She C, Zhong H, Hu XM, *et al*. Investigating the effects of moxibustion on serum metabolism in healthy human body based on the ^{1}H NMR metabolomics technology. J Acupunct Tuina Sci, 2016, 14(2): 93-100
DOI: 10.1007/s11726-016-0907-5

Special Topic for 973 Program

Investigating the effects of moxibustion on serum metabolism in healthy human body based on the ^{1}H NMR metabolomics technology

基于 ^{1}H NMR 代谢组学技术探讨艾灸对正常人体血清代谢的影响

She Chang (佘畅)[1,2], Zhong Huan (钟欢)[1], Hu Xiao-min (胡晓敏)[3], Wang Hou-lian (汪厚莲)[1], Guo An-lin (郭安林)[1], Gu Jin-ping (顾金萍)[3], Huang Cai-hua (黄彩华)[3], Ge Jun-yun (葛君芸)[1], Liu Mi (刘密)[1], Liu Mai-lan (刘迈兰)[1], Lin Dong-hai (林东海)[3], Chang Xiao-rong (常小荣)[1]

1 School of Acupuncture, Moxibustion & Tuina, Hunan University of Chinese Medicine, Changsha 410007, China

2 Changsha Hospital of Chinese Medicine Affiliated to Hunan University of Chinese Medicine, Changsha 410002, China

3 Xiamen Univeristy, Xiamen 361005, China

Abstract

Objective: To investigate the effects of moxibustion on the serum metabolism in healthy human body based on the ^{1}H nuclear magnetic resonance (^{1}H NMR) metabolomics technology, and to find the differences in metabolites, as well as to elucidate the effects of moxibustion on healthy human body from the viewpoint of global metabolism.

Methods: Sixty subjects of healthy young men from the enrolled students were randomly divided into a moxibustion group and a control group using random number table, with 30 cases in each group. Subjects in the moxibustion group accepted mild moxibustion on the right Zusanli (ST 36), once a day, 15 min for each time, and continuous treatment for 10 d; those in the control group did not receive any intervention. There were 28 cases in the moxibustion group and 23 cases in the control group after interventions. On the 1st day, 5th day and 10th day of the intervention, serum samples were collected from subjects of the two groups, and metabolic spectra were obtained by the ^{1}H NMR technology.

Results: Before and after the intervention, serum ^{1}H NMR of the moxibustion group was significantly different, while the difference was insignificant in the control group. Metabolite changes in the moxibustion group were mainly in low density lipoprotein (LDL)/very low density lipoprotein (VLDL), valine, isoleucine, leucine, lactic acid, glutamine, citric acid, polyunsaturated fatty acids, creatine, glycine, glycerol, glucose, tyrosine, histidine, formic acid, alanine, lysine, acetic acid, and glutamic acid.

Conclusion: Moxibustion can cause changes of serum metabolic patterns in healthy human by influencing the concentrations of branched-chain amino acids, polyunsaturated fatty acids, and other metabolites to strengthen body's metabolisms of amino acids and fatty acid.

Keywords: Moxibustion Therapy; Moxa Stick Moxibustion; Point, Zusanli (ST 36); Metabolomics; Healthy Volunteers; Men

【摘要】目的：基于核磁共振氢谱代谢组学技术(^{1}H nuclear magnetic resonance, ^{1}H NMR)探讨艾灸对正常人体血清代谢的影响，并寻找差异性代谢物，从整体代谢的角度阐述艾灸对健康人体的影响。**方法：**将 60 例在校健康青年男性采用随机数字表随机分成艾灸组和对照组，每组 30 例。艾灸组予温和灸右侧足三里，每天 1 次，每次 15 min，连续治疗 10 d；对照组不予任何干预。干预结束后艾灸组剩余 28 例，对照组剩余 23 例。在干预第 1 d、第 5 d 和第 10 d，采集两组受试者的血清样品，运用 ^{1}H NMR 技术获取代谢图谱。**结果：**艾灸组干预前后血清 ^{1}H NMR 有明显差异，对照组干预前后 ^{1}H NMR 无明显差异。艾灸组代谢物的变化主要是低密度脂蛋白(low density lipoprotein, LDL)/极低密度脂蛋白(very low density lipoprotein, VLDL)、缬氨酸、异亮氨酸、亮氨酸、乳酸、谷氨酰胺、柠檬酸、多不饱和脂肪酸、肌酸、甘氨酸、甘油、葡萄糖、酪氨酸、组氨酸、甲酸、丙氨酸、赖氨酸、乙酸、谷氨酸。**结论：**艾灸能够引起正常人体血清代谢模式变化，通过影响支链氨基酸、多不饱和脂肪酸等代谢物浓度加强机体的氨基酸、脂肪酸代谢。

【关键词】灸法；艾条灸；穴，足三里；代谢组学；健康志愿者；男性

【中图分类号】R245.8　　**【文献标志码】**A

Author: She Chang, 2014 grade doctorial degree candidate, attending physician

Joint Corresponding Authors: Chang Xiao-rong, bachelor, professor. E-mail: xrchang1956@163.com; Lin Dong-hai, doctor, professor. E-mail: dhlin@xmu.edu.cn

Metabolomics (metabonomics), an important branch of research in the field of systems biology after the genomics, transcriptomics and proteomics[1], as well as a type of global, high-throughput, and unbiased analytical technology for the study of metabolic pathways in vivo, it can reflect the body's early subtle metabolic changes after external stimuli, and reflect the body's global health by high-throughput metabolomics analysis[2-3]. Moxibustion, one of the commonly used external therapies of traditional Chinese medicine (TCM), is an approach to disease treatment, disease prevention and health care by acting on the body's meridians and acupoints through the thermal stimulation during burning moxa. Moxibustion has a unique role which is different from acupuncture and drugs. Previous clinical and mechanism researches mainly concentrated upon studies of moxibustion on disease vectors, such as irritable bowel syndrome[4-5], primary osteoporosis[6], osteoarthritis of the knee[7], and asthma[8]. However, few studies conducted the global explanation of the role of moxibustion from the perspective of metabolomics.

We believe that the effect of moxibustion on the human body is a multi-angle and multi-target effect. Given that, from metabolite levels, metabolomics can globally reflect the effects of external stimuli on human bodies, we used ^{1}H nuclear magnetic resonance (^{1}H NMR) metabolomics technology to study the influence of moxibustion on healthy human, which are as follows.

1 Clinical Information

1.1 Inclusion criteria

Males aged 18-30 years old; usually in good health, regular dietary; without smoking, alcohol, tea, and coffee; normal sleep, moderate build [body mass index (BMI) ＞18.50, ＜23.91]; did not receive acupuncture therapy in the last one month; signed the informed consent; no hemophilia and other contraindications of acupuncture.

1.2 Exclusion criteria

Those who failed to cooperate with the test (such as refusal to sign an informed consent, and unreasonable rejection of the tests); not feeling well within one week; those who could not complete because of consciousness, severe vision, hearing and aphasia disorders, and other health assessments; with surgical history; physical examination of circulatory system, respiratory system or nervous system was abnormal; with severe primary diseases in liver, kidney, hematopoietic system and endocrine system; psychosis, epilepsy, diabetes or cancer patients; heavy alcohol drinking or drug abusers.

1.3 Subjects

A total of 60 subjects were included, who were all 2014 class healthy male undergraduate or graduate students in Hunan University of Chinese Medicine. All subjects signed the informed consent, and were randomly divided into a control group and a moxibustion group using a random number table, with 30 subjects in each group. Nine subjects dropped out during the intervention, 2 cases in the moxibustion group, and 7 cases in the control group.

2 Intervention Methods

2.1 Moxibustion group

Those in the moxibustion group received moxibustion during fixed time period (18:00-19:00). Moxibustion was operated by trained professionals of the project members.

Acupoint: Zusanli (ST 36) on the right side.

Methods: Ignited one end of the pure moxa stick (Huatuo Brand) and kept 2-3 cm from the skin to perform the mild-warm moxibustion, to make the subjects have tolerable warm, sour, numbness, distension or pain. Once a day, 15 min for each time, and continued for 10 d.

2.2 Control group

Those in the control group didn't receive any interventions during the observation process.

3 Results

3.1 Test items

3.1.1 Collection and pretreatment of serum samples

Blood of subjects in both groups were collected at the university hospital by professional nurses during the fixed time period (7:00-8:00) on the 1st day, 5th day and 10th day of the intervention. On the day before blood collection, subjects were asked for fasting of water and food from 21:00 to the time of blood collection, prohibiting alcohol or coffee, chocolate, tea and other stimulating beverage or spicy foods. Subjects in both groups avoided strenuous exercise within 10 d of the test. Blood collection tubes were dry tubes without any additives. Centrifuged for 10 min at 4 ℃ and 3 000 r/min after agglutination at room temperature. Time between sampling and centrifugation was controlled within 2 h. Upper layer serum was collected and kept at －80 ℃ for preservation.

The serum samples stored at －80 ℃ were thawed and centrifuged (10 000 r/min, 4℃, 10 min). Took out 300 μL serum and added in 200 μL PBS (0.1 mol/L K_2HPO_4 and NaH_2PO_4) prepared by the heavy water (D_2O), and added into the NMR tube of 5 mm in diameter after centrifugation.

3.1.2 ^{1}H NMR data capture and processing

Serum one-dimensional hydrogen spectra of nuclear magnetic resonance was captured by the Bruker AV600 Ⅲ NMR spectrometer. The molecular signals of macromolecular and other restriction molecular motion were filtered out using Carr-Purcell-Meiboom-Gill [RD-90-(τ-180°-τ) n–ACQ (Bruker Biospin Pulse Program Library, Germany)].

The τ of the spin-echo was set to 500 μs, cycle number was set to 20, so the total spin-echo time was 20 ms. Pre-saturated water peak pressing was conducted to eliminate the wide packet signal from proteins and other high molecular weight substances during relaxation delay (RD). Time domain (number of raw data points, TD) was 64 K, the spectral width (SW) was 20 part per million (ppm), the number of scans (NS) was 128, and number of dummy scans (DS) was 16 times.

MestReNova 6.1 software was used for correction of the baseline and phase of NMR spectra, peak alignment and calibration. Segment integration was carried out for the region from 0.002 ppm to 0.60-8.50 ppm, the aberrant area due to pressing water peak was excluded. The integration of the chemical shift δ4.52-6.00 was set to 0, the remaining integral values were normalized. The resonance signals of metabolites were identified using NMR Suite software by combining with the published data for the compound chemical shift.

SIMCA software was used to process the segment integrated NMR spectra, firstly conducted the unsupervised principal component analysis (principal component analysis, PCA) to confirm the classification of samples, remove the extreme point, and identify the change trends of metabolic patterns. Then the supervised orthogonal partial least-squares discriminate analysis (OPLS-DA) method was used for model orthogonal correcting process, to maximally highlight the internal model differences among groups, and find the important metabolites which have contributed significantly to differentiate the metabolic patterns. Seven-fold cross-validation method was used in the PLS-DA analysis. Used the robustness (robustness) of the discriminant model established by arrangement experimental verification with 200 cycles and analyzed the correlation coefficient combining the importance ranking value of variable weigh to summarize the statistically significant variables (Bin), and identify specific metabolites with significant differences.

3.2 Test results

3.2.1 Serum samples

Eighteen, 19 and 21 blood samples without hemolysis were selected respectively on the 1st day, 5th day and 10th day of the intervention in the moxibustion group. And 22, 23 and 22 blood samples without hemolysis were selected respectively on the 1st day, 5th day and 10th day of the observation in the control group. There were 28 cases in the moxibustion group and 23 cases in the control group after interventions. Selected numbers of samples without hemolysis at three time points were showed in Table 1.

Table 1. Comparison of the detection results for serum samples without hemolysis from subjects in the two groups (sample number)

Group	*n*	1st day	5th day	10th day
Moxibustion	28	18	19	21
Control	23	22	23	22

3.2.2 ^{1}H NMR spectra and metabolite identification of serum from subjects

Typical ^{1}H NMR spectra of serum samples at 3 time points in the two groups was shown in Figure 1. Metabolites were identified by the metabolite chemical shift (ppm), valine: 1.05; isoleucine: 1.02; leucine: 0.95; lactic acid: 1.36; alanine: 1.48; lysine: 1.72; acetic acid: 1.92; glutamic acid: 2.05; glutamine: 2.45; glucose: 3.92; polyunsaturated fatty acids (PUFA): 2.75; creatine: 3.01; glycine: 3.57; histidine: 7.08.

3.2.3 PCA analysis

1A and 2A, 1A and 3A could be better distinguished when the samples were pairwise compared at 3 time points (moxibustion intervention for 1 d, 5 d, 10 d) in the moxibustion group. The results showed that different time of moxibustion therapy could significantly alter the serum metabolic patterns of healthy human; 2A and 3A could also be better differentiated, which indicated that long time moxibustion intervention could further affect the changes of metabolic patterns, as the extension of treatment (Figure 2).

3.2.4 PLS-DA model validation

Based on PCA analysis, we used PLS-DA method to analyze the metabolic patterns serum samples at the 3 time points in the moxibustion group, which showed that 1A and 2A, 1A and 3A, 2A and 3A were able to be better distinguished. Eventually, we performed model validation for the results. Cross-validation was conducted using arrangement experiment with a cycle of 600 times. The established discriminant model was reliable according to the plot features of PLS-DA score and model validation. It can be seen that the metabolic pattern changes caused by different moxibustion intervention time are reliable (Figure 3).

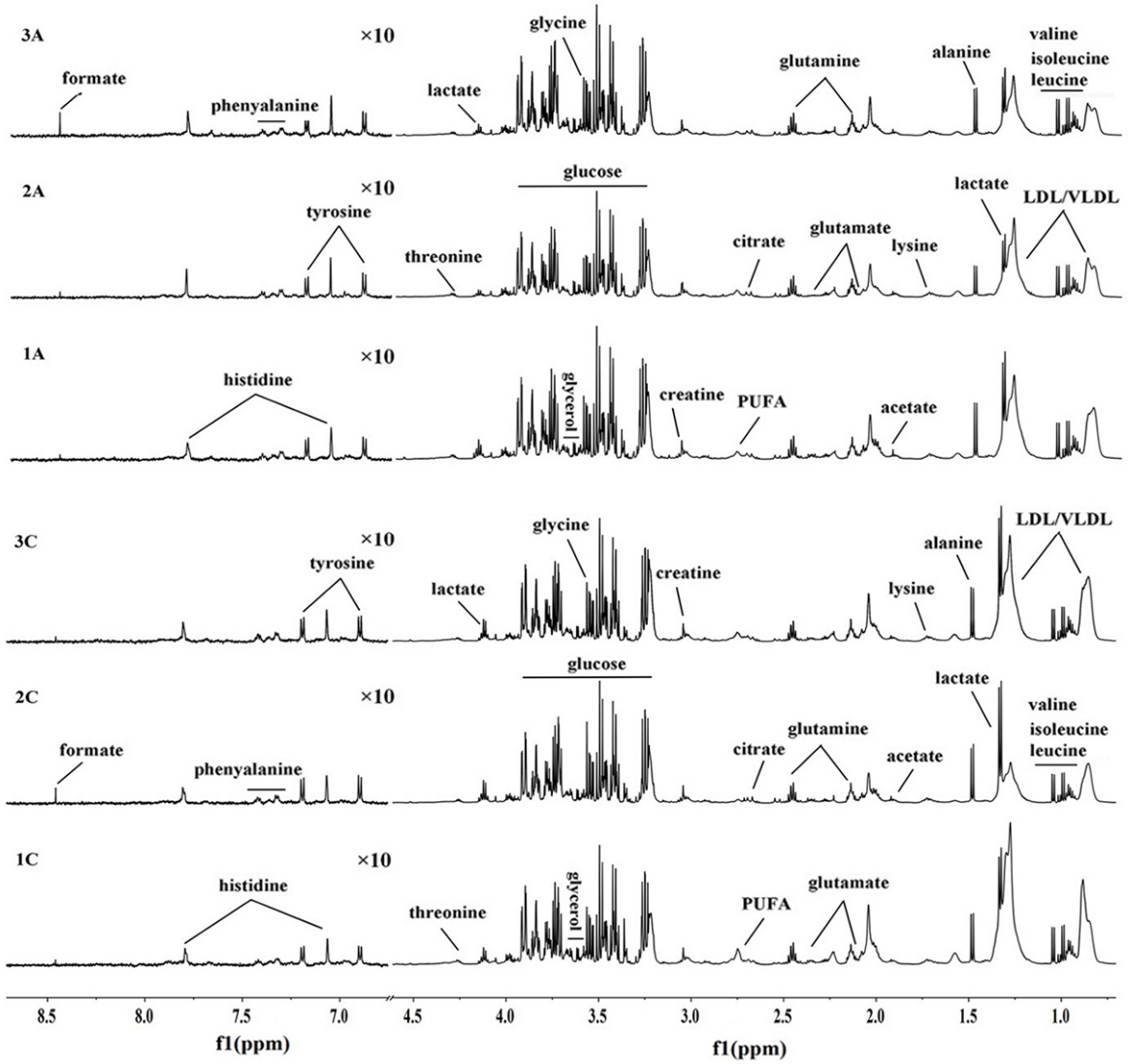

Figure 1. Typical ^{1}H NMR spectra and metabolite identification of serum samples (Note: 1A, 2A and 3A represent moxibustion for 1 d, 5 d, 10 d in the moxibustion group; 1C, 2C and 3C represent the corresponding time in the control group)

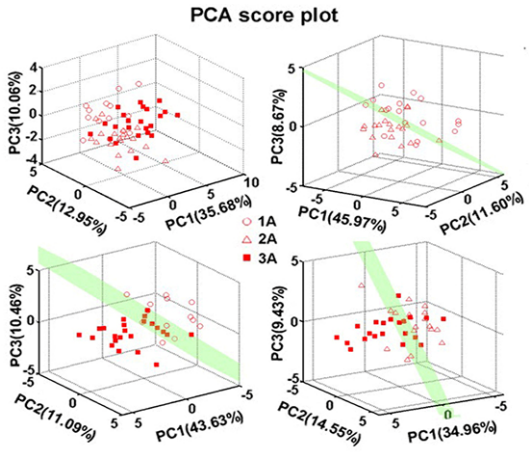

Figure 2. PCA analysis score plot of serum ^{1}H NMR data at the 3 time points in the moxibustion group (Note: 1A, 2A and 3A represent moxibustion for 1 d, 5 d, 10 d, respectively)

3.2.5 Difference of metabolites

Moxibustion interfered with the healthy human body could cause changes of metabolism patterns, and moxibustion intervention for 5 d or 10 d had some differences on serum metabolites. Five days after the moxibustion, metabolite concentrations of valine, leucine, isoleucine, alanine, lysine, acetic acid, glutamate, glutamine, glucose, creatine and glycine were significantly increased; concentrations of lactic acid, histidine and unsaturated fatty acid were significantly reduced. Ten days after moxibustion, concentrations of low density lipoprotein (LDL)/very low density lipoprotein (VLDL), lactic acid, unsaturated fatty acid, tyrosine and histidine were significantly increased, while metabolite concentrations of valine, leucine, isoleucine, glutamate, citric acid, creatine, glycine, glycerol, glucose and formic acid were significantly reduced (Figure 4 and Figure 5).

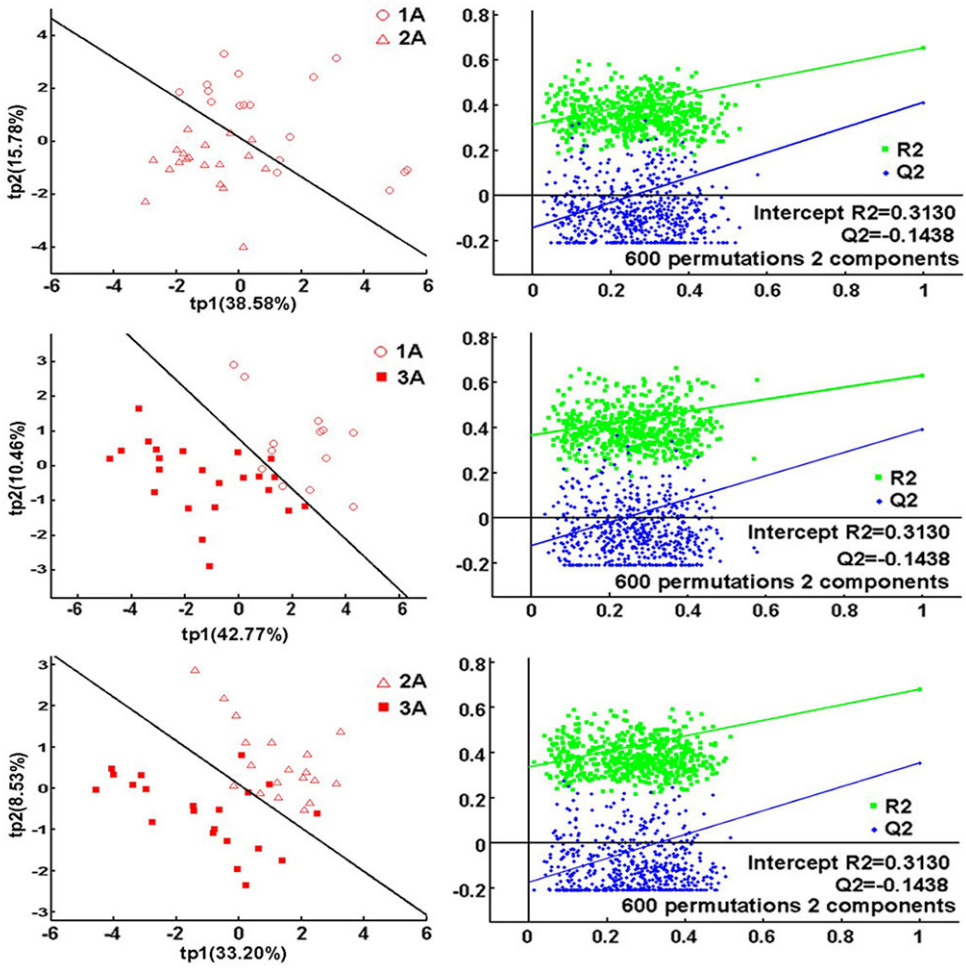

Figure 3. PLS-DA and model validation at three time points in the moxibustion group (Note: 1A, 2A and 3A represent the moxibustion for 1d, 5 d, 10 d, respectively)

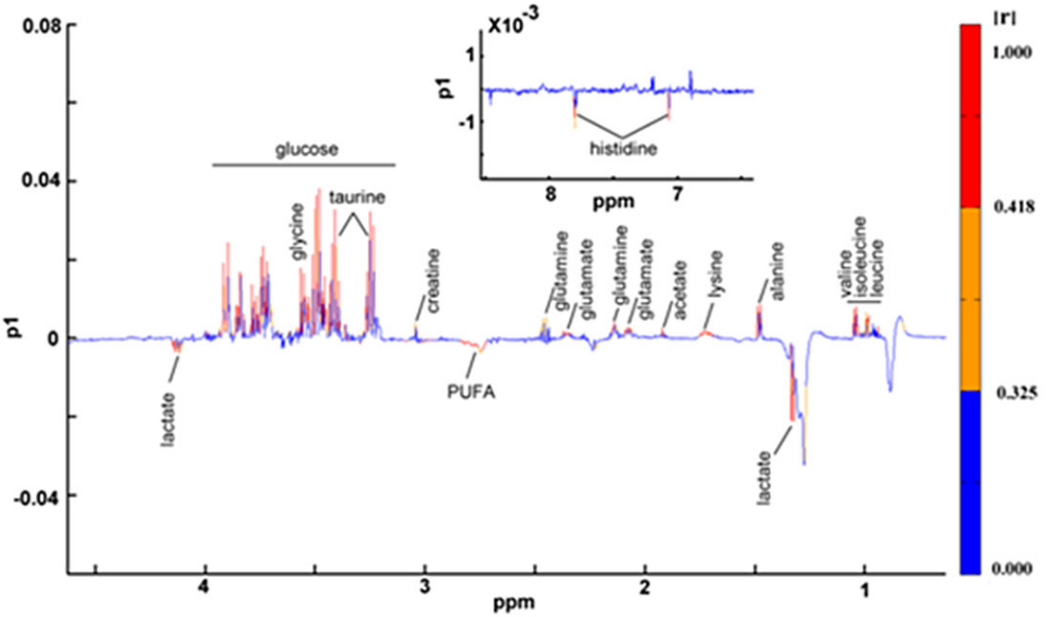

Figure 4. OPLS-DA correlation coefficient loading plot of serum ^{1}H NMR data of moxibustion for 5 d, 2A vs. 1A (Note: The yellow and red value on the color column was the critical value of the correlation coefficient absolute at *P*= 0.01 and *P*=0.05, respectively)

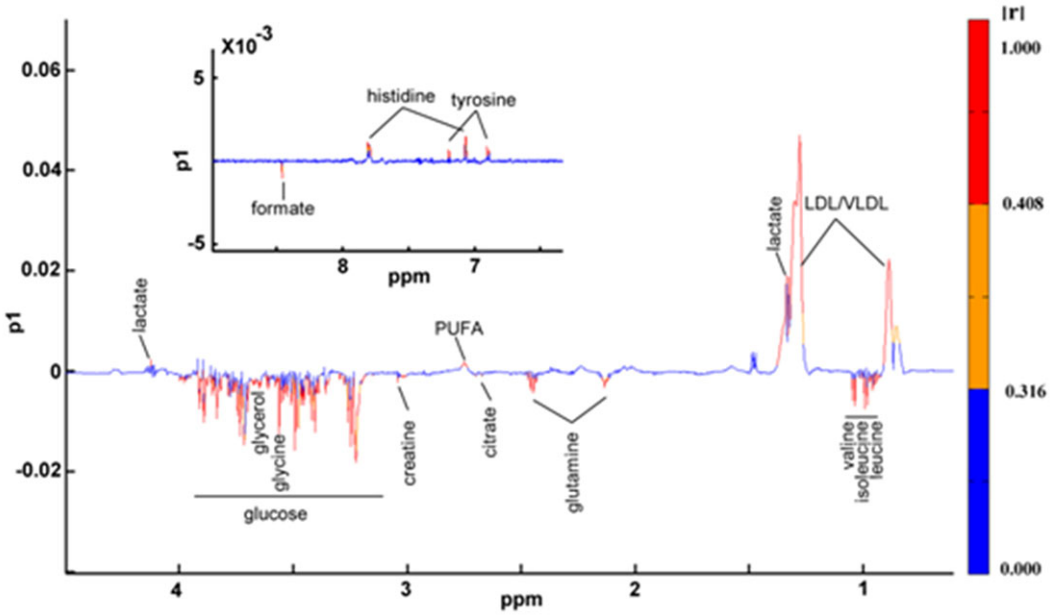

Figure 5. OPLS-DA correlation coefficient loading plot of serum ^{1}H NMR data of moxibustion for 10 d, 3A vs. 1A (Note: The yellow and red value on the color column was the critical value of the correlation coefficient absolute at *P*=0.01 and *P*=0.05, respectively)

4 Discussion

Moxibustion therapy, one of the most unique TCM therapies, and a commonly used method to prevent disease, maintain health and achieve longevity, it is an important component of acupuncture therapy. Studies have shown that the effectiveness of acupuncture is a multi-path, multi-link, multi-level and multi-target global regulation. The overwhelming majority of studies currently only stay on the research levels of certain substances, some parts and some paths; it cannot commendably interpret the characteristics and mechanisms of acupuncture action. The metabolomics is a science to reveal the metabolic nature of body's life activities by extracting the relevant biomarkers, which can more accurately reflect the states at metabolic level after the biological systems experience the external disturbance. The metabolomics also has the dynamic, complete and holistic characteristics which are coincided with characteristics of acupuncture action and adapt to the need of acupuncture study[9]. In this study, serum metabolite changes of healthy young men caused by moxibustion at Zusanli (ST 36) were measured using ^{1}H NMR metabolomics technology. This study found that the ^{1}H NMR spectra was significantly different before and after moxibustion treatment, and serum metabolites had also undergone significant changes with the extension of treatment time. This study also found that concentrations of 11 kinds of metabolites increased on the 5th day of moxibustion, while concentrations of 3 kinds of metabolites decreased; concentrations of 6 kinds of metabolites increased on the 10th day of moxibustion, while concentrations of 10 kinds of metabolites decreased. As could be seen from the metabolites being influenced, 5 d of moxibustion mainly affected the ammonia recycling, propanoate metabolism, biotin metabolism, and lysine degradation pathways; and 10 d of moxibustion mainly affected propanoate metabolism, valine/leucine/isoleucine degradation, protein biosynthesis, and citric acid cycle metabolic pathways.

Judged by the two time points of moxibustion intervention, metabolite concentrations showed the opposite trend with 5 d of moxibustion and 10 d of moxibustion. Eg., valine, leucine, isoleucine, glutamate, glucose, creatine and glycine showed a increasing trend on the 5th day of moxibustion, while showed a decreasing trend after 10 d of moxibustion; lactic acid, histidine and unsaturated fatty acids showed a decreasing trend on the 5th day of moxibustion, while showed an increasing trend after 10 d of moxibustion. It could be seen that different intervention time played different role in the human body.

Valine, leucine and isoleucine are the most important metabolites that showed differences. Because all of them have branched structures and are called branched-chain amino acids, and belong to the essential amino acids[10-12]. Majority of amino acid metabolism is in the liver in vivo, and branched-chain amino acid is the only amino acid that its degradation is not limited in the liver. Studies have shown that adipose tissue possesses dissimilation of circulating branched-chain amino acids and coordinate regulation of branched-chain amino acid enzymes in adipose tissues, which can regulate the level of circulating branched-chain amino acids[13]. Moxibustion intervention could cause increase of branched-chain amino acid, which suggested that moxibustion was associated with the

metabolism of adipose tissue, or could promote skeletal muscle protein degradation[14]. Another study showed that supplement of branched-chain amino acid could reduce the activities of muscle-damage-associated enzymes (such as creatine kinase and lactate dehydrogenase), thereby could improve the muscle damage caused by overtiredness[15]. Supplement of branched-chain amino acid in vitro also can promote liver albumin synthesis by mammalian target of rapamycin (mTOR) signaling pathway[16], and also can promote glucose metabolism of skeletal muscle, adipose tissue, and liver tissue[17-19]. A low level of serum branched-chain amino acids due to 10 d of moxibustion intervention may be caused by promoting the use of branched-chain amino acids in the body with moxibustion, and thus it is one of the mechanisms playing a certain role in enhancing the metabolism and protecting the body. Polyunsaturated fatty acids are also important metabolites that showed differences, and the body's essential substances. Many studies have shown that polyunsaturated fatty acids had potential anti-inflammatory effects on the human body, as they could commendably balance the pro-inflammatory cytokines and anti-inflammatory factors in the body[20], and could inhibit the inflammatory response of many chronic degenerative diseases, and thus played a protective role[21]. Low level of polyunsaturated fatty acids on the 5th day of moxibustion indicated that polyunsaturated fatty acids involved in the anti-inflammatory response induced by moxibustion in the early stage of moxibustion intervention; while the increased concentrations after 10 d of moxibustion intervention may also indicate that moxibustion can improve the unsaturated fatty acid concentrations in the body, which reflected the protective effect of moxibustion on the body.

This study found that the changes of metabolite concentrations showed a reversal phenomenon during moxibustion. Analysis revealed that changes in metabolite concentrations after 10 d of moxibustion tended to benefit the body, while whether the changes in metabolite concentrations, appearing on the 5th day of moxibustion, are related to the body's reaction to thermal stress needs to be further studied. More importantly, even the body showed a significant benefit on the 10th day during moxibustion intervention, however, continued development of metabolite concentrations in one direction will inevitably lead to the body's metabolic disorder, while in clinic, the healthy population who used long-term moxibustion therapy for health care did not show the disease state of metabolic disorders, how the metabolite concentrations maintain continuous benefits without causing pathological phenomenon during moxibustion intervention and whether this is related to the body's feedback regulatory mechanism also needs to be further verified by tests.

Conflict of Interest

The authors declared that there was no potential conflict of interest in this article.

Acknowledgments

This work was supported by National Basic Research Program of China (973 Program, 国家重点基础研究发展计划项目, No. 2015CB554502); National Natural Science Foundation of China (国家自然科学基金项目, No. 81202770, No. 81574082); Special Research Fund for the Doctoral Program of Higher Education of China for New Teachers (高等学校博士学科点专项科研基金新教师类资助课题, No. 20124323120002); Foundation for the Author of Excellent Doctoral Dissertation of Hunan Province (湖南省优秀博士学位论文获奖作者科研资助项目, No. YB2013B037); Fund Project of Hunan Province Education Office (湖南省教育厅资助科研项目, No. 14B129); 2015 Graduate Student Research Innovation Project of Hunan Province (2015 湖南省研究生科研创新项目, No. CX2015B320).

Statement of Informed Consent

Informed consent was obtained from all individual participants included in this study.

Received: 18 October 2015/Accepted: 25 November 2015

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Translator: Yang Yan-ping (杨燕萍)

