Effects of aminooxyacetic acid on hippocampal mitochondria in rats with chronic alcoholism: the analysis of learning and memory-related genes

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The incidence of chronic alcoholism leading to central and peripheral nervous system damage has been increasing year-to-year. The purpose of this study is to explore the effects of aminooxyacetic acid on hippocampus mitochondria in rats with chronic alcoholism and analyze learning and memory-related genes. Sixty male Sprague Dawley rats were randomly divided into three groups. Except for the control group, each group was fed with the water containing (v/v) 6% alcohol for 28 days. After 14 days, rats in the treatment group were intraperitoneally injected daily for 14 days with aminooxyacetic acid. High throughput sequencing was combined and tested for learning and memory abilities, Hydrogen sulfide content, catalase activity, and by playing a protective role in neuronal function by reducing the formation of H2S. Physiological doses of H2S, acting as a neurotransmitter, have been shown to relax blood vessels and increase microcirculation (Zhao et al., 2001), directly antagonize oxidative stress damage (Saleewong et al., 2012), induce long-term potentiation in the hippocampus CA1 region (Gadalla and Snyder, 2010), strengthen the excitatory postsynaptic potential and improve learning and memory processes. However, high concentrations of H2S have a toxic effect in vivo.

On the one hand, it exerts an inhibitory influence on the central nervous system because of specific but reversible suppression of the excitatory postsynaptic membrane potential; this blocks synaptic transmission, thereby inhibiting neuronal function (Chen et al., 2012; Ren et al., 2008). On the other hand, it enhances calcium overload otherwise mediated by N-methyl-D-aspartic acid (NMDA) receptors to cause swelling of the mitochondria. This, in turn, results in disorders of energy metabolism and progressive cell necrosis (Liang et al., 2016; Shang et al., 2008). Aminooxyacetic acid (AOAA) is an inhibitor of CBS activity, which reduces the concentration of H2S, inhibits the influx of Ca2+, and reduces intracellular Ca2+ overload, thus improving the function of a damaged central nervous system. However, there are currently few studies that address the use of AOAA treatment for chronic alcoholism. Here it is speculated that AOAA relieves nerve cell damage caused by chronic alcoholism and may improve the central nervous system function by reducing the formation of H2S, increasing the content of F-actin, and by playing a protective role in neuronal mitochondria.
The cytoskeleton is an important structure for maintenance of the normal morphology and function of cells, and F-actin is one of the most important cytoskeleton components. Long-term synaptic potentiation (LTP) in the hippocampus is a manifestation of synaptic plasticity and putatively provides the neurocytological basis for learning and memory. There is evidence that F-actin is an important substrate for LTP induction and maintenance (Fukazawa et al., 2003), and the role of the neuronal cytoskeleton in the induction and maintenance of LTP has been receiving increased attention.

Based on previous research (Du et al., 2014), bioinformatics methods were applied to analyze the gene expression profiles of Sprague Dawley (SD) rats that exhibited chronic alcoholism, which suggested damage to the mitochondrial electron transfer chain complex I. In this study, a rat model of chronic alcoholism was investigated, and the effect of AOA treatment was observed for changes in H2S levels, mitochondrial structure, and F-actin levels in the hippocampus. The learning and memory capabilities of rats subjected to the reported model of alcoholism were also tested.

2. Materials and methods

2.1 Laboratory animals and groups

Sixty clean-grade healthy SD male rats, weighing 120-160 g, were provided by the Animal Center of Xinxiang Medical University [SCXK (Yu) 2015-0004]. The animals were fed in the laboratory for four to six days to adapt them to the laboratory environment. They were then randomly divided into three groups of 20 rats to form normal control (NC), chronic alcoholism model (M), and amino hydroacetic acid remedy (AR) groups. The Animal Ethics Committee approved the experiment of Xinxiang Medical College of People’s Republic of China (No.4107000081551).

2.2 Model establishment and drug administration

The NC group was given access ad libitum to pure water changed every morning at 9:00 am. To establish a model of chronic alcoholism (Li et al., 2006), the model and AOA remedy groups were given ad libitum access to a 6% (v/v) alcohol solution for 28 days, also changed every morning at 9:00 am. From days 15-28, rats in the AR group were intraperitoneally injected once daily with 5 mg/kg AOA (Geel, Antwerp, Belgium) dissolved in 1 ml of saline. The NC and M groups were intraperitoneally injected with 1 ml saline once daily from day 15-28. After the establishment of the model, a Y maze test was performed to verify whether the model was successful. A significant difference in learning and memory abilities between M and NC groups indicated the model was successfully established.

2.3 Major instruments and reagents

ACROS, Co., Ltd. (USA) provided nHS and AOA; the total ATPase assay kit, total protein assay kit and mitochondrial extraction kit were provided by the Institute of Bioengineering in Nanjing, China; the ultra-low temperature refrigerator is produced by Thermo Forma Company of the United States, and micro-adder was purchased from Eppendorf Company of Germany. The high-speed refrigerated centrifuge was purchased from Heraeus company of Germany; FA1004 electronic analytical balance was purchased from Shanghai LuoMing Scientific Instrument Co., Ltd.; Shanghai shift number Mdt InfoTech Ltd produces the Y type electric maze; SDS Gel Kit, ECL luminous solution, rat anti-GAPDH protein primary antibody, and Anti-Western Blot II antibody were purchased from Shanghai Biyuantian Biotechnology Co., Ltd. Other reagents are domestic or imported analytical grade.

2.4 Assessment of learning and memory ability

Rats in each group were tested in a Y-type electric maze (Tang, 2015). This maze was divided into three arms: I, II, and III, which were each randomly used as a safety zone. A signal lamp was set at the top of each arm. When the lamp was illuminated, it indicated that the arm was a safe area. All the learning and memory tests were conducted at night under low light. One rat was placed in the maze. The three-arm indicator lights were turned on to allow the rat to adapt to the environment. These lights were turned off after three minutes. The indicator light of one arm was then illuminated at random, and the two unlit arms were automatically delayed for five seconds, after which a rat was electrically shocked until they escaped to the two-safety zone. The light was then extinguished after 15 seconds, and the test was completed. Rats moving directly to the safe area within 10 seconds was the correct reaction; any alternative response was assessed as incorrect. If a rat responds correctly in nine out of ten trials, this was interpreted as the rat learning the correct response. The learning and memory performance of the spatial discrimination response was assessed as the number of training sessions a rat required to learn the correct response. The smaller the number of training sessions, the stronger the learning ability of rats.

2.5 Sample preparation and sequencing

Rats were sacrificed, the brain quickly extracted, and 6 μg total RNA removed from the hippocampus. After a series of steps such as purification, obtain, cDNA synthesize, and so on. Genome samples were sequenced by the method of sequencing by synthesis. Clean tags were obtained with the following six steps:

1. 3’ (means the 3 terminal of cDNA) adaptor sequence removal: Tags were only 21 nucleotides long while sequencing reads were 49 nucleotides long, raw sequences are with 3’ adaptor sequences.
2. Empty read with only 3’ adaptor sequences, but no tags were removed.
3. A low-quality tag with unknown sequences was removed.
4. Tags longer or shorter than 21 nucleotides were removed.
5. Tags with only a single copy were removed as they implied a sequencing error.
6. Clean tags were generated.

2.6 Data processing and differentially expressed genes (DEGs) screening

If it is assumed that the total number of tags in a gene A is x and in a large library it is known that the amount of each gene product expressed (ρ(x)) is only a fraction of total gene expression, the distribution of ρ(x) should follow a Poisson distribution. The following formula can be used to select the differential gene between two different samples (Audic and Claverie, 1997):

\[ p(x) = \frac{e^{-\lambda} \lambda^x}{x!} \]

(λ is the true transcription number of gene A)
If the total number of clean tags in each sample is known \(N_1, N_2\) respectively) and the number of clean tags from gene A (first sample) is \(x\), and the corresponding number for the second sample is \(y\), then the probability of the equal expression of the genes in two samples can be calculated from:

\[
p(y|x) = \left( \frac{N_2}{N_1} \right)^y \frac{(x+y)!}{x!y!} \left( 1 + \frac{N_2}{N_1} \right)^{(x+y+1)}
\]

After obtaining the preliminary \(P\) (Probability)-value data, results must be checked and corrected if necessary. \(P\)-Value corresponds to differential gene expression test. FDR (False Discovery Rate) is a method to determine the threshold of \(P\)-Value in multiple tests and analysis through manipulating the FDR value. Assume that we have picked out \(R\) differentially expressed genes in which \(S\) genes really show differential expression, and the other \(V\) genes are false positive. If we decide that the error ratio \(Q = V/R\) must stay below a cutoff (e.g., 1%), we should preset the FDR to a number no larger than 0.01. Refer to (Benjamini and Yekutieli, 2001) for details. We use “FDR < 0.001 and the absolute value of log2Ratio ≥ 1” as the threshold to judge the significance of gene expression difference. More stringent criteria with smaller FDR and bigger fold-change value can be used to identify DEGs.

### 2.7 Significant enrichment analysis of gene function

Gene ontology (GO) is an extremely important method in bioinformatics and is widely used for gene function classification. Term is the basic unit of GO analysis. It employs three components to describe gene function, including molecular function, cellular components, and biological processes. GO is gradually changing the way biological data is organized and understood. The lower the \(P\) value, the higher the reliability. In GO enrichment, terms with low \(P\) value are the main research objects. GO enrichment analysis was performed for all differentially expressed genes. The \(P\) value can be calculated with the following equation:

\[
P = 1 - \sum_{i=0}^{m-1} \left( \frac{M}{i} \right) \left( \frac{N-n}{n-i} \right) \left( \frac{N}{n} \right)
\]

where: \(N\) is the number of genes with a GO annotation in the genome; \(M\) is the number of genes annotated as a particular GO term in the genome; \(n\) is the number of differentially expressed genes in \(N\); \(m\) is the number of genes differentially expressed in a specific GO term. After obtaining preliminary \(P\)-value results, a Bonferroni calibration step is necessary to reset the significance threshold for statistical testing to 0.05. A GO term satisfying this condition is defined as a GO term that is significantly enriched in differentially expressed genes.

### 2.8 Analysis of significant enrichment of pathway

The genes do not work in isolation; multiple genes often interact and influence each other. The results of signaling pathways enrichment must, therefore, be analyzed to obtain a deeper understanding of gene function. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a major public database of pathways (Liu et al., 2008). It provides a powerful source of gene function, the data are extensively covered, and it allows a detailed analysis of high throughput sequencing data. Significant enrichment analysis of Pathway is based on KEGG Pathway. Through internal calculation, we found signaling pathways that are significantly enriched, which are the same as GO enrichment analysis. Then we drew the pathway enrichment bubble map with the help of the ggplot2 package using R software. The vertical coordinate is the pathway, and the abscissa is the \(P\)-value of each path. The lower the \(P\)-value corresponding to each path is, the higher the reliability is. The larger the bubbles are, the more differentially expressed genes involved in the pathway are. If the color is close to red, it means higher enrichment.

### 2.9 PPI network construction

The "Search Tool for Retrieval of Interacting Genes" (STRING) is a database that provides possible interactions predication between a known protein and a predicted protein. It can analyze and score the possible interactions between proteins to show the likelihood of protein interaction. In general, if the protein value is more than 0.4, then it is considered that there is a significant interaction between the two proteins. First of all, we imported DEGs into the STRING database to build a PPI network and set the threshold to be greater than 0.9 to get the final PPI network. The PPI network consists of nodes and degrees. Nodes represent the protein, and the borderline represents the protein's interaction. The number of edges connected by a single node is called the degree of the node. By calculating the degree of each node in a PPI network, it is possible to obtain a protein with a higher degree of node called hub proteins. We then downloaded the protein interaction files from the STRING database and imported them into Cytoscape (an open-source software platform for visualizing complex networks and integrating these with any attribute data) for further analysis.

### 2.10 H2S 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). The levels of \(H_2S\) in the hippocampi were measured.

### 2.11 Observation of mitochondrial morphology and structure under an electron microscope

The hippocampal tissues of rats in each group were taken for electron microscopic observation, and electron microscopic pictures were taken. Twelve hours after euthanasia, 1 cubic millimeter pieces of the 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. 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 P. R. China).
2.12 Extraction of mitochondria from the hippocampus

According to the instructions of the kit for mitochondrial extraction, the cells were first broken by a mechanical method, then debris and giant organelles were removed by centrifugation at 4 °C for 5 minutes at 800 × g, and finally, mitochondria were obtained by centrifugation at 4 °C for 10 minutes at 15000 × g.

2.13 Determination of catalase activity

According to the instruction of the catalase kit, the absorbance was measured at 405 nm by spectrophotometer, and the catalase activity was calculated according to the equation defined at the end of this 13. 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 Catalase 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 405 nm. The Catalase Activity was calculated by (measured OD value-control OD value) / (standard OD value - blank OD value) × standard concentration × 6 × 7.8/sample protein concentration.

2.14 Quantitative detection of F-actin protein expression by Western blotting

The abdominal cavity anesthetized the rats. The brain was taken off, and the fresh hippocampal tissue was separated. The fresh hippocampal tissue was immediately put into a liquid nitrogen tank for reserve. The whole protein was extracted by centrifugation after 30 minutes of static storage with the appropriate proportion of tissue lysate and protease inhibitor. BCA protein quantitative method was used to detect the protein concentration. The extracted protein was diluted to the same concentration and stored at -80 °C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (separation gel 12%, concentrate gel 5%) 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). 5% skim milk powder was sealed for 90 min; The membrane was incubated with primary antibody (diluted antibody concentration 1 : 250) overnight at 4 degrees; TBST (Tris-Buffered Saline and Tween 20) buffer was used to wash for 3 times, and then the membrane was incubated with secondary antibody (Goat anti-rabbit, antibody diluted to 1 : 1000); TBST rinsed, ECL. Strip analysis was then performed using an imaging system (UVP, Upland, CA, USA). The results were analyzed by the image-Pro Plus image analysis software. 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.

2.15 Statistical analysis

Data were expressed as the mean ± SD 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.

3. Results

3.1 AOAA treatment significantly improves study and memory performance of rats with chronic alcoholism

As shown in Fig. 1 and Table 1, compared with the control group, the rats of the model group and the AR group needed significantly more training time for exploration of space ($P < 0.05$). After the intervention of AOAA, the number of training times required for the spatial resolution of rats in the AR group was significantly reduced ($P < 0.05$), When compared with the model group. The results suggest that AOAA can improve the learning and memory ability of rats with chronic alcoholism, but the learning ability of the AR group is worse than that of the control group.

Figure 1. The learning and memory ability of rats in the model group was significantly reduced compared with the NC group. However, after the AOAA intervention, the learning and memory ability of the rats in the AR group was significantly improved compared with the model group. NC: control group M: model group AR: AOAA remedy group. ▲ vs. NC Group $P < 0.05$; ★ vs. M Group $P < 0.05$.

3.2 Data preprocessing and screening of DEGs

After processing the data, we found that there were 208 DEGs in the model group compared with the control group, of which 51 genes were up-regulated, and 157 genes were down-regulated. The red dots indicate the up-regulated genes, the green dots indicate the down-regulated genes, and the blue dots indicate non-differential genes.

3.3 GO enrichment analysis and pathway analysis of differentially expressed genes

The results showed that most GO Terms and parts of pathways had no statistical difference, which was related to the number of differentially expressed genes and KEGG databases. These GO terms, however, are related to this study's goal. Therefore, we chose 10 GO Terms with higher enrichment for analysis. Among the comparison between the AOAA treatment group and the chronic alcoholism model group, the biological process analysis of GO enrichment Table 2 shows that the differential genes mainly mediate humoral immune response mediated by circulating immunoglobulin, regulation of type I hypersensitivity, regulation of inflammatory response to antigenic stimulus, regulation of hypersensitivity, immunoglobulin mediated immune response, type II hypersensitivity, B cell-mediated immunity, oxidation-reduction process, respiratory electron transport chain, energy derivation by oxidation of organic compounds, and so on Table 3 Molecular function of GO enrichment indicates that the differential genes mainly involves in sulfur amino acid transmembrane
Table 1. The learning and memory ability of rats from three groups

| Group      | n  | Mean ± SD  | F    | P for ANOVA | P values for LSD post-hoc test |
|------------|----|------------|------|-------------|-------------------------------|
|            |    |            |      |             | NC Group vs. M Group          |
| NC Group   | 16 | 38.94 ± 2.57 | 482.786 | 0.000 < 0.05 | 0.000 < 0.05                 |
| M Group    | 16 | 82.25 ± 4.23 | 0     | 0.000 < 0.05 | 0.000 < 0.05                 |
| AR Group   | 16 | 63.25 ± 4.73 | 0     | 0.000 < 0.05 | 0.000 < 0.05                 |

transporter activity, Rho GTPase binding, NADH dehydrogenase activity, oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor, Amino acid transmembrane activity, Ras GTPase binding, sequence-specific DNA binding RNA polymerase II transcription factor activity, sequence-specific DNA binding transcription factor activity, amine transmembrane transporter activity, and other functions. The results of KEGG enrichment analysis (Fig. 2; Table 4) show that the first 10 marked enriched signaling pathways are metabolic pathways, pathways in cancer, Huntington's disease pathway, Alzheimer's disease pathway, B cell receptor signaling pathway, Oxidative phosphorylation pathway, Phagosome pathway, Fc gamma R-mediated phagocytosis pathway, Parkinson's disease pathway, and Ribosome pathway. The following table shows differentially expressed genes involved in signaling pathways (Table 5).

3.4 Analysis of the interaction of proteins encoded by differentially genes

We imported these 208 differentially expressed genes into the online tool-STRING database. Only 198 genes can be searched for relevant information. We removed proteins that did not interact with other proteins to get a protein-protein interaction map (Fig. 3). And we imported it into the Cytoscape for further analysis and visualization, removing the degree-1 protein and divergent local protein interaction network to achieve a final network of central protein interaction (Fig. 4). The larger the circle, the more complex the interaction, and the greater the role it plays. The proteins, such as Rpsa, Rps11, Rps9, Mrt04, Nduba2, Wdr31, Mrp135, Rpl6, Dap3, Ndub8, Ndub6, Nduba4, Rela, Mrps28, Snpd2, Mtrf11, interact with other multiples (≥ 3) proteins. They are the central proteins of the interaction network (Table 6). If these central proteins were removed, the network structure was fragmented.

3.5 AOAA treatment significantly reduced the excessive H2S level in hippocampus

As shown in Fig. 5 and Table 7, the H2S content in the hippocampus of the model group was significantly higher than that in the NC group, with significant difference (P < 0.05), which proved that alcoholism could increase the content of hydrogen sulfide in the hippocampus of rats. After the intervention of AOAA, the content of H2S in the hippocampal tissue of the AR group was significantly reduced compared with that in the model group (P < 0.05), but is significantly higher than that of control group (P < 0.05). AOAA is a cystathionine-beta-synthase inhibitor and can reduce H2S formation in the brain by inhibiting cystathionine-beta-synthase activity, which agrees with our experimental results. The results showed that AOAA might protect the learning and memory abilities of rats by reducing excessive hydrogen sulfide produced by chronic alcoholism.
Table 2. Biological processes involved in differential genes

| GO Terms for NC Group vs. M Group                                      | Count | Corrected P-Value |
|---------------------------------------------------------------------|-------|-------------------|
| humoral immune response mediated by circulating immunoglobulin       | 4     | 0.12343           |
| regulation of type I hypersensitivity                               | 2     | 0.48256           |
| regulation of inflammatory response to antigenic stimulus           | 2     | 0.48256           |
| regulation of hypersensitivity                                      | 2     | 0.48256           |
| immunoglobulin mediated immune response                              | 4     | 0.61783           |
| type II hypersensitivity                                            | 2     | 0.71904           |
| B cell mediated immunity                                            | 4     | 0.96818           |
| oxidation-reduction process                                         | 4     | 1                 |
| respiratory electron transport chain                                 | 3     | 1                 |
| energy derivation by oxidation of organic compounds                 | 3     | 1                 |

Table 3. The molecular function involved in differential genes

| GO Terms for NC Group vs. M Group                                      | Count | Corrected P-Value |
|---------------------------------------------------------------------|-------|-------------------|
| sulfur amino acid transmembrane transporter activity                 | 2     | 0.09918           |
| Rho GTPase binding                                                   | 2     | 0.97862           |
| NADH dehydrogenase (quinone) activity                               | 3     | 0.23421           |
| NADH dehydrogenase activity                                          | 3     | 0.23421           |
| oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor | 3     | 0.28857           |
| Amino acid transmembrane activity                                    | 3     | 0.72336           |
| Ras GTPase binding                                                   | 3     | 0.77513           |
| sequence-specific DNA binding RNA polymerase II transcription factor activity | 4     | 0.21999           |
| sequence-specific DNA binding transcription factor activity          | 4     | 0.28371           |
| amine transmembrane transporter activity                             | 4     | 0.3391            |

Figure 3. A network of differentially expressed genes protein-protein interaction (PPI) was constructed by STRING online database. Each circle represents a protein, and the line indicates the interaction between the two proteins: the more the line, the more important the position of the protein in the network.

3.6 AOAA treatment significantly alleviates mitochondrial damage in hippocampal neurons

Mitochondrial electronic microscopy in the hippocampal tissue cells of the control group was intact and occasionally defective. Most of the rats in the model group had vacuoles in the mitochondria, and the crest was ruptured and swollen. After treatment with aminohydroxyacetic acid, mitochondrial morphology improved.

The mitochondrial morphology of the hippocampal tissue cells was observed under the electronic microscope, as shown in Fig. 6A-C, with the black arrow pointing to the normal mitochondria and the white arrow pointing to the pathological mitochondria.
Figure 4. Differentially expressed genes protein-protein interaction (PPI) network was visualized using Cytoscape software. We delete the simple isolated network and only keep the most important subnetworks. The blue, green, and yellow labeled proteins constitute three important subnetworks, of which the yellow protein is the most important one, which is mainly related to energy metabolism disorder, mitochondrial damage, and oxidative phosphorylation.

Table 4. Analysis of Pathway enrichment related to differential genes

| Pathway ID   | Pathway                     | Count | P-value       |
|--------------|-----------------------------|-------|---------------|
| ko01100      | Metabolic pathways          | 22    | 0.0827525     |
| ko05200      | Pathways in cancer          | 8     | 0.2000734     |
| ko05016      | Huntington’s disease        | 8     | 0.0159559     |
| ko05010      | Alzheimer’s disease         | 7     | 0.0272287     |
| ko04662      | B cell receptor signaling pathway | 7 | 0.01873595 |
| ko00190      | Oxidative phosphorylation   | 7     | 0.0015559     |
| ko00563      | Phagosome                   | 1     | 0.2774405     |
| ko04666      | Fcgamma R-mediated phagocytosis | 6 | 0.0807513 |
| ko05012      | Parkinson’s disease         | 6     | 0.0166793     |
| ko03010      | Ribosome                    | 5     | 0.7093742     |

3.7 AOAA treatment significantly improves catalase activity in mitochondria

As shown in Fig. 7 and Table 8, compared with the control group, mitochondrial catalase activity in the hippocampi of the model group decreased, with significant difference ($P < 0.05$). After the intervention of AOAA, the activity of mitochondrial peroxidase activity in the hippocampi of the AR group was significantly higher than that of the model group ($P < 0.05$), but is significantly lower than that of control group ($P < 0.05$).

3.8 AOAA treatment significantly improves the expression of F-actin in hippocampal tissue

As shown in Fig. 8 and Table 9, compared with the control group, the content of F-actin in the hippocampus of the model group was significantly decreased ($P < 0.05$). After the intervention of AOAA, the content of F-actin in the hippocampal tissue of the AR group was significantly increased compared with that in the model group ($P < 0.05$). However, the content of the F-actin of the AR group was significantly lower than that of the NC group ($P < 0.05$).

4. Discussion

Chronic alcoholism is a progressive, potentially fatal disease. Excessive drinking can cause severe damage to the normal functions of the central nervous system, lead to cognitive dysfunction with regards to learning and memory and will cause a series of social problems. However, the mechanisms underlying the cognitive impairment and other related conditions caused by chronic alcoholism are still not clear. Its pathogenesis may involve the changes of expression of multiple genes and signal transduction pathways. Using bioinformatics analysis, we analyzed the sequencing data of hippocampal samples from rats with chronic alcoholism and found that the metabolic pathway, oxidative phosphorylation pathway, and other signaling pathways were significantly enriched. The differentially expressed genes, such as Cox6c, Ndufb8, Ndufa4, Ndufa2, and Ndufb6 are involved in the oxidative phosphorylation pathway, Huntington’s disease signaling pathway, Parkinson’s dis-
Table 5. Differential genes involved in the first 10 pathways with the highest concentration

| Pathway                          | Differentially expressed genes                                                                 |
|---------------------------------|-------------------------------------------------------------------------------------------------|
| Metabolic pathways              | Cyp4f6, Cox6c, Ndufb8, B3gat2, Acer2, Gsta4, Tcirg1,                                          |
| Pathways in cancer              | Tecl2, Bcr, Gsta4, Tr, Mmp9, Dv11, Raf1, Rela                                                  |
| Huntington’s disease            | Cox6c, Ndufb8, Tcb, LOC679739, Ndufa4, Pacsin3, Ndufa2, Ndufb6                                |
| Alzheimer’s disease             | Ppp3cb, Cox6c, Ndufb8, LOC679739, Ndufa4, Ndufa2, Ndufb6                                      |
| B cell receptor signaling pathways | LOC100363671, LOC100363606, Ppp3cb, RGD156339, Raf1, Igsf1, Rela                             |
| Oxidative phosphorylation       | Cox6c, Ndufb8, Tcirg1, LOC679739, Ndufa4, Ndufa2, Ndufb6                                      |
| Phagosome                       | LOC100363671, LOC100363606, RT1-Ba, Tcirg1, RGD1306991, Sec61g                               |
| Fc gamma R-mediated phagocytosis | LOC100363671, LOC100363606, Prkcd, LOC689074, Arpc1b, Raf1                                  |
| Parkinson’s disease             | Cox6c, Ndufb8, LOC679739, Ndufa4, Ndufa2, Ndufb6                                              |
| Ribosome                        | Rps11, Mrto4, Rpsa, Rpl6, Rps9                                                               |

Figure 6. Ultrastructure of mitochondria in 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 containing 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: Amino-oxyacetic acid.

ease signaling pathway, and Alzheimer’s disease signaling pathway; additionally, their expression showed a significant change, and the proteins they encode comprised the central proteins in the PPI network. Among these genes, Ndufb8, Ndufa4, Ndufa2, and Ndufb6 are subunits of complex I. The downregulation of Ndufa4, Ndufa2, and Ndufb6 may be closely related to the deficiency of complex I, which suggests that alcoholism leads to oxidative stress and mitochondrial damage in hippocampal neurons.

Many studies have shown that oxidative stress and mitochondrial dysfunction play an important role in cognitive impairment. Our transmission electron microscopy analysis showed that compared with the rats of the control group, the number of mitochondria in the neurons of rats from the model group was low, and structural changes such as swelling and crest dissolution were observed. However, the morphology of the mitochondria in rats treated with aminoglycolic acid improved, suggesting that AOAA has a protective effect on mitochondrial function.

In a related study carried out by Zheng (2017), it has been shown that cognitive impairment is closely related to energy metabolism, respiratory electron transport, the chemical coupling of ATP synthesis, the heat generated by the uncoupling of proteins, and electron transport chains. Several previous studies (Bowling and Beal, 1995; Swerdlow, 2012) have reported that energy metabolism disorders are one of the key reasons for the development of cognitive impairment.

The electron transfer chain in mitochondria is mainly composed of the following four parts: (i) the flavoprotein; (ii) the iron-sulfur protein; (iii) cytochrome; and (iv) ubiquinone. These are all hydrophobic molecules. All these components, except ubiquinone, are proteins and transfer electrons through the reversible oxidation-reduction of their subunits, forming four complexes on the membrane surface: complex I (NADH dehydrogenase complex or Mitochondrial complex I, CI), complex I (succinate dehydrogenase complex), complex I (cytochrome reductase complex), and complex I (Cytochrome oxidase complex). NADH passes through the following order: CI coenzyme Q complex I cytochrome C complex IV to transfer electrons to oxygen; at the same time, the proton is transferred to the mitochondrial intermembrane space. CI is involved in the first part of mitochondrial oxidative phosphorylation (Ugalde et al., 2004), and its role is to
Table 6. PPI network central protein

| Gene   | log2 Ratio | P         | FDR        | Degree |
|--------|------------|-----------|------------|--------|
| Rpsa   | -1.37420703| 3.65E-15  | 2.34E-13   | 8      |
| Rps11  | 1.154600764| 2.39E-40  | 7.44E-38   | 7      |
| Rps9   | -1.1669571 | 1.77E-09  | 6.06E-08   | 6      |
| Mrto4  | -1.41124393| 4.24E-05  | 0.00063154 | 6      |
| Ndufa2 | -1.38231729| 8.74E-23  | 9.77E-21   | 5      |
| Wdr31  | -1.28379297| 6.11E-15  | 3.84E-13   | 5      |
| Mrpl35 | -1.53349838| 5.47E-26  | 7.71E-24   | 3      |
| Rpl6   | -1.18204578| 1.75E-67  | 1.31E-64   | 4      |
| Dap3   | -1.09459493| 9.83E-11  | 3.92E-09   | 4      |
| Ndufb8 | 1.16355994 | 5.56E-26  | 7.1E-24    | 3      |
| Ndufb6 | -1.08073127| 1.82E-25  | 2.41E-23   | 3      |
| Ndufa4 | -1.47559198| 1.27E-21  | 1.26E-19   | 3      |
| Rela   | -1.03891322| 6.27E-05  | 0.00089583 | 3      |
| Mrps28 | 1.21759435 | 4.17E-05  | 0.00062449 | 3      |
| Snrpd2 | 1.46872537 | 5.1E-05   | 0.00079742 | 3      |
| Mtrf1l | 1.78362703 | 1.92E-05  | 0.00032028 | 3      |

log2 Ratio < 0 indicates that the gene is down-regulated, log2 Ratio > 0 means that the gene up-regulated.

Figure 7. The mitochondrial catalase activity in the hippocampus of the model group was significantly reduced when compared with the NC group. Compared with the model group, the mitochondrial catalase activity in the hippocampus of the AR group was significantly increased. NC: control group M: model group AR: AOAA remedy group. ✱ vs. NC Group P < 0.05; ✱✱ vs. M Group P < 0.05.

that the defects of CI are an important cause of human diseases. There is strong evidence showing that if suppressed, CI can accumulate large amounts of damage-causing substances, i.e., the combination of reactive oxygen species, nitrogenous materials, and environmental toxins; this plays a key role in the development of common neurodegenerative diseases, including Parkinson’s disease (Carroll et al., 2006; Mizuno et al., 1989), Alzheimer’s disease (Kim et al., 2001), Huntington’s disease (Arenas et al., 1998), amyotrophic lateral sclerosis (Vielhaber et al., 2000), Down’s syndrome, and schizophrenia (Ben-Shachar et al., 2000), and even the aging process (Lenaz et al., 2000).

Hydrogen sulfide (H₂S), long known for its peculiar odor and toxic properties in rotten eggs, has recently been identified as the third endogenous gas transmitter after carbon monoxide and nitric oxide (Carroll et al., 2006). H₂S plays an important role in signal transduction and the regulation in various tissues of the whole body, including the mammalian central nervous system, digestive system, and urogenital system (Du et al., 2004; Huang et al., 2016; Yuan et al., 2015). H₂S is synthesized from L-cysteine in the body by the action of CBS (Zhang et al., 2017). CBS, which is highly expressed in the nervous system (Chen et al., 2005), is a major enzyme in the brain that is responsible for the production of H₂S. In CBS-knockout mice, altering the activity of CBS has been shown to regulate the production of H₂S (Eto et al., 2002). In addition, H₂S participates in the pathophysiological processes of central nervous system-related diseases, such as epilepsy, stroke, neurodegenerative diseases, and neurotoxicity; its neuroprotective effects have been shown to depend heavily on its concentration and location (Yakovlev et al., 2017). AOAA has been shown to inhibit the expression of CBS, thereby reducing the formation of H₂S, inhibiting Ca²⁺ influx, and alleviating the intracellular Ca²⁺ overload, which alleviates cell damage. Therefore, AOAA may reduce...
Table 7. The hydrogen sulfide content of three groups

| Group     | n  | Mean ± SD | F    | P for ANOVA | P values for LSD post-hoc test |
|-----------|----|-----------|------|-------------|-------------------------------|
| NC Group  | 8  | 28.55 ± 1.86 |      | 0           | 0.000 < 0.05                  |
| M Group   | 8  | 45.22 ± 2.92  | 102.344 | 0           | 0.000 < 0.05                  |
| AR Group  | 8  | 34.69 ± 2.17  | 0    | 0.000 < 0.05 | 0.000 < 0.05                  |

Table 8. The mitochondrial catalase activity in the hippocampus of three groups

| Group     | n  | Mean ± SD | F    | P for ANOVA | P values for LSD post-hoc test |
|-----------|----|-----------|------|-------------|-------------------------------|
| NC Group  | 8  | 8.46 ± 0.36 |      | 0           | 0.000 < 0.05                  |
| M Group   | 8  | 4.71 ± 0.34  | 219.37 | 0           | 0.000 < 0.05                  |
| AR Group  | 8  | 6.89 ± 0.38  | 0    | 0.000 < 0.05 | 0.000 < 0.05                  |

The excessive hydrogen sulfide produced by alcoholism and alleviate the intracellular Ca\(^{2+}\) to protect mitochondria and improve mitochondrial catalase activity.

The catalase can effectively decompose H\(_2\)O\(_2\) (a mitochondrial active free-radical product) into H\(_2\)O and O\(_2\) (Yang, 2013). Increased catalase activity can prevent cells from entering the apoptosis stage, and is an indicator of mitochondrial damage. Previous studies have shown that catalase activity decreases when the mitochondria are damaged (Fu, 2010).

The cytoskeleton is a dynamic fiber network; it can cause cell shrinkage, maintain the shape, movement, adhesion, and energy consumption of cells, enable the transmission of signals among cells, and perform other functions. Microfilaments in the cytoskeleton are an important part of the postsynaptic structure, dendritic spine. Microfilaments are mainly composed of actin, which can be divided into two types: globular monomer (G-actin) and fibrous polymer (F-actin). The transformation between them determines the morphology and function of dendritic spines. There is evidence that F-actin is an important substrate for LTP induction and maintenance (Fukazawa et al., 2003). A certain degree of actin aggregation can be used as a marker of dendritic spine activation in the LTP process. Similar changes have been observed in the learning process of rats. Therefore, actin remodeling characterized by F-actin alteration may play an important role in the synaptic mechanism (LTP). It also participates in the occurrence and development of some diseases (Valensin et al., 2002). Actin is one of the most important cytoskeletal components in eukaryotic cells, and its monomer (G-actin) and fibrous (F-actin) forms have been shown to transform into one other (Yan et al., 2011).

Actin is present in the form of F-actin when it functions because F-actin can change its spatial structure and perform specific functions; thus, it can better reflect the changes of the cytoskeleton (Tang et al., 2004). Therefore, the integrity of F-actin is necessary for the maintenance of the normal functions of cells. Intracellular calcium ions play an important role in regulating the assembly and decomposition of the cytoskeleton. A high concentration of calcium ions can also degrade the cytoskeleton, membrane proteins, and enzymes in neurons, thereby impairing the structures and functions of cells. Relevant studies (Carloni et al., 2004; Franco and Huttenlocher, 2005; Goll et al., 2003; Li et al., 2009) have shown that the increase in the levels of ethanol and its metabolite acetaldehyde, oxygen-containing free radicals, and the toxic effects of high concentrations of H\(_2\)S can not only activate NMDA receptors, open receptor-gated calcium channels, and continuously increase the concentration of cytosolic Ca\(^{2+}\), but also intensify the deficiency of assembly energy and decomposition of F-actin. The decrease of the intracellular F-actin content results in the dissolution, remodeling, and collapse of the cytoskeleton, alteration of the synaptic structure, blockade of synaptic transmission, neuronal dysfunction, and decrease of learning and memory ability (Xu et al., 2015).

In summary, chronic alcoholism can activate CBS, catalyze the formation of H\(_2\)S, enhance the calcium overload mediated by the NMDA receptors, cause the swelling of the mitochondria, impede energy metabolism, aggravate the inadequacy of assembly energy and decomposition of F-actin. Besides that, it can cause the dissolution and collapse of the cytoskeleton, alter the synaptic structure, and block synaptic transmission; this, in turn, reduces the learning process.
ability and memory. When chronic alcoholism occurs, mitochondrial damage can lead to the upregulation of Ndufb8 and downregulation of Ndufa4, Ndufa2, and Ndufb6, which may inhibit the function of CI through the oxidative phosphorylation pathway. Huntington's disease pathway, Parkinson's disease signaling pathway, and the Alzheimer's disease signaling pathway. On the one hand, the levels of the free radicals produced by the body increase and damage the nerve cells. On the other hand, they also inhibit the electron transport chain activities in the mitochondria, resulting in the reduction of the ATP levels and damaging the cells. Also, combined with bioinformatics research methods, we screened 208 differentially expressed genes and the related signaling pathways, and further screened out 18 important central proteins of the PPI network. These differentially expressed genes, pathways, and proteins are closely related to the mechanisms underlying chronic alcoholism, which will enable the development of new strategies for the treatment of chronic alcoholism and related diseases. Also, as our experiments were carried out earlier, many genes could not be found in the KEGG database at that time, which resulted in the corrected P-value of our GO enrichment analysis and KEGG enrichment analysis larger than 0.05. But we can't simply assume that the results are meaningless, at least our experiments prove that there is some correlation. This is a flaw in this study, and more research is needed to support it further.

AOAA is an active CBS inhibitor that regulates the H₂S content. To explore the protective effects of AOAA in the brains of rats with chronic alcoholism, this study examined the H₂S content and protein expression of F-actin in the brain tissues. The results showed that compared to the rats in the control group, the CBS activity and H₂S content of rats from the group increased, but the F-actin protein content decreased, showing a significant difference (P < 0.05). However, when compared with the rats from the model group, the CBS activity and H₂S content in the brain tissues of rats from the low-, medium-, and high-dose AOAA treatment groups were reduced, and the hippocampal F-actin expression increased, showing a significant difference (P < 0.05). It is suggested that AOAA may reduce the alcoholism-induced increase in the CBS activity, thereby reducing the increase in the H₂S concentration caused by alcoholism, inhibiting the influx of Ca²⁺, reducing the intracellular Ca²⁺ overload, alleviating the alcohol-induced damage of F-actin, protecting the structure and function of neurons and mitochondria, and ultimately, improving the learning and memory ability of rats with chronic alcoholism. This experiment only measured the CBS activity, H₂S contents, and F-actin contents in the hippocampal tissues of rats. Further studies will provide more information regarding the scientific basis for the mechanisms underlying the damage caused by chronic alcoholism and the therapeutic effects of AOAA.

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Conflict of interest

The authors declare no competing interests.

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