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Effects and mechanisms of acetyl-L-cysteine in rats with chronic mountain sickness with H^1 -NMR metabolomics methods

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Background: We established a rat model of chronic mountain sickness using acetyl-L-cysteine. Then we studied the effects and mechanisms of acetyl-L-cysteine (Da) in rats with chronic mountain sickness using nuclear magnetic resonance (H^1 -NMR) metabolomics methods.

Material/Methods: Using NMR spectroscopy combined with pattern recognition and orthogonal partial least squares discriminant analysis, we analyzed the impact of Da on blood metabolism in rats with chronic mountain sickness by determining different metabolites and changes in metabolic network in the blood of rats with mountain sickness after the intragastric administration of different doses of Da suspension.

Results: Increased levels of amino acids (valine, tyrosine, 1-methyl-histidine, leucine, phenylalanine, and methionine) were detected in the blood of rats in the chronic mountain sickness group, yet significantly decreased levels were detected in control rats. At the same time, β -glucose and α -glucose levels were markedly elevated in the blood of rats in the model group but decreased in the chronic mountain sickness group, which indicated a statistically significant difference compared with the chronic altitude sickness model group ($P < 0.05$).

Conclusions: Da has a significant impact on the metabolism of rats with chronic mountain sickness. Da may act on the disturbed glucose metabolism and amino acid metabolism in rats triggered by chronic mountain sickness, resulting in the treatment and prevention of this disease.

MeSH Keywords: **H^1 -NMR • Acetylcysteine • Altitude Sickness**

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Background

Chronic mountain sickness (CMS), a symptom of an individual's lack of adaptation to living at high altitude, often develops in low elevation inhabitants who move to an altitude >3000 m, and is characterized by an excessive increase of red blood cells and severe reversible tissue damage [1]. Rapid recovery upon returning to a lower altitude is characteristic [2]. This disease usually attacks individuals at ≥ 3000 m above sea level. Causal factors are mainly lack of oxygen, fatigue, cold, dry conditions, and solar radiation, although malnutrition can also promote the incidence of this disease [3].

Altitude sickness, also known as CMS or Highland maladaptation, refers to an idiopathic disease that occurs in the human body in a hypoxic high altitude environment. The international diagnostic criteria for CMS (named "Qinghai standards") and the guidelines for its management resulted from the 2004 International High Altitude and Low Oxygen Physiology Conference. CMS is considered as a clinical syndrome caused by the maladaptation of long-term low elevation residents to altitudes >3000 m above sea level to the hypoxic highland environment and is mainly characterized by polycythemia (≥ 190 g/L hemoglobin in females, ≥ 210 g/L hemoglobin in males). When patients move to lower elevations, their clinical symptoms gradually disappear, but if they return to the highland environment, the disease relapses [4].

CMS is caused by multiple factors: low oxygen, low temperature, low humidity, solar radiation, fatigue, and malnutrition. However, hypoxia is the main causative factor because it reduces the amount of oxygen-carrying hemoglobin, resulting in insufficient oxygenation throughout the body and gradually affecting organ function. To date, there have been no major medication breakthroughs for its treatment. Drugs such as acetazolamide [5], endothelin receptor antagonist, nitric oxide [6], prostaglandin, and calcium antagonists have been reported to effectively alleviate some symptoms of hypoxia, while Chinese herbal medicines such as rhodiola [7], ginseng, and ginkgo biloba, as well as Tibetan medicine, also have a very good effect on its prevention and treatment.

Acetyl-L-cysteine (Da), a thiol compound, contains the active-SH radical that is commonly used as an expectorant in clinical settings [8,9]. It is also commonly used to reduce acetaminophen-induced liver toxicity. In recent years, many studies have reported that Da is an antioxidant that interferes with free radicals, generates free radical scavenging, and regulates the metabolic activity of cells [10,11]. It has been widely used in clinical and experimental research of the respiratory, cardiovascular, and nervous systems, as well as acquired immunodeficiency syndrome [12]. Our animal model of chronic mountain sickness is based on the 2004 International High Altitude and Low

Oxygen Physiology Conference "Qinghai standard". There is no research on the effects of altitude sickness drugs by use of the metabolic approach. We wanted to understand the impact of chronic mountain sickness on body metabolism after administration of small molecules and metabolic changes. This study examines the role of Da in improving some metabolic markers in rats with CMS and discusses the mechanism of DA in preventing and treating CMS by establishing a CMS model using rats.

Material and Methods

Materials

Laboratory animals

A total of 32 male and female Specific Pathogen-Free Sprague-Dawley rats weighing 200 ± 30 g each were purchased from Xinjiang Medical University Laboratory Animal Center (Urumqi China).

Instruments

Instruments used in this experiment included Superspeed centrifuges at low temperature (Beckman Corporation, USA), an Inova 600 nuclear magnetic resonance (NMR) spectrometer (Varian, USA), and a -80°C ultralow-temperature refrigerator. Acetylcysteine effervescent tablets (batch number: 28002193) were produced in Italy by the Zambon S.p.A Company.

Low-pressure oxygen cabin: peacetime and wartime medical service support the artificial test chamber in the Northwest Territories (located in the Urumqi, Lanzhou Military Region General Hospital). The environment is a plateau 5000 m above sea level with a temperature of $18\text{--}26^{\circ}\text{C}$, 40–60% humidity, pressure of 54.1 KPa, and oxygen partial pressure of 10.84 KPa.

Plains environment: The environment of the plains 720 m above sea level with a temperature of $18\text{--}26^{\circ}\text{C}$, 40–60% humidity, pressure of 93.2 KPa, and oxygen partial pressure of 19.54 KPa.

Main reagents

The main reagents included heavy water (Cambridge Isotope Laboratories, Inc.); NaCl, K_2HPO_4 , and NaH_2PO_4 (Tianjin Guangfu Fine Chemical Research Institute); an NMR tube, and distilled water.

Experimental methods

Animal groups and feeding

We selected 32 (16 males and 16 females) SD rats, weighing 200 ± 30 g and randomly divided them into the CMS group and

the control group after lab-stable breeding for 3 days. The CMS group was fed in the low pressure oxygen warehouse environment and was weighed daily for 30 days, while the control group was fed in the plains environment and was weighed daily for 30 days.

We randomly divided 16 rats in each model into 2 groups of 8 each (using the layered random method in which 8 male rats and 8 female rats in each model were randomized; we used random numbers generated from a randomized list into 2 groups to reach equal group numbers) as follows:

- (Pn) plains control group: The 8 SD rats (4 males and 4 females) were fed in the plains environment and weighed daily for 30 days. No medication; continued feeding for 15 days.
- (Pu) high altitude control group: The 8 SD rats (4 males and 4 females) were fed in the low pressure oxygen warehouse environment and weighed daily for 30 days. No medication; continued feeding for 15 days.
- (Da-pn) plains Da medication group: The 8 SD rats (4 males and 4 females) were fed in the plains environment and weighed daily for 30 days. They received 16 mg acetyl-L-cysteine as well as daily intragastric administration for 15 days.
- (Da-pu) Da medication group at high altitude: The 8 SD rats (4 males and 4 females) were fed in the low pressure oxygen warehouse environment and weighed daily for 30 days. They received 16 mg acetyl-L-cysteine as well as daily intragastric administration for 15 days.

We collected blood samples from all groups of rats, separated blood and plasma 1 hour after the last dose, and stored samples at -80°C until needed for measurements.

Sample preparation and plasma ^1H -NMR tests

We took 200 μL of serum from each blood sample and added 400 μL of phosphate buffer prepared with heavy water, let the mixture rest for 10 min and then separated the components by centrifugation at 10 000 r/min for 10 min. We then transferred 550 μL of serum to a 5-mm NMR tube.

The Inova 600 NMR spectrometer (NOESYPRESAT-1D; RD-900-T1-90 0-TM-90 0-ACQ) delivered hydrogen gas chromatographic pulse sequences with the following parameters: scan time, 1.64 s; 500.13 MHz frequency; total scans, 64; sample data points, 32 768; spectral width, 20 mg/L; sample delay, 2 s; test temperature, 25°C ; and presaturation method to suppress the water peak. To solve the spectrum for this purpose, some samples were used to test the J-decomposition spectrum (J-Res), ^1H - ^1H homonuclear correlation spectrum (COSY spectrum), Proton total correlation spectroscopy (NOESY), and two-dimensional nmr.

Map processing and analysis

The ^1H atlas of NMR in plasma (^1H -NMR spectrum) was selected for automatic integration in the 10.0–0.5 ppm range after baseline and phase adjustment and the integration interval was set to 0.003 ppm. To eliminate the effects of water intake on metabolites, integral normalized values (every score value added to the sum of all points) and SMICA-P+ (Switzerland) to least-squares discriminant analysis software (partial least square-discriminant analysis [PLS-DA]) for statistical analysis and orthogonal least-squares discriminant analysis (orthogonal partial least-squares discriminant analysis [OPLS-DA]). The PLS-DA was mainly used to observe the differences among groups, while the OPLS-DA was used to judge integral differences between groups. Metabolites of the correlation coefficient represented by each of the scores obtained by OPLS-DA were used to identify the different metabolic components in the blood plasma of the male and female rats, and values of $P=0.05$ functioned as the inspection standard.

In this study, according to the Pearson correlation coefficient significant difference test (Pearson's product moment correlation coefficient), we determined that the metabolites represented by $|r|>0.707$ ($n=6$) was the threshold for the metabolites that were statistically different ($P<0.05$). The larger $|r|$ value represents larger differences, while smaller values represent smaller differences.

Statistical analysis

Metabolomics data processing: The ^1H -NMR spectrum of the original rat plasma data was calibrated using the proton chemical signal of shifted α -glucose of 5.233 ppm as the standard. We used the 0.003 ppm spectrum and divided the atlas into 2668 fields to automatically score and made each field 0.003 ppm by using Topspine software (Germany) in the range of 10.0–0.5 ppm and the score values were normalized. We removed the water peak signals in the range of 5.20–4.66 ppm and performed the OPLS-DA using SMICA-P+11 software. We then performed the statistical analysis using the PLS-DA.

Results

Groups of blood ^1H -NMR spectra

Figure 1 shows the ^1H -NMR spectrum in the blood of model control group of chronic mountain diseases, plains Da delivery group, plains model group, and Da medication for the chronic mountain disease at high altitude group.

The three-dimensional spatial distribution map was obtained by segmenting the ^1H -NMR spectrum of the integrated score value

Figure 1. The plateau model group (Pu), plains Da medication group (Da-pu), the plains model group (Pn), and the Da plateau medicine group (Da-pu).

for the PLS-DA analysis (Figure 2). This analysis showed $R^2X=0.88$, $R^2Y=0.38$, and $Q^2=0.20$. Figure 2 shows that the distribution areas are completely separate in each group and that the metabolic components in the blood of rats in each group are significantly different. We determined the degree of difference based on the chemical shift of the different metabolic components in the 2 groups received from the coefficients of correlation that were gained from the comparison and analysis of the 2 sets of OPLS-DA data. We then determined the different metabolic components

in the blood of the rats in each group using NMR of 2 spectrum technologies, such as 1H - 1H associated with the nuclear spectra (COSY Spectra), J-decomposition of the spectrum (J-Resolved Spectra), and proton spectroscopy (TOCSY spectrum) (Table 1).

The results of each group show that the various metabolic components in the blood of rats differ markedly. The metabolites with positive correlation coefficients among the metabolites with differences are those that are increased in the plains model group, while the metabolites with negative correlation coefficients are those that are decreased in the plains model group.

Compared with the CMS model group, the metabolites with positive correlation coefficients among the metabolites with differences were increased in the plains Da medication group, while the metabolites with negative correlation coefficients were decreased in the plains Da medication group. Compared with the CMS model group, the metabolites with positive correlation coefficients among metabolites that have differences are the increased metabolites in the plateau Da medication group, while the metabolites with negative correlation coefficients are the decreased metabolites in the plateau Da medication group.

Compared with the plains Da medication group, the metabolites with positive correlation coefficients among the metabolites that had differences were the increased metabolites in the plains model group, while the metabolites with negative correlation coefficients were the decreased metabolites in the plains model group. Compared with the plains Da medication group, the metabolites with positive correlation coefficients among the metabolites that had differences were the increased metabolites in the plateau Da medication group, while the metabolites with

Figure 2. Analysis of the 3D space maps by blood samples from each group 1H -NMR spectrum PLS-DA. Note: for the highland model groups (plains Da medication, plateau Da medication, and plains model groups), the model parameters were $R^2X=0.88$, $R^2Y=0.38$, and $Q^2=0.20$, respectively.

Table 1. The main metabolites with differences and their coefficients of correlation in the blood ^1H -NMR spectrum of rats in each group are achieved according to OPLS-DA analysis.

No.	Metabolite	Chemical shift (ppm)	Assignment	Coefficient (r)					
				Pu vs. Pn	Pu vs. DA.Pn	Pu vs. DA.Pu	DA.Pn vs. Pn	DA.Pn vs. DA.Pu	Pn vs. DA.Pu
				R^2X	0.75	0.76	0.87	0.89	0.84
				Q^2	0.4	0.56	0.18	0.44	0.45
1	Isoleucine	0.93(t), 1.00(d), 1.96(m)	$\delta\text{-CH}_3$, $\beta\text{-CH}_3$					0.75	
2	Valine	0.98(d), 1.03(d)	CH_3 , CH_3 , $\alpha\text{-CH}_2$	-0.74				0.78	
3	Lactate	1.33(d), 4.11(q)	NHCO-CH_3	-0.71	0.87	0.92		0.77	0.73
4	Glycoprotein	2.03(s)	CH_3 , $\alpha\text{-CH}$	-0.8				-0.76	
5	Glycine	3.55(s)	CH_2			0.69			
6	Tyrosine	6.89(d), 7.18(d)	$\alpha\text{-CH}$, H3/H5, H2/H6	-0.68				-0.75	
7	Phenylalanine	7.32(m), 7.42(m)	H_2/H_6 , H_4 , H_3/H_5					0.78	0.68
8	Acetone	2.22(s)	CH_3	-0.78				-0.77	
9	Pyruvate	2.36(s)	CH_2			0.85			
10	1 – methyl-histidine	7.05(s)	H_4 , H_2	-0.68		0.77		0.81	0.79
11	α -glucose	3.53 (dd), 3.72(dd), 5.23(d)	C-H_2 , half $\text{CH}_2\text{-CH}_6$, C-H_1	0.94			0.7		-0.69
12	β -glucose	3.24(dd), 3.40(t), 3.47(ddd), 3.49(t), 3.70(dd), 3.90(dd), 4.64(d)	C-H_2 , C-H_4 , C-H_5 , C-H_3 , C-H_6 , half $\text{CH}_2\text{-CH}_6$, C-H_1	0.98	-0.67	-0.9	0.78		-0.71
13	Lipids (VLDL)	0.85(m), 0.88(m),	$\text{CH}_3(\text{CH}_2)_n$, $\text{CH}_3\text{CH}_2\text{CH}_2\text{C}$	-0.69			-0.74	-0.78	-0.69
14	Carnitine	2.46(dd)	$\text{CH}_2(\text{COO})$				0.76	0.69	
15	Scyllo-inositol	3.35(s)	$6\times\text{CH}$					0.83	
16	Creatine	3.03(s)3.93(s)	CH_3CH_2	-0.7		0.67		0.71	0.69

s – humped; d – double peaks; t – triple peaks; q – four peaks; m – multiple peaks; dd – double-double peak; ddd – double-double-double peak.

negative correlation coefficients were the ones with decreased metabolites in the plateau Da medication group. Compared with the plains model group, the metabolites with positive correlation coefficients among metabolites with differences were the increased metabolites in the plateau Da medication group, while the metabolites with negative correlation coefficients were the decreased metabolites in the plateau Da medication group.

The levels of many kinds of amino acids in the blood of rats with CMS significantly decreased, including valine, tyrosine, 1-methyl-histidine, leucine, phenylalanine, and methionine, the latter of which was statistically significant ($P<0.05$) compared with that in the plains model group. In addition, lactic acid, glucose, very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), protein, acetone, creatine, and β -hydroxybutyrate contents increased significantly ($P<0.05$). The α -glucose and β -glucose

levels in the blood of rats in the CMS group decreased significantly compared to that in the plains model group ($P<0.05$).

After gavage with Da, the levels of several amino acids (acetic acid, L-methyl-histidine, lactate, and creatine) significantly decreased in the blood of rats in the CMS model group, the difference of which was statistically significant compared with the CMS group ($P<0.05$). After gavage with Da, β -glucose levels in the blood of the rats in the CMS model group decreased significantly compared those in the CMS model group ($P<0.05$).

Discussion

In the hypoxic high altitude environment, the body's normal metabolism will certainly make some adjustment to adapt to

the environment and reaches a steady state in the dynamic equilibrium. Such a steady-state environment definitely has some differences in metabolite content compared with the plains environment. Metabolism is fundamental to body function; it ensures the metabolism level is maintained at a normal level and has great significance for the body's resistance to a hypoxic high altitude environment. This study demonstrated that compared with the plains model group, lactic acid levels in the plateau model group were increased, showing that the body's energy metabolism converts aerobic oxidation into anaerobic glycolysis. In general, when an individual's energy needs cannot be met by aerobic respiration, levels of the main anaerobic fermentation products such as lactic acid continue to rise [13]. Anaerobic glycolysis can greatly improve adenosine triphosphate production speed to ensure the body's energy supply remains intact in a low oxygen environment [14]. In this case, pyruvate dehydrogenase is unable to convert pyruvate into acetyl coenzyme A; therefore, pyruvate begins to accumulate in the blood. This finding, to an extent, supports the pathological condition of CMS. At the same time, α -glucose and β -glucose content significantly decreased in the blood of rats in the chronic mountain model group and demonstrated a statistically significant difference ($P < 0.05$) compared with the plains model group. Despite low oxygen levels, anaerobic glycolysis can effectively ensure the supply of energy, but this mode of production is extremely inefficient, so it greatly increases the demand for glucose and results in decreased blood glucose levels. When living in a low-oxygen environment for a long time, due to the discrepancy between energy needs and energy supply, energy supply had to change the way the body use glycolysis for energy [15]. Due to changes in the environment, the supply of oxygen is insufficient, and the cells are in hypoxia; hypoxia induction of hypoxia-inducible factor can stimulate the generation of the blood erythropoietin produced by stimulating red blood cell formation, and enhance the ability to increase the supply of oxygen to the blood. This can promote glycolysis by increasing glucose transporters and glycolytic enzyme gene expression to adapt to hypoxia when there is an energy shortage [16].

Compared with the plains model group, the levels of various amino acids (valine, tyrosine, 1-methyl group of alanine, leucine, phenylalanine, and methionine) increased significantly in the blood of rats in the CMS group. This finding indicates that under low oxygen conditions, the massive consumption of glucose leads to increased levels of hepatic gluconeogenesis due to the increased blood content of amino acids. Those

amino acids are transported to the liver for use in the gluconeogenesis process, which provides glucose to the blood and ensures a continuous energy supply. Levels of esterified fatty acids were elevated in the rats in the CMS group as well.

As mentioned above, levels of blood VLDL and LDL increased to improve the energy supply, and in low-oxygen conditions, the ketone body is the final product of β -oxidation, and levels of both acetone and β -hydroxybutyrate increased. Changes of these metabolites reflect the effects of high-altitude hypoxia on body metabolism, while the long-term accumulation of these metabolites will certainly affect organ function. If the administration of Da can improve the metabolic level and return the metabolite levels to those seen in the plains environment, then it will be considered an effective treatment for CMS.

In this study, levels of acetic acid and 1-methyl-histidine decreased in the control group compared with the CMS model group. After the intragastric administration of Da, lactic acid levels in the blood were also significantly reduced, indicating that Da can decrease the degree of anaerobic glycolysis and improve the body's oxygenation, resulting in significantly decreased blood β -glucose and α -glucose levels in rats and returning gluconeogenesis and fatty acid levels to those seen in animals in the plains environment. These results suggest that after the administration of Da, the metabolism of rats with CMS was effectively improved. Hypoxia-induced proliferation of free radicals inhibits the transcription of genes to reduce the activity of antioxidant enzymes in the body and the oxidative modification effect, so that the content of antioxidant enzyme activity and non-oxidative enzymes and antioxidants reduce further, increasing the *in vivo* the concentration of free radicals and increasing tissue damage. In body tissue, hypoxia-induced ROS production and clearance mechanisms are unbalanced, further promoting the development of oxidative stress, and ultimately leading to tissue damage. Da, as an exogenous antioxidant [17], can stop the vicious cycle of oxidative stress, allowing the ROS metabolism to restore the equilibrium state, thereby reducing free radical damage to the body.

Conclusions

In summary, Da plays a role in improving the metabolism of rats with CMS, which provides a theoretical basis for the prevention and treatment of CMS.

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