippocampus of each group, we found that compared with the control group, ATPase activity was remarkably decreased in the model group. Compared with the model group, ATPase activity was dramatically increased in the AOAA remedy group.

MBP is the main protein of central nervous system myelin and can promote axonal regeneration (Luo et al., 2001; Zhou et al., 2017). In addition, MBP can also inhibit the calcium-induced degradation effect of free radicals on membrane

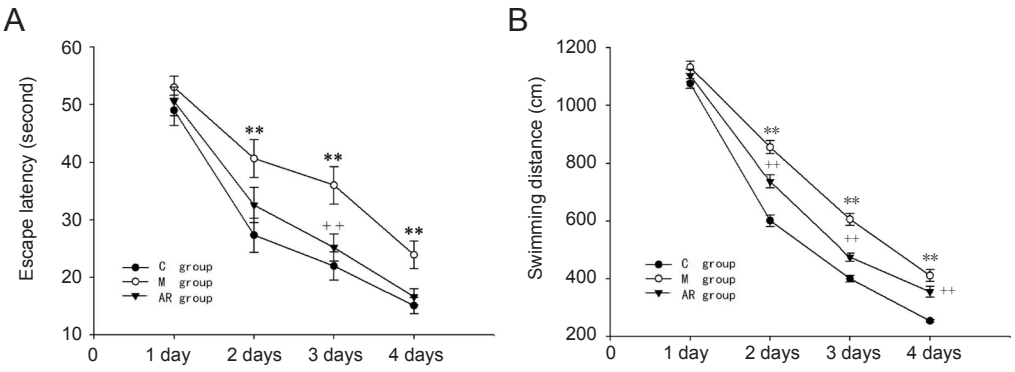


Figure 1 Morris water maze navigation test.
(A) Latency to the platform for the three groups; (B) swimming distance to the platform for three groups. The data are presented as the mean ± SEM. Comparisons between groups were conducted by one-way analysis of variance followed by the least significant difference *post hoc* test. $^{**}P < 0.01$, *vs.* C group; $^{++}P < 0.01$, *vs.* M group. C group: Control group; M group: model group; AR group: AOAA remedy group (chronic alcoholism model + aminooxyacetic acid remedy). AOAA: Aminooxyacetic acid.

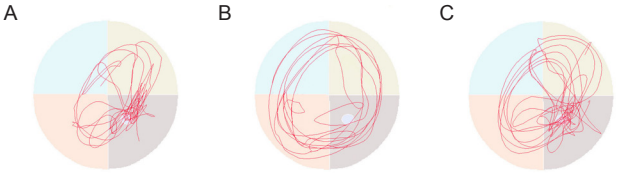


Figure 2 Representative trajectories of rats in the Morris water maze spatial probe test on day 5.
(A–C) Representative trajectories of rats in the control (A), model (B) and AOAA remedy groups (C) (chronic alcoholism model + AOAA remedy), respectively. The more trajectories in the target quadrant (lower left quadrant), the better the memory of the rat; the more platform crossings (circle in the lower left quadrant), the better the memory of the rat. The trajectories of rats in the model group were significantly more evenly distributed in each quadrant compared with the control group, and the number of platform crossings was reduced. The trajectories of the rats in the control and the AOAA remedy groups were significantly concentrated in the platform quadrant and many crossed the platform. AOAA: Aminooxyacetic acid.

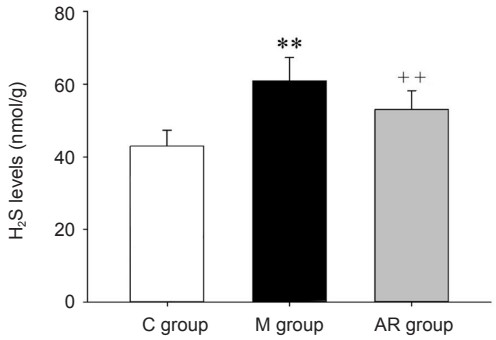


Figure 3 H_2S levels in the hippocampus.
The data are presented as the mean ± SD ($n = 8$). Comparisons between groups were conducted by one-way analysis of variance followed by the least significant difference *post hoc* test. $^{**}P < 0.01$, *vs.* C group; $^{++}P < 0.01$, *vs.* M group. C group: Control group; AR group: AOAA remedy group (chronic alcoholism model + aminooxyacetic acid remedy); M group: model group. AOAA: Aminooxyacetic acid.

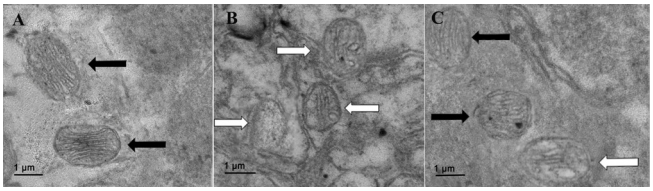


Figure 4 Ultrastructure of hippocampal mitochondria.
(A–C) Ultrastructure of mitochondria in the control (A), model (B) and AOAA remedy groups (C), respectively (scanning electron microscopy, original magnification, 10,000 ×). Black arrows indicate intact mitochondria without vacuoles, cristae cracks, swelling or deformation. White arrows indicate damaged mitochondria. They contain vacuoles and the cristae are broken and deformed. The mitochondria in the control group were intact without vacuoles, cristae cracks or swelling. The mitochondria in the model group showed vacuoles, cristae cracks and swelling. Mitochondrial ultrastructure was improved after AOAA treatment. AOAA: Aminooxyacetic acid.

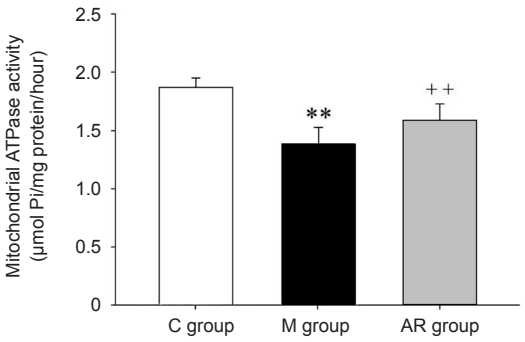
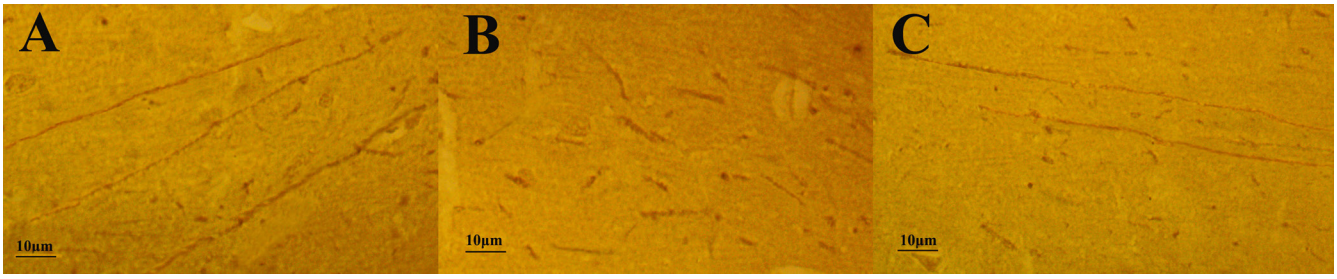


Figure 5 Mitochondrial ATPase activity in the hippocampus.
The data are presented as the mean ± SD ($n = 8$). Comparisons between groups were conducted by a one-way analysis of variance followed by the least significant difference *post hoc* test. $^{**}P < 0.01$, *vs.* C group; $^{++}P < 0.01$, *vs.* M group. C group: Control group; M group: model group; AR group: AOAA remedy group (chronic alcoholism model + aminooxyacetic acid remedy). AOAA: Aminooxyacetic acid.

lipids (Xu et al., 2005). Sandell and Peters (2003) found that cognitive and memory function defects are associated with structural changes of myelinated myelin sheath in the central nervous system. High levels of H_2S can have effects on the central nervous system. Accumulation of ethanol can cause oligodendrocyte damage (Hu et al., 2016) and reduce MBP levels, causing the myelin sheath to come off axons, leading to impairment of information transmission among synapses (Bai et al., 2017).

In summary, AOAA can ameliorate the effects of chronic alcoholism on brain tissue, which might be achieved by reducing H_2S levels. Thus, AOAA protects mitochondrial



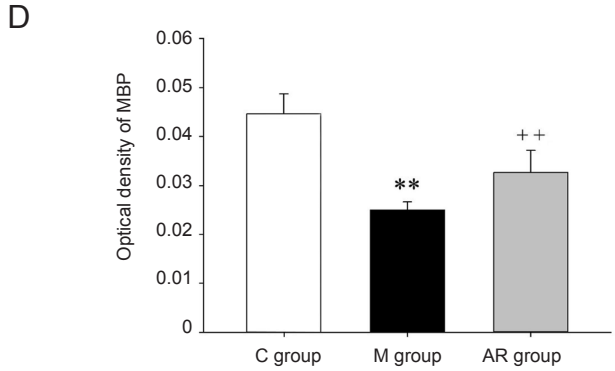


Figure 6 Mean optical density of MBP in the hippocampus.
(A–C) Light microscopy images of MBP immunohistochemistry in the hippocampus of the control (A), model (B) and AOAA remedy groups (C), respectively. (D) Mean optical density of MBP in the hippocampus of each groups (C). The data are presented as the mean ± SD (n = 8). Comparisons between groups were conducted by a one-way analysis of variance followed by the least significant difference *post hoc* test. **P < 0.01, *vs.* C group; ++P < 0.01, *vs.* M group. C group: Control group; M group: model group; AR group: AOAA remedy group (chronic alcoholism model + aminooxyacetic acid remedy); MBP: myelin basic protein. AOAA: Aminooxyacetic acid.

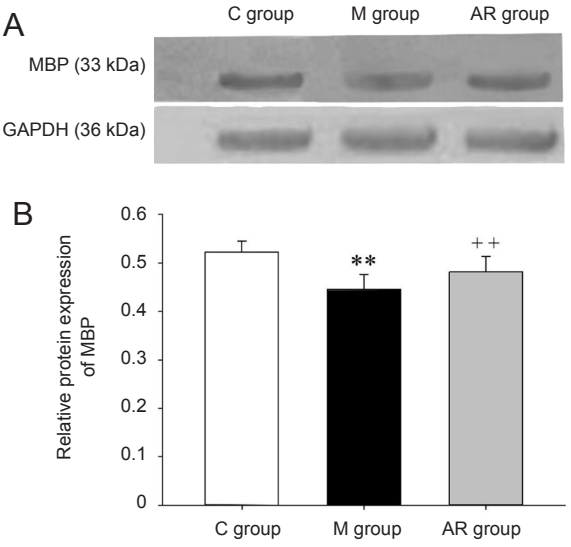


Figure 7 MBP levels in the hippocampus (western blot assay).
(A) Protein bands of MBP and GAPDH in the hippocampus of each group. (B) The levels of MBP in the hippocampus of each group. Data are presented as the mean ± SD (n = 8). Comparisons between groups were conducted by a one-way analysis of variance followed by the least significant difference *post hoc* test. **P < 0.01, *vs.* C group; ++P < 0.01, *vs.* M group. C group: Control group; M group: model group; AR group: AOAA remedy group (chronic alcoholism model + aminooxyacetic acid remedy); MBP: myelin basic protein. AOAA: Aminooxyacetic acid.

function, reduces alcohol-induced MBP damage, and protects the structure and function of neurons, thereby improving the learning and memory of chronic alcoholism model rats. However, in this study, we only focused on the learning and memory capabilities of rats, and H_2S levels, mitochondrial ATPase activity and MBP levels in the hippocampus. Therefore, further investigations are needed to support AOAA treatment for chronic alcoholism.

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