Metabolomic Study on Electroacupuncture Treatment of Chronic Alcoholism in Rats with Learning and Memory Disorders

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Abstract

In recent years, electroacupuncture has been widely used in clinic, especially in the treatment of learning and memory dysfunction caused by chronic alcoholism, but its internal mechanism is not yet clear. In order to find the possible mechanism of electroacupuncture treatment for learning and memory disorders caused by chronic alcoholism, we exposed SD rats to alcohol to build a model. After successful modeling, electroacupuncture stimulated Baihui and Dazhui points. Metabolic profile of the brain tissue of the needle group. Using LC-MS / MS technology for research, we found that electroacupuncture treatment can effectively improve the learning and memory ability of rats with chronic alcoholism, and at the same time can reduce the endoplasmic reticulum by affecting the levels of metabolites such as glutamate metabolism and lipid metabolism in the hippocampus. Neurotoxicity such as stress and oxidative stress promotes local energy metabolism, improves intracellular homeostasis, enhances the synaptic plasticity of the hippocampus, protecting learning and memory ability.

Introduction

According to statistical research, related diseases caused by alcohol consumption worldwide are the seventh major risk factor for death and disability-adjusted life years, accounting for 2.2% of age-standardized female deaths and 6.8% of age-standardized male deaths^{1,2}. Chronic alcoholism is a disease that causes damage to the mental state, nervous system and internal organs due to long-term excessive drinking. Among the various pathological changes caused by it, brain damage is the most prominent and can lead to cognitive impairment³. Current research suggests that chronic alcoholism may be a complex process involving the central nervous system and multiple neurotransmitters⁴⁻⁶.

Alcoholism causes a variety of neurological complications. A survey of 550 adults, such as Topiwala⁷, revealed that there is a new type of dose-dependent relationship between alcohol intake and hippocampal atrophy. Zhou only drinking 14–21 units (112–168 grams) and the hippocampal atrophy rate are nearly three times, and the study population's cognitive ability of simple vocabulary decreased, and cognitive ability such as memory and recall decreased faster⁸. In another long-term prospective study from the United States⁹, people with long-term alcohol use were more likely to develop severe memory disorders than the control group (OR 2.21, 95% CI 1.27–3.85). Studies in animals with chronic alcoholism have shown that the hippocampus is continuously affected, and other hypothalamus nuclei, especially visual and paraventricular are also affected, leading to cognitive dysfunction¹⁰⁻¹².

The brain is the high-level life center of human beings. In addition to regulating daily life activities, it also has the function of regulating learning and memory. The hippocampus participates in the key areas of learning and memory formation. The main function of synapses is the transmission of information between nerve cells and synapses. Plasticity is closely related to learning and memory. Among them, synaptic plasticity is mainly affected by long-term potentiation (LTP), which is considered to be important of information processing and hippocampal neuron processing, synaptic regulation is also an important basis for learning and memory formation. Cell types and nerve cell information transmission in

hippocampal tissue are extremely complex. Many studies have shown that it can achieve cognitive and other related brain functions, including learning and memory, through multiple signal transduction pathways¹³⁻¹⁵. The learning and memory mechanism depends not only on the regulation and plasticity of synaptic transmission in neural networks, but also on the modification of existing synaptic proteins.

The generation and formation of learning and memory in the brain involves many biochemical processes involving very complex neuronal protein molecule interactions. External stimulus signals are enhanced or weakened through multi-phase synaptic transmission, and eventually converted into the synthesis of a variety of new proteins. And modification, and cause corresponding biochemical reactions and signal pathways. Multiple studies have confirmed that learning and memory functions are closely related to the regulation of multiple genes and their function through their corresponding protein expression products. At present, there are more and more studies on NMDA receptor-related pathways and protein molecules related to CREB (cAMP-response element binding protein) -related pathways, all of which play a key role in learning and memory formation¹⁶⁻¹⁹.

Electro-acupuncture is used to treat systemic diseases through the conduction of meridians and acupuncture points. The main treatment principle is to pierce the needle into the acupuncture point to lead the microcurrent that the human body can tolerate, and stimulate the acupuncture point with the microcurrent to achieve the purpose of treatment. Numerous clinical experiments and animal studies have shown that electricity has a good effect on the relief of various brain diseases. Studies by Lin Zhicheng et al.²⁰ and others have shown that electroacupuncture can improve patients' cognitive dysfunction, Liu et al.²¹ Animal experiments have been conducted, and the results show that electroacupuncture can improve learning and memory dysfunction, which may be because electroacupuncture relieves LTP inhibition in hippocampal neurons in rats, and is therefore widely used in clinical practice to treat a variety of diseases, including chronic diseases. Compared with other clinical treatment methods, cognitive decline caused by alcoholism and peripheral neuropathy caused by chronic alcoholism have the advantages of simplicity, convenience, and no toxic side effects.

The effect of electricity on learning and memory is a very complex multi-dimensional biological network process, which necessarily involves single or multiple pathways, and one or more groups of specific targets interacting in biochemical reactions and signaling. In experimental research, it only affects a certain signal pathway or protein expression in the brain, and the results of these experimental studies often come from independent experiments, which study the function of a certain protein molecule individually. The experiments cannot be performed at the same time. It is impossible to obtain as much biological information as possible in the living body. As far as the network complexity and overall characteristics of brain cognitive functions are concerned, similar experimental studies are relatively single, time-consuming and inefficient, and various problems cannot be achieved. The purpose of large-scale and efficient analysis is also unable to grasp the brain function mechanism of acupuncture on cognitive functions such as learning and memory. Based on the above reasons, the study of hippocampal metabonomics on the mechanism of cognitive function in rats with chronic alcoholism has become inevitable.

Therefore, this project aims to start from the multi-dimensionality and complexity of cognitive function mechanisms, and use LC-MS / MS metabolomics methods to screen out the signaling pathways and biological processes involved in significantly different metabolites. And further verified the accuracy of the results by Western Blot technology, preliminary exploration and analysis of differential protein expression changes in hippocampus tissues after electroacupuncture intervention, and the mechanism of electroacupuncture on cognitive function in chronic alcoholism rats from the perspective of metabolomics,And provide clues for future research.

Results

Behavioral analysis of rats after electroacupuncture treatment

Before modeling, there was no significant difference between the rats in the blank group, model group and electro-acupuncture group; after modeling, the learning and memory abilities of rats decreased significantly (Table 1, Table 2); after 6 weeks of electro-acupuncture intervention, compared with the model rats, the learning and memory abilities of rats in the electroacupuncture group were significantly improved ($P < 0.05$), as shown in Figs. 1 and 2.

Table 1
Comparison of escape latency of rats in each group at the same time (s,±S)

Group	Before the modeling	6 weeks after drinking	After 6 weeks of treatment
Control group	12.7 ± 4.26	11.77 ± 5.8	9.85 ± 3.81#
Modeling group	11.71 ± 3.88	24.16 ± 3.47*	27 ± 4.76*
EA group	12.05 ± 3.69	24.53 ± 3.56*	15.86 ± 3.03*#
Note: * $P < 0.05$ compared with the blank group; $P < 0.05$ compared with the model group			

Table 2
Fourth quadrant exploration time of each group of rats at the same time

Group	Before modeling	6 weeks after drinking	After 6 weeks of treatment
Control group	42.97 ± 3.92	46.37 ± 3.13	50.32 ± 2.68#
Modeling group	42.6 ± 3.06	26.11 ± 3.16*	24.27 ± 2.59*
EA group	41.65 ± 3.66	26.12 ± 3.34*	33.25 ± 2.95*#
Note: * $P < 0.05$ compared with the blank group; $P < 0.05$ compared with the model group			

Mass spectrometry analysis of hippocampal tissue metabolism in model group and electroacupuncture group

Metabolic mass spectrometry was performed on the hippocampus of the model group and the electroacupuncture group. The total ion current (TIC) chromatogram was obtained after UPLC-Q-TOF (LC-MS/MS) detection and analysis, as shown in Fig. 3. Mass spectrometry analysis showed that different metabolites were observed in the positive and negative ionization modes, and different metabolites could be analyzed and identified according to the two ion modes. We observed significant differences in some peaks in the chromatograms of the model group and the electroacupuncture group, suggesting that electroacupuncture treatment may significantly alter the distribution of metabolites in the brain.

OPLS-DA model analysis of hippocampal metabolism in model group and electroacupuncture group

In order to identify the metabolomics differences between the model group and the electroacupuncture group, a supervised orthogonal partial least squares discriminant analysis (OPLS-DA) model was established to observe the distribution of the two groups. The score map of the OPLS-DA model in the hippocampus showed a significant difference between the model group and the electroacupuncture group (Fig. 4A). The fitness and goodness (R^2) and predictability (Q^2) of the model were used to evaluate the quality and effectiveness of the model. The figure shows that the model's R^2 value is greater than 0.5, indicating a good fit. Therefore, variable importance (VIP) ≥ 1 is considered to be a significant change m/z that mediates population clustering. In the hippocampus, in all the m/z ratios detected in the positive ion mode and the negative ion mode, compared with the electroacupuncture group, a total of 53 ratios changed significantly ($p < 0.05$) (Fig. 4B). These results suggest that chronic alcoholism can cause dramatic changes in the metabolic profile of hippocampal tissues in rats, and electroacupuncture has a significant effect on the metabolic profile of hippocampal tissues in chronic alcoholism rats.

Bioinformatics analysis of metabolic differences

Hierarchical cluster analysis (Figure 5A) was performed on the expression levels of the 53 significantly different metabolites that were screened to assist in the study of the relevant metabolic processes of the two groups of metabolites in the electroacupuncture group and the model group. The Pearson correlation analysis method was performed, Calculated the correlation coefficient between the significantly different metabolites (Figure 5B), according to the figure, there is a significant correlation between the different metabolites.

Analysis of KEGG metabolism-related pathways

The related metabolite data information obtained in the comparison between the electroacupuncture group and the model group was used for enrichment of metabolic pathways and analysis of related pathways, so as to further analyze the metabolic pathways involved in the regulation of hippocampus by electroacupuncture in chronic alcoholism rats. Figure 6 shows the analysis of KEGG-related pathways and metabolic pathway enrichment analysis, mainly involving 10 pathways, including glutamate metabolism, lipid metabolism, neurotransmitter signaling, protein digestion and absorption, amino acid biosynthesis and Metabolism, aminoacyl-tRNA biosynthesis, abc transporter; significant differential metabolites are mainly concentrated in lipid metabolism and glutamate metabolism pathways.

Discussion

A large number of literature studies have shown that long-term abuse of alcohol can lead to irreversible changes in brain structure and function.^{22,23} Especially the hippocampus tissue related to memory storage and processing is more susceptible to neurotoxic damage.^{24–26} And in most neuropathology, the hippocampus area has been shown to be the most affected brain area.²⁷ So far, electroacupuncture has been widely used in the treatment of brain injury in patients with chronic alcoholism and has achieved good results, but its underlying molecular mechanism has not been fully determined.

The study of Johnson et al.²⁸ showed that patients with chronic alcoholism and cognitive impairment are often accompanied by white matter and gray matter brain structure defects on brain CT. In many studies, the relationship between the degree of brain atrophy and alcohol consumption It is widely known that the modification of synapses in the brain, especially in the hippocampus, may be the cellular mechanism of brain memory storage and learning and memory.²⁹

In the metabolomics results, we found that after electroacupuncture at Baihui and Dazhui points, the lipid metabolism process in the hippocampus area has undergone significant changes, and the lipid metabolites of glycerophospholipids, glycerolipids and fatty acyl in the hippocampus have all occurred A significant change. Glycerophospholipids are a key component of the membrane structure of mammalian mitochondria. Due to their different biological functions, their composition is very different in the different cell types, organelles and inner and outer membranes of mammalian mitochondria.^{30,31} Most glycerophospholipids are synthesized through two synthetic pathways, namely the Kennedy pathway and the CDP-DAG (cytidine diphosphate-diacylglycerol) pathway³². In the CDP-DAG pathway, phospholipid synthesis strongly depends on the transport of phospholipids between the endoplasmic reticulum and mitochondria and between the outer and inner mitochondrial membranes³³, and glycerophospholipids are the most abundant type of phospholipids. These lipids are biological Part of the membrane is the active substance on the bile and the membrane surface. Glycerophospholipids also act as messengers in the process of cell signal transmission³⁴, providing stability, fluidity and permeability to the nerve membrane, so they are required to have Intact membrane protein, receptors and normal biological functions of ion channels, electroacupuncture at Baihui and Dazhui points has a very important effect on the regulation of the nervous system and immune system, and has a regulatory effect on organ function,

neuroinflammatory response, etc.³⁵ Studies have shown that electroacupuncture can inhibit the expression level of pro-inflammatory factors and play a neuroprotective effect. The effectiveness of cognitive improvement in AD and vascular dementia has been confirmed in different clinical and animal studies^{36–38}. Therefore, our research results indicate that electroacupuncture at Baihui and Dazhui points may affect brain function by changing the composition of glycerophospholipids. In fact, changes in the lipid composition of nerve membrane glycerophospholipids have been reported to be related to neurological diseases³⁹. In addition, since the myelin sheath contains the highest content of glycerophospholipids in the meninges⁴⁰, we speculate that electroacupuncture treatment of chronic alcoholism rats may inhibit most of the changes in glycerophospholipids, thereby reducing the breakage of myelin fibers and preventing neurons from more serious injuries.

In experimental studies, we found that chronic alcoholism can significantly change glycerophospholipids and glycerophospholipids-related phospholipids, which indicates that alcohol may activate lipogenesis-related enzymes, thereby affecting lipid synthesis pathways, and electroacupuncture can reduce lipid abnormal accumulation of metabolites. As we all know, the endoplasmic reticulum membrane is mainly composed of lipids. It is also the central organelle of protein and lipid synthesis, membrane biogenesis, exogenous detoxification, and calcium homeostasis. It is responsible for maintaining various aspects of fatty acid and lipid homeostasis⁴¹, and Disorders of lipid metabolism can interfere with the normal operation of endoplasmic reticulum function, the strength of cell membrane structure and the basic functions of organelles, such as mitochondria, Golgi apparatus, and nucleus, causing endoplasmic reticulum stress, and PERK (Protein kinase RNA-like ER kinase) is activated, which damages memory-related functions^{42,43}. Therefore, electroacupuncture at Baihui and Dazhui points may change the lipid distribution in the hippocampus and reduce the effect of alcohol on nerves in the brain. Damage and inflammatory stress response. At the same time, it also shows that electroacupuncture can inhibit the endoplasmic reticulum stress caused by chronic alcoholism in the brain, maintain the homeostasis and cell function of central cells, and protect learning and memory.

Therefore, we speculate that after 6 weeks of alcohol gavage, the alcohol-induced effect in the hippocampus disrupts lipid metabolism in the brain, leading to oxidative damage to brain DNA, proteins, lipids, and even organelles and mitochondria; on the other hand, for 6 consecutive weeks of alcohol gavage may cause the up-regulation of acid sphingomyelinase (ASMase) expression. ASMase can induce endoplasmic reticulum stress and activate the lipid pathway by synthesizing ceramide. Ceramide has an important contribution to the structure of cell membranes and exerts many basic cell processes, including cell growth, proliferation, movement, adhesion, differentiation, aging, and apoptosis. Increased ceramide can lead to lipid toxicity and endoplasmic reticulum stress. It is found in the pathogenesis of several neurodegenerative diseases^{44–48}. In this experiment, the metabolomic analysis results are consistent with the recent research literature. The results show that in the brain tissue of chronic alcoholism, we noticed that the level of ceramide in the hippocampus is up-regulated, suggesting that the brain endoplasmic reticulum may respond to chronic alcoholism. After 6 weeks of intervention with electroacupuncture at Baihui and Dazhui points, electroacupuncture may inhibit the occurrence of

the endoplasmic reticulum stress process, thereby reducing the level of ceramide, and changing the metabolic profile to maintain the stability and enhancement of cell membranes. Neuroplasticity protects the integrity of brain tissue from damage and improves learning and memory functions.

In summary, by using metabolomics methods, we found that chronic alcoholism can cause profound changes in hippocampal metabolism. Electroacupuncture at Baihui and Dazhui points can reverse and promote the levels of neurotransmitters and lipid metabolites, suggesting electroacupuncture It can improve the inhibitory state of nerve signal transduction at an early stage, and improve the function of brain neurotransmitter and lipid metabolism. It is of great significance to reveal the pathophysiological mechanism of electroacupuncture intervention in the cognitive impairment of learning and memory in rats with chronic alcoholism.

Materials And Methods

Ethics statement

The animal care and experimental procedures used in this study have been approved by the Animal Care and Use Committee of Experimental Animal Center of Southwest Medical University (License No. :SWMU201803147).

Chemicals

Alcohol was purchased from Beijing Red Star Co., Ltd. Acetonitrile, methanol, and ammonia were purchased from Merck, Germany, and ammonium acetate was purchased from Sigma, USA.

Animals

Sprague-Dawley rats weighing 180-220 g. Purchased from the Experimental Animal Center of Southwest Medical University (Yinzhou, China), all SD rats were adaptively fed for one week under the conditions of indoor temperature of $(23 \pm 2)^\circ\text{C}$ and relative air humidity of $(65 \pm 5)\%$. They were able to eat and drink freely. All experimental procedures and animal use were in accordance with guidelines developed by the Laboratory Animal Care Evaluation and Accreditation Association and the Animal Care Use Committee of Southwestern Medical University.

Model preparation and treatment

SD rats were modeled after 1 week of adaptive feeding. The model was prepared based on Xu's²⁴ model of learning and memory impairment in rats at 10 ml/kg/d caused by chronic alcoholism. The standard was to irrigate with 56% alcohol for 6 weeks. The control group was given an equal volume of 10 ml/kg/d saline. Electroacupuncture Intervention

According to "Animal Acupoint Atlas" written by Hua⁴⁹ and "Experimental Acupuncture" written by Li⁵⁰ the acupuncture points were simulated based on the comparison of anatomy and osteometry. At the intersection of the two ears with the cranial vertex midline, the Dazhui acupoint is located between the 7th cervical spine and the 1st thoracic spinous process posterior midline, electroacupuncture was performed 6 weeks after modeling to locally disinfect the skin of the Baihui and Dazhui points of the rat. The rat Dazhui acupoint was used for 0.35 mm × 25 mm milli-needle vertical needle 5 mm; the rat Baihui acupoint was used for 0.35 mm × 25 mm milli-needle horizontal needle 2 mm, voltage 3 V, current 1 mA, frequency 3 Hz and continuous wave. Process for 30 min.

Morris water maze experimental behavior test

Through Morris water maze spatial memory measurement and positioning navigation experiments, the rats' learning and memory abilities were measured before modeling, 6 weeks after modeling and 6 weeks after electroacupuncture intervention.

Sample preparation

After the modeling, rats were anesthetized with 60 mg/kg pentobarbital sodium intraperitoneally at the end of administration. After quickly excising the brain, hippocampus were immediately dissected according to the brain atlas⁵¹ and frozen in liquid nitrogen before storage at -80 °C.

Metabolite extraction

According to the experimental procedure, all samples were homogenized in MP at low temperature, 200 µL of pre-cooled water and 800 µL of pre-cooled methanol / acetonitrile (1: 1, v / v) were added, mixed, and sonicated in an ice bath for 60 min. Incubate at -20 °C for 1 hour to precipitate the protein, and centrifuge at a rate of 16000 g for 20 min at 4 °C to centrifuge out the supernatant. Take equal amounts of metabolites according to the weight of each sample and dry them in a high-speed vacuum concentration centrifuge. During mass spectrometry detection, add 100 µL of acetonitrile-water solution (1: 1, v / v) for reconstruction, then 14000g. Centrifuge at 4 °C for 15 min. Remove the supernatant for analysis.

Mass spectrometric analysis of metabolites

The samples were separated by UPLC and analyzed by the Triple-TOF 5600 mass spectrometer⁵². The ESI source conditions were as follows: ion source gas 1 (Gas1): 60, ion source gas 2 (Gas2): 60, curtain gas (CUR): 30, Ion source temperature: 600°C, ion levitation voltage (ISVF) ±5500 V (both positive and negative modes); set 60-1200 Da as TOF MS scan m/z range, set 25-1200 Da as product ion scan m/z

Fan: TOF MS scan accumulation time 0.15 s/spectrum, product ion scan accumulation time 0.03 s/spectrum; the secondary mass spectrum is acquired by information-dependent acquisition (IDA), and adopts high sensitivity mode, aggregation potential (DP): ± 60 V (both positive and negative modes), collision energy: 30 eV, IDA settings are as follows: the isotope is within 4 Da, candidate ions to be monitored in each cycle: 6^{53,54}.

Data processing analysis

First, use XCMS software to extract metabolite ion peaks, and then use SIMCA-P 14.1 software (Umetrics, Umea, Sweden) for pattern recognition and multi-dimensional statistical analysis to test the repeatability of the experiment and find the overall distribution trend of possible discrete points; Finally, the metabolites with VIP>1 and univariate statistical analysis were used to screen the significant difference metabolites.

Statistical Analysis

Data analysis and processing were performed using SPSS17.0 statistical software, and the measurement data was expressed as mean $\pm$ standard deviation (indicating $\pm$ s). Comparison of means within groups was performed by paired t test, and comparison of multiple sets of sample data was performed by one-way ANOVA, after the variances were equal, grouped paired t test was used; those with different variances used rank And test, the difference is statistically significant as $P < 0.05$.

Declarations

Author Contribution

TTD performs NMR experimental data analysis, which is helpful for the manuscript preparation and the preparation of all graphs.PYZ helps with data analysis and manuscript preparation;BC contributed to the interpretation and revision of the manuscript;BC revised the manuscript;TTD is designed and tested.TTD and PYZ were performed in animal experiments and electroacupuncture.

Notes

The authors declare no competing financial interest.

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Figures

Figure 1

Escape latency of rats in each group Note: * $P < 0.05$ compared with the control group; $P < 0.05$ compared with the model group

Figure 2

Exploration time of the fourth quadrant of rats in each group at the same time Note: * $P < 0.05$ compared with the blank group. $P < 0.05$ compared with the model group

Figure 3

TIC diagram of positive and negative ions of the model group and electroacupuncture group samples.(A) Positive ion mode TIC spectrum of model group samples (B) Positive ion mode TIC spectrum of model

group samples(C) TIC spectrum of model group sample negative ion mode (D) TIC spectrum of model group sample negative ion mode

Figure 4

Analysis of OPLS-DA model comparison between model group and electroacupuncture group.(A) OPLS-DA score map of model group and electroacupuncture group. (B) Volcanic map of model group and electroacupuncture group. The x-axis shows the fold change in expression value and the y-axis significance level (log of p value).Significant changes in metabolites ($p < 0.05$) are marked in color, and metabolites without significant changes are shown in gray dots.Red and green indicate that the metabolites in the model group and the electroacupuncture group were significantly increased and decreased, respectively.

Figure 5

Metabolic analysis of metabolic differences in model group and electroacupuncture group.(A) Comparison of significant difference metabolite hierarchical clustering results between model group and electroacupuncture group (B) Comparison between model group and electroacupuncture group

Figure 6

Analysis of KEGG-related metabolic pathways (A) Bar chart of KEGG metabolic pathway enrichment analysis of significantly different metabolites.(B) Bubble chart of KEGG metabolic pathway enrichment analysis of significantly different metabolites

Supplementary Files

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- [supplementaryinformation.docx](#)