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ORIGINAL RESEARCH

UHPLC-qTOF-MS-Based Nontargeted Metabolomics to Characterize the Effects of Capsaicin on Plasma and Skin Metabolic Profiles of C57BL/6 Mice-An In vivo Experimental Study

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Background: Capsaicin is the main compound found in chili pepper and has complex pharmacologic effects. This study aimed to elucidate the mechanism of the effect of capsaicin on physiological processes by analyzing changes in metabolites and metabolic pathways.

Methods: Female C57BL/6 mice were divided into two groups(n = 10/group) and fed with capsaicin-soybean oil solution(group T) or soybean oil(group C) for 6 weeks. Ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC-qTOF-MS) based metabolomics was undertaken to assess plasma and skin metabolic profile changes and identify differential metabolites through multivariate analysis.

Results: According to the OPLS-DA score plots, the plasma and skin metabolic profiles in the group T and group C were significantly separated. In plasma, 38 significant differential metabolites were identified. KEGG pathway enrichment analysis revealed that the most significant plasma metabolic pathways included pyruvate metabolism and ABC transporters. In skin, seven significant differential metabolites were found. Four metabolic pathways with p values < 0.05 were detected, including sphingolipid metabolism, sphingolipid signaling pathway, apoptosis, and necroptosis.

Conclusion: These findings will provide metabolomic insights to assess the physiological functions of capsaicin and contribute to a better understanding of the potential effects of a capsaicin-rich diet on health.

Keywords: capsaicin, UHPLC-QTOF-MS, metabolomics, plasma, skin

Background

Peppers have been an essential element of culinary traditions across the world in recent years, and they have a long history of usage for flavoring, coloring, preserving food, and medicinal purposes.[1] The rising use of peppers in food is a worldwide trend.[2] The impact of chili peppers on health is a subject of growing interest. Capsaicinoids are a group of molecules unique to fruits of plants from the genus Capsicum (chili peppers). Although more than ten structures exist, the most prominent forms are capsaicin and dihydrocapsaicin, accounting for almost 90% of capsaicinoids. Capsaicin(8-METHYL-N-Vanillyl-6-Nonenamide) harbors many benefits extensively documented in many studies. Capsaicin consumption and supplementation positively affect glucose and insulin levels in people[3–5] and may help reduce body mass index in obese patients.[6,7] There is evidence that capsaicin can activate transient receptor potential vanilloid subfamily member 1, which may benefit the cardiovascular system.[8,9] Besides, in vivo studies corroborate capsaicin's anticancer action.[10,11]

On the other hand, it has been noticed that eating chili peppers often causes some pathological conditions such as sore throat, mouth ulcer, constipation, etc.[12] Besides, the increased risk of carcinogenesis with the usage of capsaicin has been reported.[13–15] Of note, evidence shows a link between the pepper diet and some skin conditions. A spicy diet rich in capsaicin may trigger and worsen rosacea symptoms.[16] In rat pups, capsaicin injection can cause recurrent pruritic dermatitis.[17]

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Metabolomics, a new "omics" technique, may be used to reflect the comprehensive, unbiased metabolic profile of biological samples, providing details of the metabolic responses of the living organism to external stimuli. Unlike genomics, transcriptomics, and proteomics, metabolomics represents the physiological condition of a biological system most directly since metabolites are most closely related to an organism's phenotype.[18] Analyzing all the metabolites in biological samples can provide information about what transpired in the organisms.[19] Recently, ultra-high-performance liquid chromatography and Q-TOF mass spectrometry (UHPLC/Q-TOF MS) offer a new dimension to metabolic investigations due to their improved reproducibility, detection limits, and higher chromatographic resolution.[20,21]

To date, no study has investigated the effect of capsaicin on the plasma and skin metabolome in C57BL/6 mice. In our study, we compared the changes in plasma and skin metabolic profiles in mice after capsaicin supplement based on UHPLC-Q-TOF MS. Our study will help to elucidate the mechanism of the effect of capsaicin on physiological processes by analyzing changes in metabolites and metabolic pathways.

Materials and Methods

Experimental Animals and Chemicals

Twenty healthy C57BL/6 female mice aged 6 weeks were purchased from Hunan SJA Laboratory Animal Co., Ltd (Hunan, China). All mice were allowed to acclimatize in cages for 1 week before treatment. Mice had free access to standard diet and water and were maintained under normal laboratory conditions (temperature of 22–23°C, relative humidity of 45%-50%, and 12 h/12 h light/dark cycle). Synthesized capsaicin (97% capsaicin) and soybean oil were obtained from Sinopharm, China. The synthetic capsaicin was dissolved in soybean oil to form a 0.5 mg/mL capsaicin-soybean oil solution. Ammonium acetate (NH4AC) and ammonium hydroxide (NH4OH) were purchased from Sigma Aldrich. Acetonitrile was purchased from Merck.

Animal Treatment and Sample Collection

After 1 week of adaptation, the mice were randomly assigned to either group C (control group, n = 10) or the group T (treatment group, n = 10). In the group T, the mice were treated with capsaicin-soybean oil solution (capsaicin of 5 mg/kg mice body weight) on Monday, Wednesday, and Friday (16:00–17:00) of each week for a total of six weeks. The mice in the group C were fed with the corresponding volume of soybean oil simultaneously. Before each gavage, their body weights were determined and recorded. Regular monitoring of animal health and well-being was done three times weekly, including the mice's overall health and their motor activity, eating, drinking, and weight records. At the end of 6 weeks, all mice were euthanized. The blood was immediately harvested from the heart and centrifuged at 1500g for 15min to obtain plasma samples. Skin samples were taken from the backs of mice and quickly frozen in liquid nitrogen. All plasma samples and skin samples were stored at −80°C. All animal experiments were performed according to the regulation of institutional guidelines for the care and use of experimental animals and approved by the Animal Ethical Committee of The Second Affiliated Hospital of Nanchang University.

Sample Preparation for UHPLC-Q-TOF MS Analysis

Plasma Sample Preparation

The plasma samples stored at −80°C were gradually thawed at 4°C. A 100μL aliquot of sample was added to a 400μL pre-cooled methanol/acetonitrile mixed solution (v/v, 1:1). The samples were sequentially vortexed (5min), static-placed (−20°C, 60min) and centrifuged (14000g, 4°C, 20min), and the supernatant was then collected and vacuum dried. For metabolomics analysis, the samples were re-dissolved in 100 μL acetonitrile/water (1:1, v/v) solvent.

Skin Sample Preparation

The frozen skin tissue (100 mg) was thawed at 4 °C and homogenized in 1 mL of precooled methanol/acetonitrile/ddH2O (2:2:1, v/v/v) with a homogenizer (FastPrep-24^{TM}, MP Biomedicals LLC., Santa Ana, California, USA) at 6.0 M/S (20 s each, three times). Then, the mixture was centrifuged for 15 min (14000g, 4°C), and the supernatant was lyophilized under a vacuum and stored at − 80 °C until redissolution in 100 μL of an acetonitrile/water (1:1, v/v) solvent for metabolomics analysis.

UHPLC-Q-TOF MS Analysis

This study detected twenty plasma samples and twenty skin samples by UHPLC-Q-TOF MS. The UHPLC-Q-TOF MS approaches were described in detail as follows. Briefly, analyses were performed using a UHPLC (1290 Infinity LC, Agilent Technologies) coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600) in Shanghai Applied Protein Technology Co., Ltd. The flow rate was 0.5 mL/min, and the column temperature was 25 °C. The quality control (QC) samples were inserted regularly and analyzed in every 5 samples to evaluate the system's stability and the experimental data's reliability.

Data Processing

The raw MS data (whiff. scan files) were converted to MzXML files using ProteoWizard MSConvert before importing them into XCMS software. For peak picking, the following parameters were used: centWave m/z=25 ppm, peak width = c (10, 60), prefilter = c (10, 100). For peak grouping, bw = 5, mzwid = 0.025, minfrac = 0.5 were used. CAMERA (Collection of Algorithms of MEtabolite pRofile Annotation) was sued for annotation of isotopes and adducts. In the extracted ion features, only the variables having more than 50% of the nonzero measurement values in at least one group were kept.

Compound identification of metabolites was performed by comparing accuracy m/z value (<10 ppm) and MS/MS spectra with an in-house database established with available authentic standards. The structural identification of metabolites in biological samples was performed by comparing with the information of retention time, molecular mass (molecular mass error <10ppm), secondary fragmentation spectra and collision energy of metabolites in the database, and the identification results were strictly checked and confirmed by manual secondary inspection. The identification level is above level 2. Level 2 is defined as matched to literature data or databases by diagnostic evidence, at least two orthogonal pieces of information, including evidence that excludes all other candidates.

Metabolites were compared with free online databases KEGG (http://www.genome.jp/kegg/) and HMDB (http://www.hmdb.ca/), and the corresponding KEGG pathways were extracted. KEGG enrichment analysis was performed using MetaboAnalyst(www.metaboanalyst.ca).

Statistical Analysis

After normalized to total peak intensity, the processed data were uploaded into before importing into SIMCA-P (version 16.1, Umetrics, Umea, Sweden), where it was subjected to multivariate data analysis, including Pareto-scaled principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). The 7-fold cross-validation and response permutation testing were used to evaluate the robustness of the model. The variable importance in the projection (VIP) value of each variable in the OPLS-DA model was calculated to indicate its contribution to the

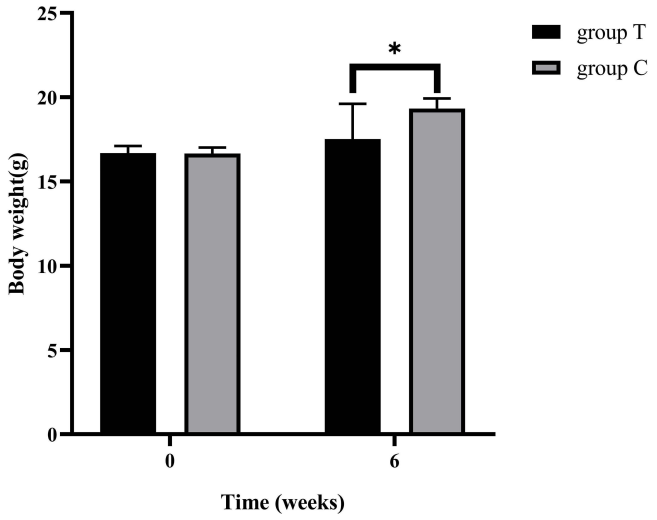

Figure 1 Comparison of body weight of mice in group C and group T. Data were presented as the mean±standard deviation, *p<0.05 vs group C.

classification. Metabolites with the VIP value >1 were further applied to Student's *t*-test at the univariate level to measure the significance of each metabolite, the p values less than 0.05 were considered statistically significant. In addition, Student's *t* test was used to compare the body weight of mice in groups T and C before and after the experiment.

Results

Effect of Capsaicin on Body Weight in C57BL/6 Mice

As shown in Figure 1, the group T significantly decreased in body weight compared with the group C after 6 weeks of capsaicin feeding ($p < 0.05$).

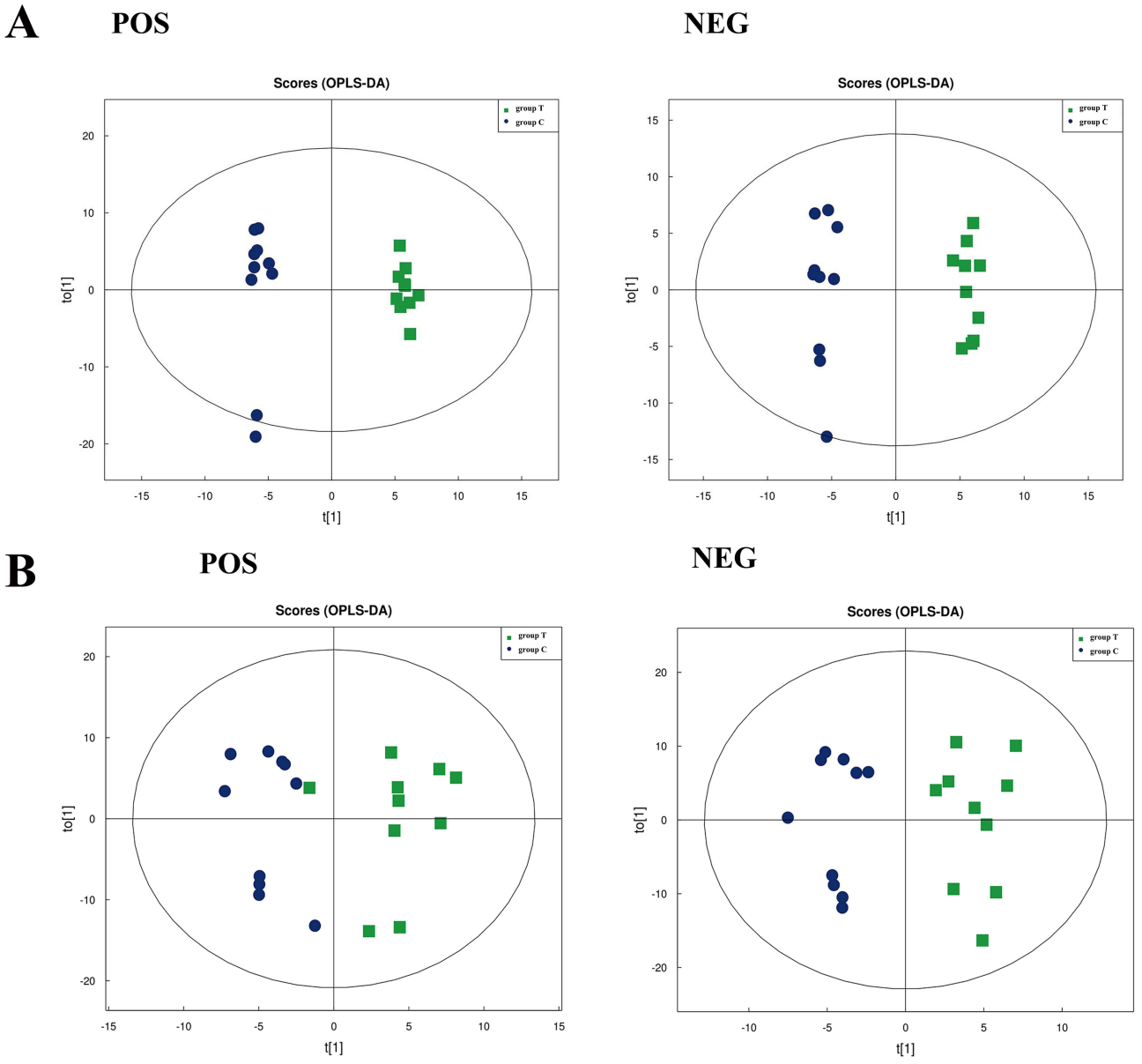

Figure 2 The score plots from OPLS-DA mode. (**A**) Plasma OPLS-DA score plots in positive and negative modes of the group C and group T. (**B**) Skin OPLS-DA score plots in positive and negative mode of the group C and group T.
Abbreviations: POS, positive mode; NEG, negative mode.

Analytical Method Assessment

QC samples were prepared to validate the metabolomic methodology. PCA analysis was performed on all extraction peaks of all experimental and QC samples after Pareto-scaling. The relatively tight clustering of the QC samples in the PCA score plots (Figures S1 and S2) suggested the experiment had good repeatability and stability.

Effects of Capsaicin on Plasma and Skin Metabolic Profiles of C57BL/6 Mice

OPLS-DA is a supervised discriminant analysis statistical method. According to the OPLS-DA score plots (Figure 2A), the plasma metabolic profiles in the group T and group C were obviously separated both in positive and negative modes. Similarly, a clear differentiation in skin metabolomic profiles between the two groups was observed (Figure 2B). These results indicated capsaicin can induce significant changes in the plasma and skin metabolic profile.

Identification of Significantly Differential Plasma Metabolites and Metabolic Pathways

In plasma, 272 metabolites were identified, including 138 metabolites in positive ion mode and 134 in negative ion mode.The chemical classification attribution map revealed that the highest proportion of differential metabolites were organic acids and derivatives(20.22%), lipids and lipid-like molecules(16.17%), and organoheterocyclic compounds(6.98%) (Figure 3).Based on an OPLS-DA model with VIP > 1 and P-value < 0.05, 38 significant differential plasma metabolites were identified (Table S1). Their relationships were revealed by correlation analysis (Figure 4A and B). The hierarchical clustering heatmap indicated that the plasma metabolites of the group T were significantly separated from the group C (Figure 4C). The ROC curves were used to assess the predictive value of the screened potential markers.AUC values between 0.7 and 0.9 have “medium”accuracy, and values greater than 0.9 have “high” accuracy. The ROC curves of the top 10 differential plasma metabolites in the VIP value were shown in Figure 5. The results indicated that, except gamma-Glutamylcysteines, other differential metabolites have good diagnostic performance.

To identify significantly affected metabolic pathways, fisher’s exact test was used to analyze and calculate the significance level of metabolite enrichment for each pathway. The smaller the p-value, the more significant the difference in the metabolic pathway. A total of 42 metabolic pathways were significantly changed ($p < 0.05$) in group C and group T. The most significant metabolic pathways included pyruvate metabolism and ABC transporters (Figure 6).

Identification of Significantly Differential Skin Metabolites and Metabolic Pathways

A total of 392 metabolites were identified in the skin. Seven significant differential skin metabolites were found (VIP >1, and $p < 0.05$) (Table S2). The differentially expressed skin metabolites of group C and group T were visualized in a clustering heatmap (Figure 7A), indicating an altered skin metabolic profile in mice after capsaicin supplementation.

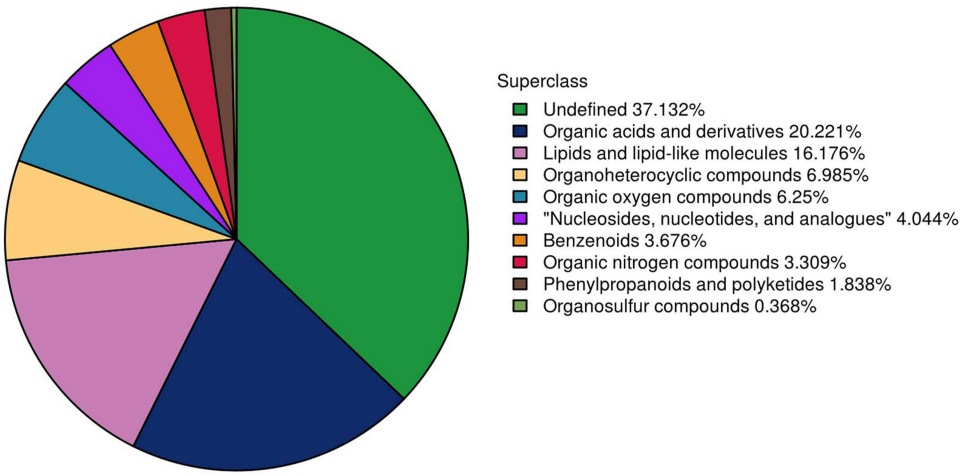

Figure 3 The proportion of identified plasma metabolites in each chemical classification.

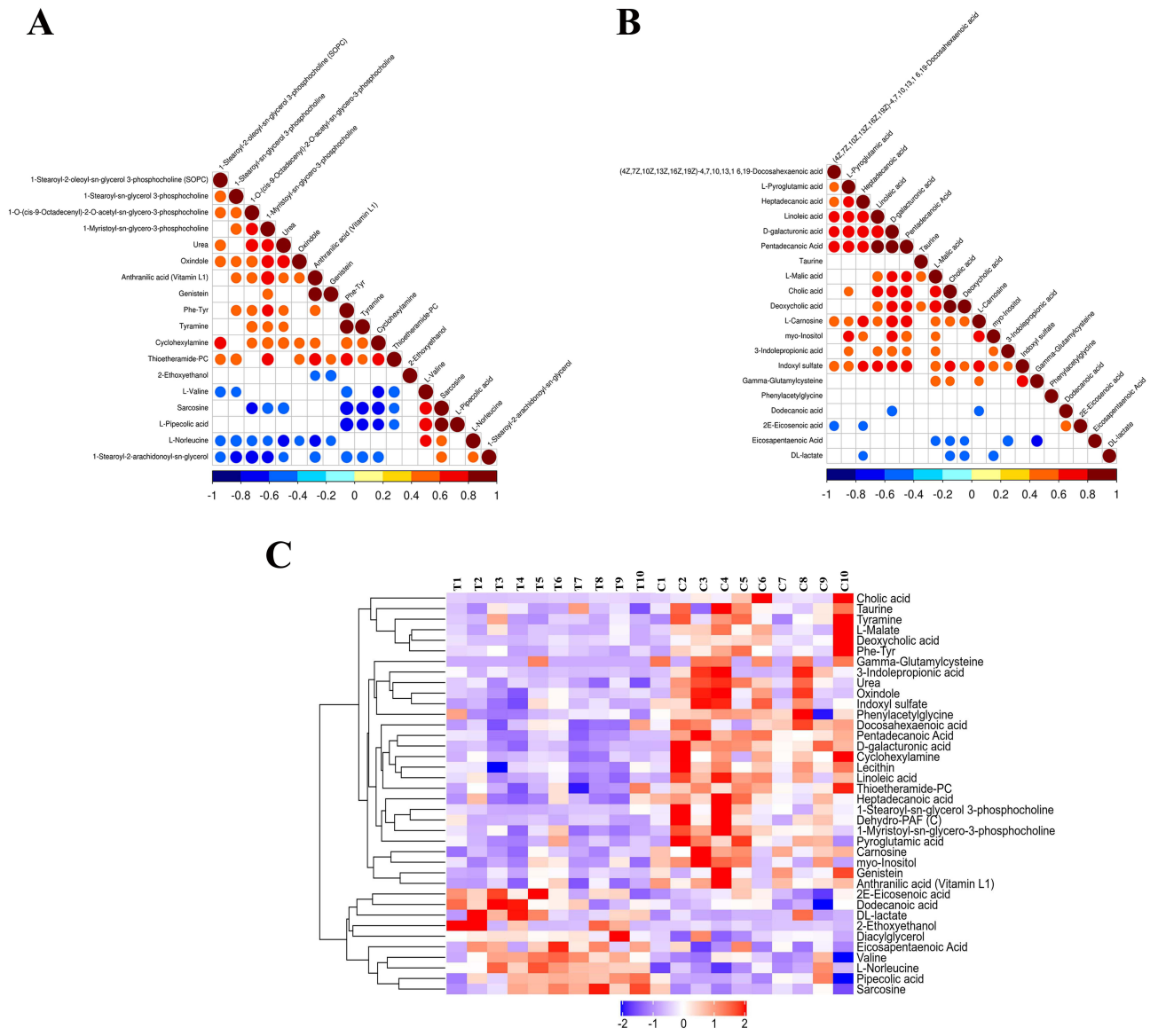

Figure 4 Correlation heatmap and hierarchical clustering heatmap. (**A**) correlation heatmap analysis of differential metabolites in plasma in positive mode. (**B**) correlation heatmap analysis of differential metabolites in plasma in negative mode. (**C**) Hierarchical clustering heatmap of 38 differential plasma metabolites between group C and group T.

Besides, four metabolic pathways with p values < 0.05 were detected, including sphingolipid metabolism, sphingolipid signaling pathway, apoptosis, and necroptosis (Figure 7B).

Discussion

In the current study, we demonstrated that the plasma and skin metabolic profiles based on UHPLC-QTOF could distinguish group T from group C. Meanwhile,38 significant differential metabolites were identified, and a total of 40 metabolism pathways were perturbed in plasma. Furthermore, seven significant differential metabolites were identified, and four metabolic pathways were detected in skin.

Numerous studies have discovered that capsaicin has a beneficial effect on weight management.For example, capsaicin can suppress obesity by modulating energy metabolism, reducing adipose tissue weight, and increasing lipid oxidation.[22] The possible mechanism is that capsaicin can activate of TRPV1, leading to a rapid increase in Ca2+, which causes changes in the expression of obesity-related factors such as PPAR-γ, SREBP-1, and FAS in cells.[23] However, the underlying mechanisms are not fully understood. In our study, we found that the body weight of group T was

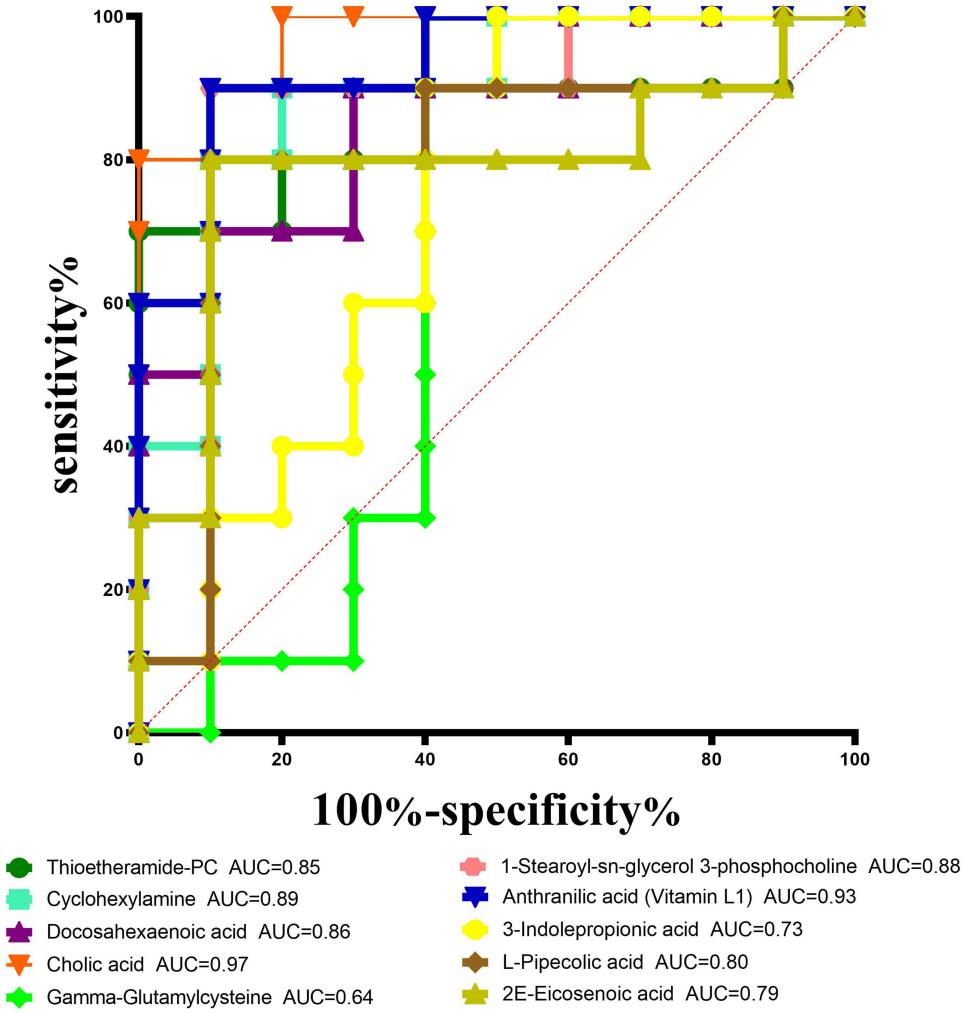

Figure 5 ROC curves for potential plasma biomarkers with VIP values in the top 10.

significantly lower than group C at the end of the 6-week intervention($P<0.05$), indicating capsaicin inhibited the weight increase of C57BL/6 mice. Of note, some differential metabolites (D-lactate, L-lactate, and L-Malate) and metabolic pathways(ABC transporters and Pyruvate metabolism) associated with energy metabolism were identified in our study. DL-lactate and L-malate are engaged in the pyruvate metabolism pathway, a critical pathway for energy metabolism.[24] L-Malate is a three shuttle acid cycle intermediate metabolite, directly involved in mitochondrial energy metabolism.[25] Besides, the ABC transporters metabolic pathway is characterized by the use of ATP-hydrolyzed energy to transport specific compounds across cell membranes. It can mediate fatty acid transport that is associated with obesity and insulin resistant states.[26] Therefore, we speculate that capsaicin may alter the energy metabolism of mice, which in turn affects their body weight.

Due to its antioxidant properties, capsaicin has been experimentally demonstrated to regulate cellular oxidative stress. It has beneficial effects in preventing or improving oxidative stress-mediated diseases (cardiovascular disease, diabetes, cancer, etc.).[27–29] In the current study, some plasma differential metabolites associated with oxidative stress were identified, such as cholic acid, deoxycholic acid, pipecolic acid, carnosine, taurine, pyroglutamic acid, gamma-Glutamylcysteines, and 3-Indolepropionic acid. Pipecolic acid can cause oxidative stress in the cerebral cortex of young rats in vitro.[30] Carnosine and taurine have antioxidant properties,[31–33] whose deficits may be detrimental to the disease's recovery from oxidative stress damage. Gamma-glutamylcysteine, pyroglutamic acid and taurine also play a role in regulating oxidative stress due to their

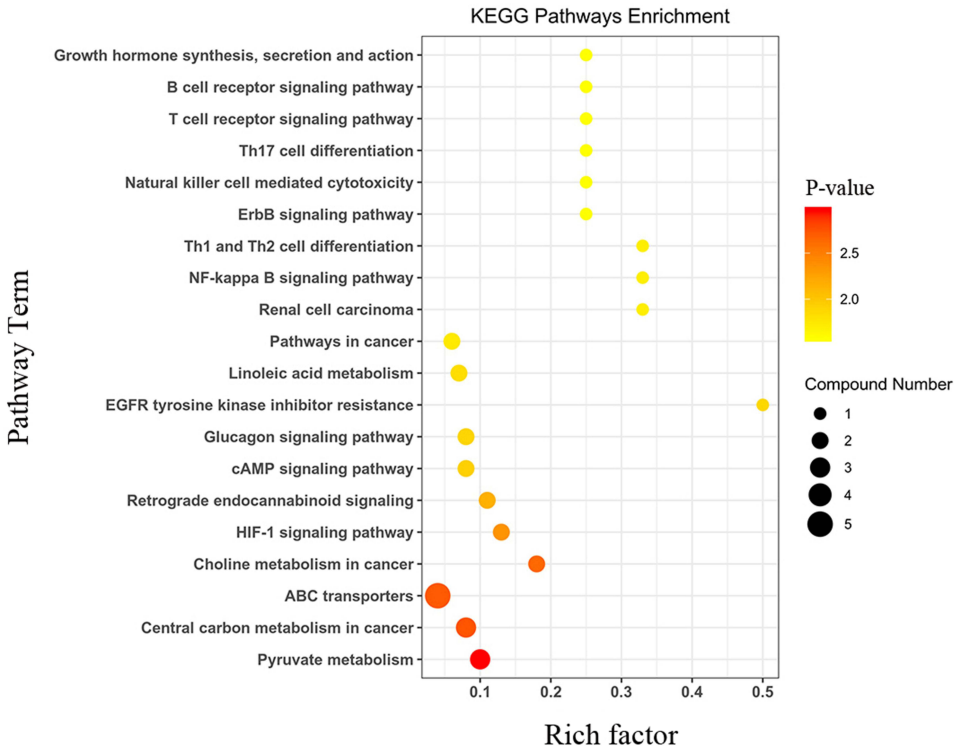

Figure 6 Statistics of KEGG enrichment in plasma. The x axis indicates the rich factor(number of significantly differentiated metabolites/total metabolites in this pathway), and the y axis indicates name of the KEGG metabolic pathway. The size and color of bubbles represent the number of metabolites and the p-value, respectively.

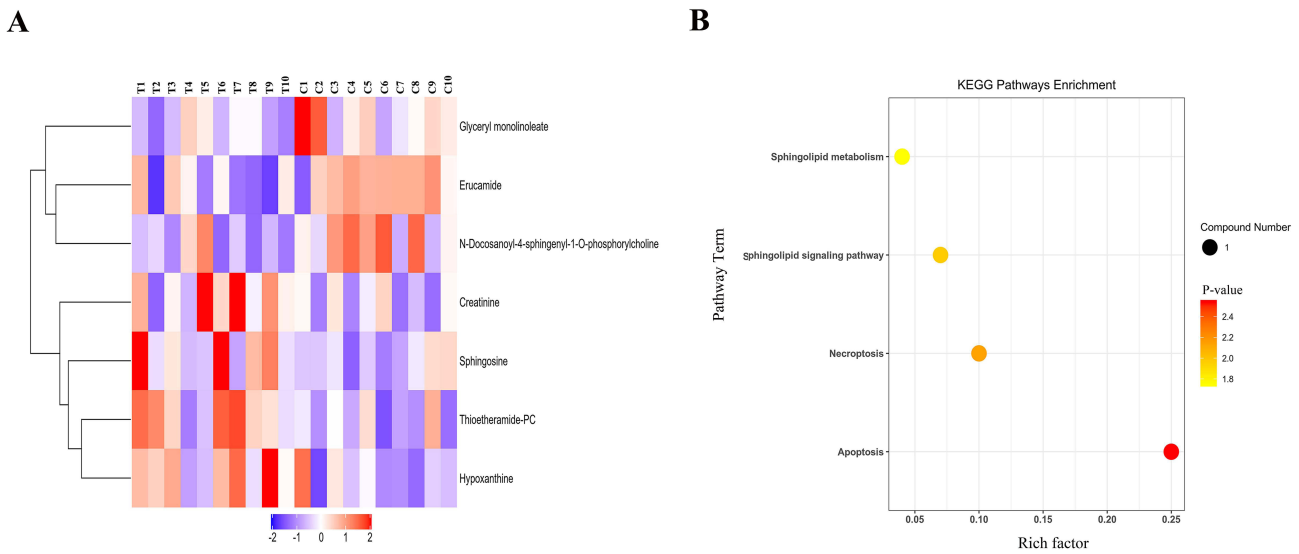

Figure 7 Hierarchical clustering heatmap and KEGG enrichment analysis chart in skin. (**A**) Hierarchical clustering heatmap of 7 differential metabolites in skin. (**B**) Statistics of KEGG enrichment in skin. The x axis indicates the rich factor corresponding to each pathway, and the y axis indicates name of the KEGG metabolic pathway. The size and color of bubbles represent the number of metabolites and the p-value, respectively.

connection with glutathione.[34–37] Hence, our results provide evidence that capsaicin may exert physiological effects by modulating oxidative stress.

According to published studies, capsaicin is effective in reducing anxiety and other emotional responses.[38] For example, dietary capsaicin improved depressive-like behavior of C57BL/6J mice.[39] However, its exact antidepressant mechanism remain unclear. Our data demonstrated that the plasma level of sarcosine was increased in group T(VIP =

1.25, FC = 1.74). Sarcosine, an N-methyl-d-aspartate receptor enhancer, can improve depression-like behavior in rodent models and depression in humans.[40] Hence, our results suggest that capsaicin may exert an antidepressant effect by regulating sarcosine. However, further researches are needed to confirm this.

There were seven metabolites were markedly altered in the skin of group T. Of these metabolites, sphingosine(VIP = 4.89, FC = 1.41) was upregulated, indicating that the sphingolipid metabolism and sphingolipid signaling pathways were significantly perturbed pathways in the skin of group T. Interestingly, our previous studies found that sphingolipid metabolism and sphingolipid signaling pathway were enriched in the rabbit ear acne model.[41] In addition, the sphingolipid signaling pathway was the most altered in the plasma of patients with moderate to severe acne.[42] It is well known that spicy foods can exacerbate acne.Hence, we speculate capsaicin may affect the progress of acne through the sphingolipid metabolism and sphingolipid signaling pathway.

This study had some shortcomings: 1) The number of unclassified metabolites was relatively large.The reason may be limited number of reliable spectral reference databases for metabolite identification; 2) It is only a preliminary exploration of the changes in plasma and skin metabolites in mice after capsaicin intervention, and the related disease models were not established.

Conclusions

In summary, our work has demonstrated that capsaicin can induce apparent plasma and skin metabolic profile changes in C57BL/6 mice. Our findings provide metabolomic insights to assess the physiological functions of capsaicin and contribute to a better understanding of the potential effects of a capsaicin-rich diet on health.

Preprint

A previous version of this manuscript was published as a preprint.[43]

Abbreviations

UHPLC/Q-TOF MS, Ultra-high-performance liquid chromatography and Q-TOF mass spectrometry; NH4AC, Ammonium acetate; NH4OH, Ammonium hydroxide; QC, Quality control; PCA, Pareto-scaled principal component analysis; OPLS-DA, Orthogonal partial least-squares discriminant analysis; VIP, Variable importance in the projection; KEGG, Kyoto encyclopedia of genes and genomes; ROC, Receiver operating characteristic; FC, Fold Change.

Institutional Review Board Statement

All animal experiments were performed according to the regulation of institutional guidelines for the care and use of experimental animals and approved by the Animal Ethical Committee of The Second Affiliated Hospital of Nanchang University.

Data Sharing Statement

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Acknowledgments

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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