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# Metabolomics Study of the Effects of Zinc Sulfate in Mild Hepatic Encephalopathy

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## ABSTRACT

**Background:** Zinc may play a role in the metabolism of minimal hepatic encephalopathy (MHE).

**Objective:** To investigate the metabolic changes in MHE rats treated by oral zinc sulfate administration.

**Methods:** A total of 24 Sprague-Dawley rats were divided into three subgroups: MHE rats (n=8), ZN rats (MHE treated by zinc sulfate, n=8), and CN rats (sham operation controls, n=8). Morris water maze (MWM) was used to evaluate cognitive and motor functions. The metabolic changes of MHE, ZN, and CN rats were investigated using 1H nuclear magnetic resonance spectroscopy (1H-NMR) based metabolomics. Sparse projection to latent structures discriminant analysis was used to identify the key metabolites in MHE vs. ZN and MHE vs. CN. A diffusion algorithm was used for enrichment analysis of the key metabolomics in MHE.

**Results:** Significant prolonged escape latency of MWM were seen in the MHE rats. Shorter escape latency is shown in ZN rats after zinc supplementation. The 1H-NMR spectroscopy of MHE, ZN and CN rats showed that 47 metabolites were identified for key metabolites analysis. The concentrations of GABA, lactate, alanine, aspartate, glutamate, and glutamine were significantly increased, while that of myo-inositol, taurine, leucine, and isoleucine were significantly decreased in MHE rats. The concentrations of myo-inositol, taurine, glycine, leucine, and isoleucine were significantly increased, while that of lactate, alanine and glutamine were significantly decreased in ZN rats.

**Conclusions:** Zinc sulfate can effectively improve cognitive impairments in MHE rats. Zn played a center role in the nitrogen metabolism of MHE by interfering alanine, glutamate, BACCs metabolisms and TCA circle.

**KEYWORDS:** Nuclear magnetic resonance; Zinc sulfate; Minimal hepatic encephalopathy

## INTRODUCTION

Hepatic encephalopathy (HE) is a central nervous system dysfunction syndrome based on metabolism disorder caused by liver dysfunction and/or portosystemic shunting [1]. Minimal hepatic encephalopathy (MHE) refers to a subtype of HE that has no typical clinical manifestations, but has abnormal signs in the cognitive field [2]. The pathogenesis of MHE is complex and has not been fully elucidated. Elevated blood ammonia is considered the main cause of HE [1]. Therefore, therapies aimed at blood ammonia reduction contribute to HE resolution [2].

Several studies showed that trace element deficiency is common in cirrhotic patients [3,4]. Zinc is a co-enzyme of a variety of many metabolic enzymes, which plays critical in biochemical processes [5]. Deficiencies in zinc may lead to metabolic and behavior and cognition disorders [6]. It has been demonstrated that oral supplementation of zinc may be effective for MHE [7]. However, the potential metabolic mechanisms of zinc in MHE have not been investigated.

Nuclear magnetic resonance spectroscopy (NMR) is a technique for continuous observing metabolites include amino acids and fatty acids, which can be found in the blood, urine, and other bodily fluids, and tissues [8]. Recently, with the development of metabolomics analysis technology, metabolomics has attracted increasing attention in the medical field as a method to search for metabolism biomarkers of diseases [9]. We developed a rat model of MHE. 1H-NMR-based metabolomics was used to detect the metabolic changes in the rat brain with and without zinc supplementation. By comparing the metabolites, we expected to clarify the therapeutic effect of zinc in MHE metabolism.

## MATERIALS AND METHODS

### Research Ethics

This study was approved by the Institutional Review Board of Jinshan Hospital, Fudan University (No. 2017-01). All procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of Fudan University.

### Laboratory animals and models

Twenty-four male Sprague-Dawley rats (weighing 150-200 g) were divided into three subgroups: the MHE rats (n=8), the ZN rats (MHE treated by zinc sulfate, n=8), and the CN rats (sham operation controls, n=8). MHE rats were modeled by partially ligating the portal vein with the surgical procedures described in a previous report [10]. The ZN rats were provided zinc sulfate with a dose of 30 mg/kg/day elemental zinc (150 mg of zinc sulfate dissolved in 500 mL of deionized water) after partially portal vein ligation [7]. The dissolved zinc sulfate doses were adjusted weekly according to the rat's body weight and water consumption. The SN rats were given deionized water without any treatment. All rats were placed in metabolic cages and housed in a well-ventilated room with temperature (25±2 ℃), air humidity (50±10%) and a light/dark cycle of 12 h. All rats were allowed to drink and eat freely for 56 days. Ain-93 experimental animal standard rat purified feed were used to fed all the rats.

### Cognitive Assessment

Morris water maze (MWM) were used for cognitive assessment on the day 57-60. A black round pool with 180 cm diameter and 50 cm height were filled with water (30 cm from the bottom). The water temperature was adjusted to 24±1 ℃. The pool was divided into four quadrants (left top, right top, left bottom, and right bottom). A circular transparent platform of 12 cm was placed in the right top with 2 cm below the water surface. The rats were randomly placed from one quadrant into the water. Each rat had 60 s to find the platform. the time of the rats climbed the platform was record as the latency. If the rats were unable to reach the platform within 60 s, they would be guided to the platform. All the rats on the platform were allowed to rest for 20 s. All the rats were trained for 4 consecutive days. The tracks of the rats were recorded by a computer equipped with a camera.

### Sample Preparation, Blood Ammonia and Brain Mn Content Measurements

After the MWM test, the rats were killed by intravenous injection of KCl (1-2 mg/kg) under deep anesthesia by isoflurane. The brain tissues were removed, cleaned by cold saline, and died by filter papers. The striatums were separated on a frozen plate according to the anatomical map of the rat brain. The samples were stored in liquid nitrogen and then transferred to a -80℃ refrigerator for further analysis.

### 1H-NMR Metabolomics

Samples were weighed (10-20 mg) and added with 0.6 mL ice-cooled extraction solution (CH3OH: H2O=2:1). After vortexing for 1 min, the sediments were broken by a tissue crusher (30 Hz, 90 s). The samples were then centrifuged (4 °C, 10,000×g, 10 min), rotary evaporated, and freeze-dried for 24 h. The solid residues were added with 600 µL buffer solution (0.1 M NaH2PO4/K2HPO4, pH: 7.4). D2O (99.96% deuteriumenriched containing 0.05% TSP) was used as a chemical shift reference. Then samples were then transferred into 5 mm NMR tubes.

The 1H-NMR was performed by a 600 MHz NMR spectrometer (AVANCE III, Bruker, German) with Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence and water suppression. The basic parameters were as follows: pulse=90 °; water presaturation during the relaxation delay=2.5 s; width=10 ms; sampling points=32 K; spectral width=20 ppm; sampling time=1.36 s; scanning times=64; empty scanning times=; temperature=298 K. 1H-NMR free induction decays data were preprocessed in a previous reported pipeline [11]. Briefly, group delay correction, solvent suppression, apodization, fourier transform, zero-order phase correction, internal referencing, baseline correction, negative values zeroing, warping, window selection, bucketing, water region removal, zone aggregation and normalization were performed in sequences. The identification and quantification of the metabolites were performed by using a spectroscopy of 191 pure metabolites as a reference. Metabolites within 2 standard deviations of the background noise were not included in the further analysis.

Supervised analysis (sparse projection to latent structures discriminant analysis, sPLS-DA with 3-fold cross-validation) was used for pattern recognition analysis of the significantly affected metabolites in MHE vs. ZN rats and MHE vs. CN rats. The metabolites with variable influence on projection (VIP) score > 1 and $P < 0.05$ were defined as the key metabolites in MHE vs. ZN rats, and in MHE vs. CN rats, respectively.

A diffusion algorithm was used for enrichment analysis of the key metabolites in MHE vs. ZN rats, and in MHE vs. CN rats. The top-ranked significant different biological pathways were also plotted based on the KEGG database.

### Statistical Analysis

All the statistical analyses were performed in R (Version 4.0.2; http://www.r-project.org/). Data met normality and variance homogeneity were measured by ANOVA followed by false discovery rate (FDR) correction. If not met normality or variance homogeneity, the data were measured by Mann-Whitney U test followed by FDR. The "mixOmics" package was used for sPLS-DA analysis and VIP score calculation; the "FELLA" package was used for enrichment and pathway analysis. P<0.05 was considered statistically significant.

## RESULTS

### General Situation

The body weight, water consumption, feed consumption and the escape latency in MWM of the three groups is shown in Table 1. No significant of the body weight, water consumption, and feed consumption is found among the three groups. A slight reduce of water consumption is shown in the ZN rats compared with the CN rats. Significant prolonged escape latency of WWM were seen in the MHE rats. Shorter escape latency is shown after zinc supplementation Table 2.

*Table 1: The body weight, water consumption, feed consumption and the escape latency in Morris water maze*

| | CN (n=8) | ZN (n=8) | MHE (n=8) |
|---|---|---|---|
| T1 (s) | 55.8 (6.2) | 58.9 (3.3) | 59.9 (0.2) |
| T2 (s) | 39.8 (10.3) | 51.0 (13.2) | 56.2 (6.7) ** |
| T3 (s) | 32.2 (10.5) | 41.3 (7.8) | 49.6 (7.7) **# |
| T4 (s) | 18.1 (11.0) | 27.5 (12.3) | 42.8 (10.8) ***# |
| Weight(g) | 369 (14.1) | 373 (21.9) | 370 (15.7) |
| Water (mL) | 35.7 (4.1) | 30.6 (2.5) | 34.1 (3.3) # |
| Feed (g) | 32.6 (4.3) | 29.9 (2.5) | 31.7 (2.8) |

**, <0.05; **, < 0.01; ***, <0.001, compared with CN; #, <0.05; ##, < 0.01; ###, <0.001, compared with CN.*

### Metabolites Identification and Quantification

After prepossessing of the 1H-NMR spectroscopy of MHE, ZN and CN rats, 47 metabolites were identified for further metabolomics analysis. All the 47 metabolites are shown in a heatmap with hierarchical clustering (Figure1).

### Key Metabolites Selection

The key metabolites between CHM and SN rats (R2x=0.47, R2y=0.98, Q2=0.84) and between CHM and ZN rats (R2x=0.52, R2y=0.98, Q2=0.86) could be well separated by sPLS-DA analysis (Figure 2). The key metabolites between ZN and CN rats were not able be separated by sPLS-DA analysis. Ten key metabolites were identified between MHE and CN rats. The concentrations of GABA, lactate, alanine, aspartate, glutamate, and glutamine were significantly increased, while that of myo-inositol, taurine, leucine, and isoleucine were significantly decreased in MHE rats. Eight key metabolites were identified between MHE and ZN rats. In which the concentrations of myo-inositol, taurine, glycine, leucine, and isoleucine were significantly increased, while that of lactate, alanine and glutamine were significantly decreased in ZN rats Supplementary Table 1.

*Table 2: Main metabolites identified in ZN and MHE rats*

| | Fold change$ ZN (n=8) | Fold change$ MHE (n=8) | P* | P# | VIP* | VIP# |
|---|---|---|---|---|---|---|
| GABA | 1.11 | 1.15 | 0.005 | <0.001 | 2 | 0.2 |
| Myo-inositol | 1.02 | 0.79 | 0.003 | <0.001 | 2.3 | 2.9 |
| Lactate | 1.28 | 1.48 | 0.005 | <0.001 | 2.2 | 1.6 |
| Aspartate | 1.16 | 1.42 | 0.002 | <0.001 | 1.3 | 1 |
| Glutamine | 1.07 | 1.57 | 0.003 | <0.001 | 2.4 | 2.7 |
| Alanine | 0.98 | 1.17 | 0.002 | <0.001 | 1.6 | 1.9 |
| Taurine | 0.96 | 0.65 | 0.002 | <0.001 | 1.8 | 2.5 |
| Leucine | 0.98 | 0.83 | 0.002 | <0.001 | 2.2 | 2.5 |
| Glutamate | 1.22 | 1.37 | 0.002 | <0.001 | 1.9 | 0.9 |
| Isoleucine | 0.93 | 0.82 | 0.002 | <0.001 | 2.4 | 2.3 |
| Glycerol | 1.08 | 0.98 | 0.002 | <0.001 | 0 | 1.1 |

*$, compared with CN; *, MHE vs. ZN; #, MHE vs. CN; VIP, influence on projection.*

### Enrichment and Pathway Analysis

The pathway analysis of the ten key metabolites from MHE vs. CN shows that alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine biosynthesis, beta-alanine metabolism, taurine and hypotaurine metabolism, pyruvate metabolism, and gabaergic synapse metabolism were evolved in the MHE metabolism. And the alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine biosynthesis, taurine and hypotaurine metabolism and pyruvate metabolism were evolved in the MHE metabolism after zinc supplementation (Figure 3).

## DISCUSSION

This study used 1H-NMR metabolomics to compare the key metabolites in MHE, zinc sulfate treated MHE and CN

**Supplementary Table 1:** *Metabolic pathways of the key metabolites involved in MHE vs.CN rats*

| KEGG ID | Entry type | KEGG name | P |
|---|---|---|---|
| rno00250 | pathway | Alanine, aspartate and glutamate metabolism -... | <0.001 |
| rno00290 | pathway | Valine, leucine and isoleucine biosynthesis -... | 0.024 |
| rno00410 | pathway | beta-Alanine metabolism - Rattus norvegicus (... | 0.043 |
| rno00430 | pathway | Taurine and hypotaurine metabolism - Rattus n... | <0.001 |
| rno00620 | pathway | Pyruvate metabolism - Rattus norvegicus (rat) | <0.001 |
| rno04727 | pathway | GABAergic synapse - Rattus norvegicus (rat) | 0.016 |
| M00027 | module | GABA (gamma-Aminobutyrate) shunt | <0.001 |
| M00029 | module | Urea cycle | 0.042 |
| M00106 | module | Conjugated bile acid biosynthesis, cholate =>... | 0.022 |
| M00119 | module | Pantothenate biosynthesis, valine/L-aspartate... | 0.001 |
| M00131 | module | Inositol phosphate metabolism, Ins (1,3,4,5)P4... | 0.023 |
| M00135 | module | GABA biosynthesis, eukaryotes, putrescine => ... | 0.018 |
| M00168 | module | CAM (Crassulacean acid metabolism), dark | 0.035 |
| M00169 | module | CAM (Crassulacean acid metabolism), light | 0.014 |
| M00170 | module | C4-dicarboxylic acid cycle, phosphoenolpyruva... | 0.035 |
| M00171 | module | C4-dicarboxylic acid cycle, NAD - malic enzym... | 0.004 |
| M00172 | module | C4-dicarboxylic acid cycle, NADP - malic enzy... | 0.025 |
| C00002 | compound | ATP | 0.044 |
| C00022 | compound | Pyruvate | <0.001 |
| C00025 | compound | L-Glutamate | <0.001 |
| C00026 | compound | 2-Oxoglutarate | 0.002 |
| C00041 | compound | L-Alanine | <0.001 |
| C00049 | compound | L-Aspartate | <0.001 |
| C00064 | compound | L-Glutamine | <0.001 |
| C00123 | compound | L-Leucine | <0.001 |
| C00137 | compound | myo-Inositol | <0.001 |
| C00245 | compound | Taurine | <0.001 |
| C00256 | compound | (R)-Lactate | <0.001 |
| C00334 | compound | 4-Aminobutanoate | <0.001 |
| C00407 | compound | L-Isoleucine | <0.001 |
| C00671 | compound | (S)-3-Methyl-2-oxopentanoic acid | 0.025 |
| C01235 | compound | alpha-D-Galactosyl-(1->3)-1D-myo-inositol | 0.034 |
| C20966 | compound | 3-{[(2E)-4-Amino-4-oxobut-2-enoyl] amino}-L-al... | 0.042 |

**Supplementary Table 2:** *Metabolic pathways of the key metabolites involved in MHE vs.ZN rats*

| KEGG ID | Entry type | KEGG name | P |
|---|---|---|---|
| rno00250 | pathway | Alanine, aspartate and glutamate metabolism -... | 0.005 |
| rno00290 | pathway | Valine, leucine and isoleucine biosynthesis -... | 0.017 |
| rno00430 | pathway | Taurine and hypotaurine metabolism - Rattus n... | <0.001 |
| rno00620 | pathway | Pyruvate metabolism - Rattus norvegicus (rat) | <0.001 |
| M00027 | module | GABA (gamma-Aminobutyrate) shunt | 0.003 |
| M00106 | module | Conjugated bile acid biosynthesis, cholate =>... | 0.011 |
| M00119 | module | Pantothenate biosynthesis, valine/L-aspartate... | 0.014 |
| M00131 | module | Inositol phosphate metabolism, Ins (1,3,4,5) P4... | 0.013 |
| M00169 | module | CAM (Crassulacean acid metabolism), light | 0.017 |
| M00172 | module | C4-dicarboxylic acid cycle, NADP - malic enzy... | 0.04 |
| C00022 | compound | Pyruvate | <0.001 |
| C00025 | compound | L-Glutamate | 0.011 |
| C00041 | compound | L-Alanine | <0.001 |
| C00064 | compound | L-Glutamine | <0.001 |
| C00116 | compound | Glycerol | <0.001 |
| C00123 | compound | L-Leucine | <0.001 |
| C00137 | compound | myo-Inositol | <0.001 |
| C00233 | compound | 4-Methyl-2-oxopentanoate | 0.04 |
| C00245 | compound | Taurine | <0.001 |
| C00256 | compound | (R)-Lactate | <0.001 |
| C00407 | compound | L-Isoleucine | <0.001 |
| C00671 | compound | (S)-3-Methyl-2-oxopentanoic acid | 0.016 |
| C01235 | compound | alpha-D-Galactosyl-(1->3)-1D-myo-inositol | 0.018 |
| C20966 | compound | 3-{[(2E)-4-Amino-4-oxobut-2-enoyl] amino}-L-al... | 0.029 |

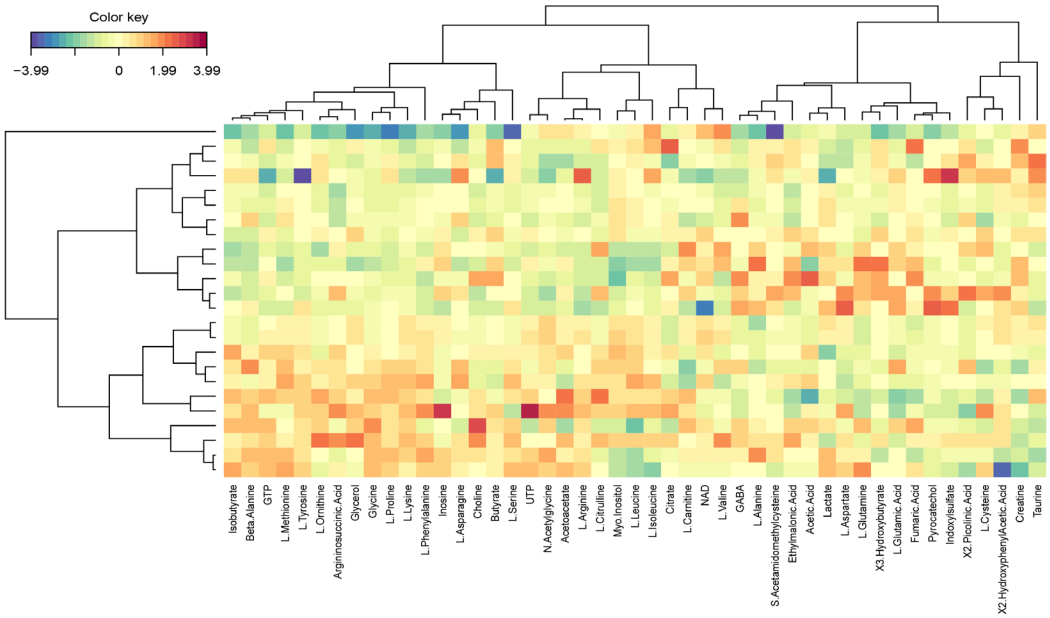


*Figure 1: Two-dimensional heatmap from hierarchical clustering shows all the 47 metabolites in the striatum in MHE, ZN and CN rats.*

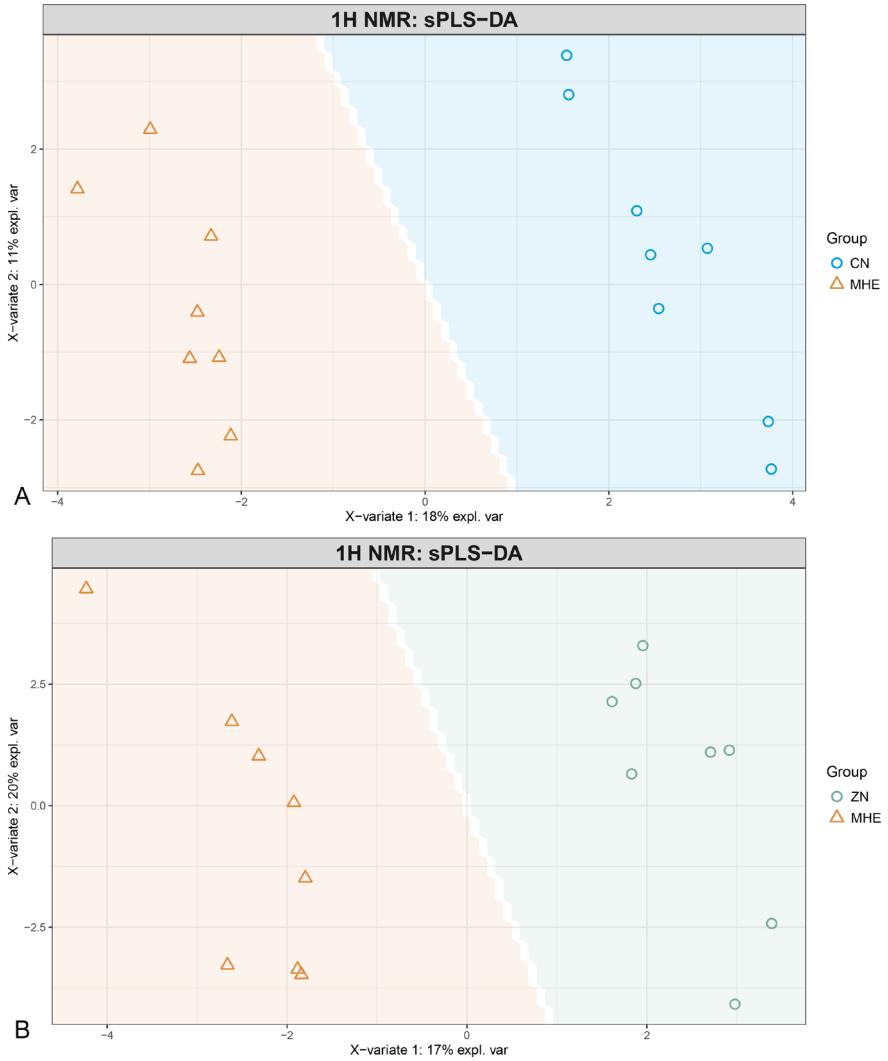


*Figure 2: Sparse projection to latent structures discriminant analysis (sPLS-DA) of the metabolites. The metabolites between CN rats and MHE rats (A), ZN rats and MHE rats could be separated by sPLS-DA.*

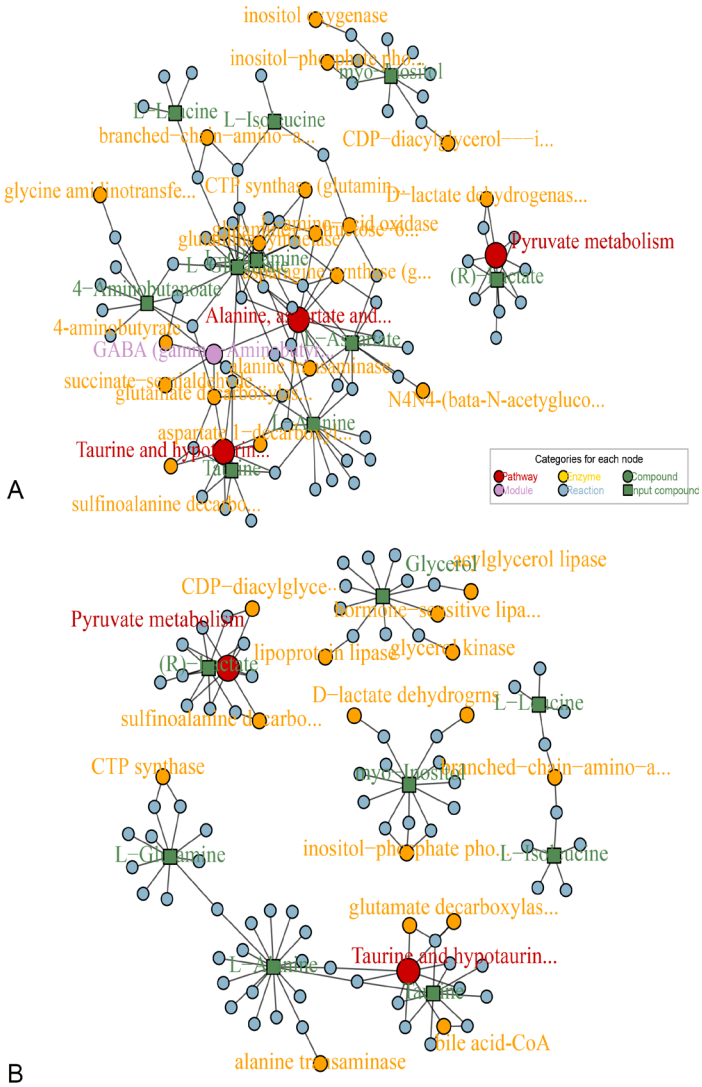


*Figure 3: Metabolic pathways of the key metabolites involved in MHE vs.CN rats and MHE vs.ZN rats.*

rats. Results indicated that cognitive impairment abnormal glycolysis, glutamine and branched chain amino acids (BCAAs) metabolism in MHE rats. The cognitive impairment and abnormal metabolism could be restored by zinc sulfate. Zinc deficiency is common in MHE patients, which is accompanied by increased serum ammonia levels [12]. Zinc supplementation was observed to reduce ammonia in both experimental animals and in human studies [13]. Elevation of blood ammonia may lead to cognitive impairment in MHE [14]. A meta-analysis demonstrated that additional zinc supplementation has a significant effect on the performance of digital connection test in cirrhotic patients with MHE [15]. The result showed that zinc supplementation restored cognitive impairment in MHE rats, which is in according to the clinical and animal studies. As was reported previously, MHE rats exhibited significantly longer escape latencies in WWM, which could be improved by oral supplementation of zinc sulfate [7].

Zinc has been reported involved in the alteration of the nitrogen metabolism [16]. In a previous study, the ammonia increase in response to alanine was observed. Although, fasting plasma ammonia was not significantly reduced by zinc treatment in cirrhotic patients [13]. Lactate and alanine are produced from pyruvate, derived in the process of glycolysis [17]. The accumulation of lactate plays an important role in the development of brain edema,which could lead to cognitive impairment in MHE[18]. Several reports describe combinations of zinc and BCAA supplementation has effective in HE, and the blood ammonia level decreased more than that treated by BCAA alone [19,20]. The contents of BCAAs were reported decreased in the brain of HE rats. Increased serum ammonia showed a "high consumption" state of BCAAs, which may be related to increased metabolism and the conversion of BCAAs into glutamate and glutamine [21]. Significant decreased BCAAs were found in MHE rats, which were restored by zinc sulfate supplementation. Results indicated that the increasing of BCAAs may be the consequences of decreasing of blood ammonia lead by zinc sulfate. Zinc deficiency was also reported to affect the activity of muscle glutamine synthetase [13]. Glutamine synthetase also catalysis glutamate and ammonia into glutamine, which is an important detoxification way of ammonia in the brain [22]. Under normal circumstances, astrocytes transporters can effectively remove extracellular glutamate and maintain the balance of glutamate/glutamine in the internal environment [23]. The concentrations of glutamate and glutamine in striatum of MHE rats were significantly higher than that of SN rats. High blood ammonia may lead to an increase of glutamate and accumulation of glutamine in the brain, which is the major cause of osmotic pressure increasing and astrocytes edema in MHE [24]. After zinc sulfate supplementation brain glutamate and glutamine were observed decrease in MHE rats Supplementary Table 2.

## CONCLUSIONS

Zinc sulfate can effectively improve cognitive impairments in MHE rats. Zn played a center role in the nitrogen metabolism of MHE by interfering alanine, glutamate, BACCs metabolisms and TCA circle.

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