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Metabolomics strategy reveals therapeutical assessment of limonin on nonbacterial prostatitis

Aihua Zhang, Huiyu Wang, Hui Sun, Yue Zhang, Na An, Guangli Yan, Xiangcai Meng, Xijun Wang*

National TCM Key Laboratory of Serum Pharmacochemistry, Laboratory of Metabolomics and Chinmedomics, Department of Pharmaceutical Analysis, Heilongjiang University of Chinese Medicine, Heping Road 24, Harbin 150040, China

* Address correspondence to:

Prof. Xijun Wang

National TCM Key Lab of Serum Pharmacochemistry, Laboratory of Metabolomics and Chinmedomics, Department of Pharmaceutical Analysis, Heilongjiang University of Chinese Medicine, Heping Road 24, Harbin 150040, China.

Tel. & Fax +86-451-82110818

Email: wangxijunomics@126.com
Abstract

Limonin has been found to possess significant anti-inflammatory properties in animal tests and with human cells, however, its precise metabolism mechanism has not been well explored. The aim of this study was to investigate the anti-inflammatory effects of limonin in a nonbacterial prostatitis (NBP) animal model. Global metabolite profiling was performed by ultra-high-performance liquid chromatography combined with time-of-flight mass spectrometry (UPLC/ESI-TOFMS) and in conjunction with multivariate data analysis and pathway analysis which were integrated to explore differentiating metabolites and clarify mechanism of limonin against capsaicin-induced NBP. Limonin has a potential protective function revealed by metabolic profiling of limonin-treated rats located closer to the normal group. Twenty potential biomarker candidates and several key metabolic pathways contributing to the treatment of NBP were discovered and identified. Among the pathways, the related glycine, serine and threonine metabolism, glycerophospholipid metabolism were acutely perturbed. The changes in metabolites were restored to their base-line levels after limonin treatment, which might be through regulating the perturbed pathways to the normal state. The results indicate that changed biomarkers and pathways may provide evidence to insight into limonin action mechanisms and enable us to increase research productivity toward metabolomics in therapeutical assessment and drug discovery.

Keywords:
Limonin; metabolomics; nonbacterial prostatitis; metabolites; biomarkers
Introduction

Nonbacterial prostatitis (NBP) is the most common urological diagnosis in men under 50 years of age and is the third most common urologic diagnosis in men over 50 years of age. An estimated 50% of all men experience prostatitis-like symptoms at some point during their lifetime [1]. Although an appropriate animal model that mimics human NBP has not yet been established, some models produced in castrated rats have been proposed to be useful for elucidating the mechanisms of the molecular pathology of NBP [2,3]. Wistar rats spontaneously develop NBP which makes them a good animal model for laboratory investigation of NBP [4]. Although, NBP has become an important clinical issue, however effective therapies for treating NBP have yet to be found. Plant-derived compounds possess a wide range of pharmacological properties and their action has been interest in recent years.

Limonoids are highly oxygenated, modified terpenoids with a prototypical structure either containing or derived from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton found abundantly in plant families such as Meliaceae and Rutaceae [5-7]. Limonin (Fig. S1), also called obaculactone or limonoic acid, is one of the most prevalent limonoids, widely used as a dietary supplement. In 1938, Highby first isolated limonin from Washington navel orange. Ongoing studies show that limonin has exhibited a range of biological activities like antibacterial, antifungal, antimalarial, anticancer, antiviral, antimicrobial, anti-HIV, and a number of other pharmacological activities on humans [8]. Limonin could inhibit chemically induced carcinogenesis and a series of human cancer cell lines, with remarkable cytotoxicity against lung, colon, oral and skin cancer in animal test and human breast cancer cells [9-11]. In recent years a large number of pharmacological studies have been carried out to indicate its beneficial effects. In vivo-tests have shown that limonin inhibits carcinogen-induced tumor growth in different organs [12]. Limonin could suppress CD4+ T-Cell proliferation and interleukin-2 production in mice by modulating nuclear factor-kB activation [13]. In vitro and in vivo tests showed limonin down-regulated T-bet expression in Th1 effector cells [14]. Limonin can also be used as an antioxidant as revealed by the b-carotene bleaching assay and can inhibit HIV-1 protease activity in infected human mononuclear cells [15,16]. Although limonin has antioxidant and anti-inflammatory activity, however, it is not yet certain that these activities of limonin are effective in the inflamed prostate. From a clinical viewpoint, it should rather be more important to be clarified whether limonin can exert its effect even after the establishment of NBP.

Recently, to explain the action mechanism of drugs, metabolomics methodology has been widely used [17-21]. Metabolomics is an important component of systems biology, especially in determining the global metabolic profile by detecting thousands of small molecules in various media ranging from cell cultures to human biological fluids such as urine, saliva, and blood [22]. It has a great impact in investigation of discovering biomarkers, and identifying perturbed pathways due to disease or drug treatment [23,24]. Ultra-high-performance liquid chromatography combined (UPLC) with mass spectrometry (MS) technique has been proven to be an effective combination for metabolites identifications and quantifications due to its excellent resolution and high sensitivity [25].
UPLC-MS-based metabolomics coupled with multivariate data tools could provide an accurate and dynamic picture of the phenotype of biosystems through the study of potential biomarkers that give new insights into the pathophysiological changes of disease and could be used for discovery of new drugs. 

Numerous studies have revealed that limonin showed effective anti-inflammatory activities and may be useful for the clinical treatment of NBP. However, the effects of limonin against NBP are yet to be fully elucidated. In this study, we are the first to investigate the beneficial effects of limonin on NBP by metabolomics approach. Rat model for NBP was developed with the use of intraprostatic injection of capsaicin, and its reliability and validity was tested by inflammatory changes of the prostate. In the present study, we performed UPLC-Q-TOF-HDMS integrated with multivariate data program and pathway analysis to investigate the therapeutic effects of limonin against NBP by revealing urinary metabolic profiling of rat models.

**Experimental Methods**

**Materials and Reagents**

Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany); methanol (HPLC grade) was purchased from Fisher Scientific Corporation (Loughborough, UK); water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used for the preparation of samples and mobile phase; leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Capsaicin (97%) was obtained from (Aladdin, USA); Tween 80 was obtained from Bodi Chemical Co., Ltd.(Tianjin, China). Limonin was purchased from the Tianjin Chemical Reagent Co. (Tianjin, China). All metabolite standards were procured from Sigma (St. Louis, MO, USA).

**Animal Handling**

Ten-month-old male Wistar rats (weighting 240±10g) were provided by the experimental animal centre of Heilongjiang University of Chinese Medicine. Rats were housed in individual cages in a room maintained at 24±1C and a relative humidity of 35–65% with an alternating 12-hr light/dark cycle (the lights came on automatically at 8:00 a.m.). Food and water were freely given. All rats were randomly divided into three groups as follows: Sham group (n=8, NBP control group), model group (NBP group, n=8), limonin group (pretreatment group, n=8). All animals were allowed to acclimatize in metabolism cages for 1 week prior to treatment. The rats in the NBP groups were castrated and NBP was induced as previously described [26]. Briefly, rats were injected subcutaneously with a daily dose of 50 ul sterile suspension of 3% capsaicin (Aladdin, USA) for 9 days. For the control group, commensurable sterile normal saline was injected. After administered orally limonin at a dose of 3.402 g/kg once daily for the following 9 days, rats in the control group were given saline alone under the same conditions. The methods were carried out in accordance with the approved guidelines of the Animal Care and Use Committee of Heilongjiang University of Chinese Medicine (HUCM-2014-05912).
Determination of Cytokines and Histopathological Evaluation

On the 10th day after castration, rats were anesthetized with pentobarbital (25mg/kg, intraperitoneally) and the prostates were rapidly removed and weighed. Parts of the ventral and dorsolateral prostate lobes were used for histological analysis. The remaining parts of the prostates were stored at -80 °C to await processing. Each prostate was homogenized in 10 volumes of ice cold phosphate-buffered saline containing 200 mM phenylmethylsulfonyl fluoride as a protease inhibitor. After further centrifugation of the 10,000g supernatant at 100,000g for 60 min, the resulting supernatant was used for determining cytokines with ELISA kits from R&D Systems (Minneapolis, MN). Protein was determined by the Bradford method (Bio-Rad protein assay kit; Nanjing, China) with bovine serum albumin as the standard protein. To confirm the presence of prostate inflammation, pieces of the ventral and dorsolateral prostate lobes were fixedin 10% neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin. The histological analysis was performed blind by a veterinary pathologist.

Metabolite Extraction

Urine samples were collected and processed as previously reported Ref 20. Briefly, urine was collected from metabolism cages at ambient temperature throughout the whole procedure. Metabolite extracts were vortexed and centrifuged at 13,000 rpm at 4°C for 10 min, and the supernatants were stored frozen at −80 °C until until further analysis. The quality control sample was used to optimize the condition of UPLC-MS, as it contained most information of whole urine samples.

Metabolic profiling platform

Chromatography

Chromatography was performed using Waters ACQUITY™ UPLC system (Waters Corp., Milford, USA) equipped with quaternary pump, online degasser, autosampler, and column compartment. The separation was performed on an ACQUITY UPLC HSS T3 column (50 mm×2.1mm i.d., 1.8µm, Waters Corporation, Milford, USA). The injection volume was fixed at 5 µL. All the samples were maintained at 45°C during the analysis and the flow rate was 0.4 mL/min. The mobile phases were composed of 0.1% formic acid in acetonitrile (solvent A) and 0.1% formic acid in water (solvent B), the gradient was used as follows: a linear gradient of 1-10% A over initial–2.0 min, 10-20% A over 2-4 min, 20-60% A over 4-7.5 min, 60-99% A over 7.5-8.5 min, 99% A over 8.5-10.5 min, 99-1% A over 10.5-11.0 min, 1% A over 11-13 min. The eluent was introduced to the mass spectrometer directly. The QC was prepared by pooling together equal volume aliquots of all individual urine samples in order to assess LC-MS system stability.
Mass spectrometry

For mass spectrometry, the Waters high-definition mass spectrometer (Waters Corp., Milford, USA) with an electrospray ionization source (ESI) in negative mode was used. The optimal conditions were as follows: the capillary voltage was 2.6kV, the sampling cone voltage was 30V, and extraction cone voltage was 3.5V, the source temperature was set at 120°C, desolvation gas temperature was 350°C, desolvation gas flow was 650L/h. The data acquisition rate was set to 0.4 s/scan, with a 0.1 s inter scan delay. Data were collected in centroid mode and the mass range was set at m/z 50–1000 using extended dynamic range. For accurate mass acquisition, a lock-mass of leucine enkephalin at a concentration of 0.2 ng/mL was used via a lock spray interface at a flowrate of 100µl·min⁻¹ monitoring for negative ion mode ([M-H]⁻ = 554.2615) to ensure accuracy during the MS analysis. The reference solution was continuously introduced into the MS system during analysis as an internal calibration of the Q-TOF system.

Quality assurance and multivariate analyses

Quality assurance was achieved by inclusion of a pooled QC sample injected during each run performed by Q-TOF-MS. The QC serves as an internal reference sample included within every separation in order to monitor long-term instrument bias and system drift that is similar to intermittent QC used in large-scale metabolomic workflows. TransOmics qualitative analysis software was used to extract molecular features—unidentified, untargeted compounds—in each of the data. It looks for mass signals (ions) that are covariant in time, considers possible chemical relationships, and generates an extracted compound chromatogram and compound mass spectrum for each molecular feature. The detailed analysis workflow of TransOmics informatics for metabolomics data from large biological data sets was shown in Rf 25. The extracted compound list for each file was exported as EXCEL file for further compound statistics by EZinfo software (Waters Corp., Milford, USA), including principal component analysis (PCA), partial least-squares-discriminant analysis (PLS-DA) and orthogonal projection to the latent structure with discriminant analysis (OPLS-DA). PCA was used to visualize general clustering, trends, or outliers among the observations. PLS-DA and OPLS-DA were subsequently conducted using the unit-variance scaled MS data as X-matrix and class information as Y-matrix. Urine metabolites were first selected as putative candidates based on their performance in VIP plot of PLS-DA. Multivariate analyses of log-transformed and auto-scaled urine metabolome data were performed using MetaboAnalyst 2.5 (http://www.metaboanalyst.ca), which aligned, normalized, visualized and filtered the molecular features, for further processing. Hierarchical cluster analysis (HCA) to check data quality, implemented in MetaboAnalyst approach commonly used for unsupervised clustering, was constructed based on the potential candidates of importance. The missing peaks were filtered according to their frequency, and compounds that appeared in more than 80% of samples in at least one group were retained.
**Biomarkers Identification**

The identification of potential biomarkers was determined by Q-TOF-ESI-MS. The MS collision energy is 35ev, and the data was obtained in the negative ion mode. MetaboLynx 4.1 software was used for data analysis. The identities of the specific metabolites were confirmed by elements information comparison of their mass spectra using the elemental composition information provided by the software. Finally, compound identification was performed by comparison with the chromatographic retention characteristics and mass-spectra of authentic standards.

**Pathway Analysis**

Pathway enrichment analysis was performed using online program Metaboanalyst 2.5 software that intended for the analysis of metabolomics data. Briefly, the potential markers that passed the criteria were input into the software and the pathway enrichment analysis in some databases, including HMDB (http://www.hmdb.org/), KEGG (http://www.genome.jp/kegg/), Metlin database (http://metlin.scripps.edu/) and LIPID MAPS (http://dev.lipidmaps.org), to discover related pathways. Pathway impact-alter was used to determine the statistical significance in the pathways.

**Statistical analysis**

The PCA was used to uncover unknown trends in the treated groups. Statistically significant differences in mean values were tested by using 2-tailed, 2-sample Student’s t-test, and p<0.05 was considered statistically significant. Prior to multivariate analysis, the resultant data matrices from two analytical techniques were mean-centered and paretoscaled.

**Results**

**Histopathological Findings**

In the first step of the present study, histologic change after establishment of NBP was investigated. Extensive infiltration of inflammatory cells in the lumina, mononuclear cells in the stroma of the gland, and epithelial degeneration were observed in the NBP group (Fig. S2). We confirmed that the stromal predominance and the macrophage infiltration were maintained or even became more striking after 9 days’ observation. The high levels of macrophage infiltration surrounding the prostate glands were observed in the NBP rats. Then, we investigated the effect of limonin on the histologic changes when it was administered after NBP was once established. Limonin treatment significantly reduced macrophage infiltration and suppressed the histologic changes of the NBP group. These effects are presumed to be the result of the anti-inflammatory effects of limonin.
Tissue Concentration of Proinflammatory Cytokines

The prostatic level of the proinflammatory cytokine TNF-a, determined as a measure of prostate inflammation, was significantly increased (p<0.001) in the NBP group compared to the sham-operated group (Table S1). Plasma protein extravasation was significantly higher in the NBP group than in the nonprostatitis control group (p<0.05). Interestingly, oral administration of limonin markedly suppressed the level of protein extravasation in the NBP rat prostates (Table S1). Limonin suppresses the stromal predominance and has certain anti-inflammatory effect on NBP rat prostates even after establishment of inflammation. It indicates that the level of cytokines, is potentially related to the suppression of NBP by limonin. To elucidate the more detailed effective mechanism of limonin, further study is needed.

Data Quality Assessment

To obtain reliable data, technical errors originating from sample collection, sample preparation, and UPLC/MS analysis must be minimized to avoid confounding multivariate data analysis. In our study, samples from each group were alternated in random order in each analysis batch. Moreover, QC sample was analyzed in parallel with the actual samples to monitor the stability of the system. For the QC sample, four selected characteristic ions were picked out to examine the drift of retention time, m/z and peak areas (table S2). Results showed that variations in the retention times were less than 0.05 min, drift values of m/z were less than 5 ppm and the relative standard deviation of each peak area was below 5.52%, demonstrating that system had excellent stability and repeatability during the analysis procedure. According to the optimized conditions of urine analysis, multivariate analysis results of the QC sample demonstrated that QC samples were gathered together to determine during the data collection and instrument operation, indicating that UPLC-Q-TOF-MS system had excellent stability during the analysis procedure (Fig.S3).

Acquisition and Processing of Metabolic Profile Data

For obtaining useful metabolomics results, data analysis strategy is as important as the analytical technique employed. In order to better visualize the subtle similarities and differences among these complex data sets, multiple pattern recognition methods were employed to phenotype the urine metabolome of rats. In this study, the UPLC-MS analysis by ESI in negative ion modes raw data was converted into TransOmics program. The representative Based Peak Intensity (BPI) chromatograms of urine samples derived from control, model, and dose groups in negative mode are presented in Fig.S4 by using the optimal UPLC/MS conditions described above. Low molecular mass metabolites could be separated well in the short time of 7 min. TransOmics (The program used available in supplemental Fig.S5) was then used to carry out peak discrimination, filtering, and retention time alignment, yielding 1894 peaks (Fig. S6) between retention times of 0.2-7 min. The resulting data were imported into EZinfo 2.0 software, and centered and pareto scaled to reduce the impact of noise and artifacts in the models.
Global Metabolomic Profiling

To investigate global metabolomic alterations, here multiple pattern recognition methods were used to classify the metabolic phenotypes and identify the differentiating metabolites and all observations acquired were integrated and coanalyzed using PCA. The PCA results are displayed as score plots indicating the scatter of the samples, which indicate similar metabolomics compositions when clustered together and compositionally different metabolomes when dispersed. With regard to information analyst of PCA in our experiment showed in Fig. 1A, the control and NBP groups were significantly divided into two classes, indicating that the model of NBP was successfully reproduced. NBP model rats deviated from control rats in their metabolic profile, indicating that the metabolic networks of NBP model rat were disordered. More subtle changes can be found by the Loading plot of PLS (Fig. 1B), which exhibits how each of these variables is responsible for the separation more intuitively. For further analysis of feature ions, S-plot (Fig. 1C) and VIP-plot (Fig. 1D) from the OPLS was carried out to select distinct variables as potential biomarkers for NBP. From the corresponding the S-plot and VIP-plot, the ions furthest away from the origin may be therefore regarded as the differentiating metabolites. We generated VIP plots from the OPLS-DA with a threshold of 3 to identify the metabolites that significantly contribute to the clustering between groups.

Identification of Potential Biomarkers

The potential markers were identified by using the ‘ID browser’ to search in Metlin database and compared with the accurate mass charge ratio in some databases, including HMDB, KEGG, LIPID MAPS, and PUBCHEM. We can know the probable name of potential biomarkers through the first step. The precise molecular mass of compounds with significant changes in the groups was determined within measurement errors (<5 ppm) by Waters QTOF/MS, and meanwhile, the potential elemental composition, degree of unsaturation and fractional isotope abundance of compounds were obtained. The presumed molecular formula was searched in Chemspider (http://www.chemspider.com/), HMDB and other databases to identify the possible chemical constitutions, and MS/MS data were screened to determine the potential structures of the ions. Finally, the structures of potential markers were determined by the use of the standards of the compounds. In the present study, 20 potential biomarkers were identified (Table S3).

Pathway Analysis

More detailed analysis of pathways influenced by NBP was performed by the ‘pathway analysis’ module within the MetaboAnalyst software. The pathways obtained shows in Table S4. We use the high-quality KEGG metabolic pathways as the backend knowledge base to identify the most relevant pathways, such as glycine, serine and threonine metabolism, glycerophospholipid metabolism and so on, in which four unique pathways (listed in Table S4)
for the model group was identified. Potential biomarkers related to glycine, serine and threonine metabolism, glycerophospholipid metabolism pathways were also conformed. Of the four distinct metabolites identified from these pathways, many are in various stages of progress of the NBP. The detailed construction of the metabolism pathways with higher score was shown in Fig.S7. Results indicate that these pathways show the marked perturbations over the formation of NBP and could contribute to the development of NBP.

**Protective Effects of Limonin Against NBP**

PCA results display that the NBP group was far away from the remaining two groups, indicating that changed metabolic pattern resulted from NBP may be significantly different from others (Fig. 2A). The position of treatment group was near to the control group, indicating that changed metabolic pattern was caused by limonin. Results manifest that limonin could change the abnormal metabolic status and may have therapeutic effects on NBP. The PLS score plot also showed that the control, NBP and limonin treatment groups were separated clearly (Fig. 2B), and the limonin treatment group was closer to the control group than the NBP group, which suggested that limonin could reverse the pathological process of NBP. This difference confirmed that limonin can play a role in regulating the abnormal metabolic network of NBP rat. Acquired data were subjected to MetaboAnalyst system analysis to further investigate the effects of limonin on the NBP metabolite profile. Hierarchical cluster analysis (HCA) of auto-scaled and log-transformed metabolomic data depicting the data structure of the crossover study that is dependent on the time course (1-9 day) of NBP after limonin intervention (Fig.2C). Results showed that the dynamic metabolic profiles after limonin treatment were closed to the control group, demonstrating that limonin had therapeutic efficacy. When we analyzed the metabolites that were regulated in NBP compared to controls, the parallel HCA heat map (Fig.2D) showed distinct segregation. The significant features of the metabolite markers as determined based on the VIP ranking was graphed in Fig. 3A. Interestingly, according to the Student’s t-test, we found that the relative concentration of these metabolites could be reversed after taking the limonin. By comparing the identified biomarker level in the NBP group, 19 of the biomarkers were completely reversed by limonin (Fig.3B). Compared with the alterations of NBP-related metabolites, most of them were reset to a normal state after limonin treated administration. It effectively repaired the metabolic network, thus exhibiting the protective effects on immune response and inflammation, ameliorating the dysfunction of the immune system during the development of NBP, thereby inhibiting the progression of NBP.

**Discussion**

In the present study, UPLC-QTOF/MS-based metabolomics method was established to study the metabolic profiles and differential metabolites associated with NBP by analyzing urine specimens collected from the NBP model group, limonin-treated group, and control group. To our knowledge, this is the first report of limonin protecting against
capsaicin-induced NBP by revealing urinary metabolic profiling of rat models. Sun and co-workers had developed the untargeted metabolomics approach based on UPLC/MS to discover the potential biomarkers to clarify mechanisms of berberine in treating a rat model of capsaicin-induced NBP [27]. Fourteen different potential biomarkers contributing to the treatment of NBP were discovered and identified. After treatment with berberine, the relative contents of the potential biomarkers were effectively regulated. Characterizing the differentiating metabolites by UPLC/ESI-Q-TOF/MS combined with pattern recognition approaches can offer a unique view of the mechanism pathways of limonin against NBP. This study was therefore designed to further elucidate the underlying mechanism of limonin on NBP regulation from the metabolic pathways in a global view.

NBP is the most common urological diagnosis in men under 50 years of age and is the third common urologic diagnosis in men over 50 years of age. It has become an important clinical issue, but the causes and treatment of NBP are largely unknown. Thus, it is imperative to study the mechanisms underlying NBP to develop effective therapeutic treatments. Natural products possess a wide range of pharmacological properties and their action has been interest in recent years. Interestingly, previous studies showed that limonin has anti-inflammatory, antibacterial, and antiviral effects, however therapeutic mechanisms on NBP remain unclear. In particular, the discovery of biomarkers could predict the risk of NBP and permit pharmacological treatment timely. Metabolomics enables the parallel assessment of the levels of a broad range of endogenous metabolites and has been shown to have a great impact in investigation of physiological status, discovering biomarkers, identifying perturbed pathways [28-30]. Small-molecule metabolites have an important role in biological systems to understand disease phenotypes, and help us in uncovering drug effects [31].

Our finding that limonin attenuated the levels of the proinflammatory cytokine TNF-α in the prostate of NBP rats. Therefore the suppression of proinflammatory cytokines by limonin in the inflamed rat prostate observed in the present study may explain the beneficial effects of limonin in the treatment of NBP. To more clearly characterize treatment effects of limonin, multiple pattern recognition methods were employed to phenotype the urine metabolome. PCA scores plot showed the control and model groups were significantly divided into two classes, indicating that the model of NBP was successfully reproduced. More subtle changes can be found by the pattern recognition approach-score plots of OPLS. For further analysis of feature ions, VIP-plot from the OPLS was carried out to select distinct variables as potential biomarkers for NBP rats. As a result, 20 differential metabolites in the NBP model were identified and these metabolites demonstrated that abnormal metabolism occurred in NBP was inferred from changes in the intermediates during substance metabolism. The related pathway of biomarker was identified by searching KEGG PATHWAY Database. We identified a total of four distinct metabolic pathways which were identified together are important for the host response to NBP.

Urine samples were analyzed by UPLC/ESI-TOF-MS and multivariate statistical analysis. The results showed that the area of dynamic metabolic profiles after limonin treatment was closed to the control group, demonstrating that
Limonin had therapeutic efficacy. According to metabolomics analysis, 20 potential biomarkers and 4 related metabolic pathways were identified in our study. The significantly down regulated lecithin, 3-dehydroxycarnitine, riboflavin, decenedioic acid, glyoxylic acid, 4-hydroxyisovaleric acid, glycolic acid, indoxyl sulfate, indoleacrylic acid, topiramate, glycine, glucosamine 6-sulfate, up regulated N-acetyl-L-phenylalanine, tetrahydrocortisone, deoxyinosine, 2-deoxyribonic acid, sebacic acid, galacturonic acid, uroporphyrin IV, hippuric acid were observed in the limonin group compared with model group. Limonin can balance the NBP disorder through decreasing the expression of N-acetyl-L-phenylalanine, tetrahydrocortisone, deoxyinosine, 2-deoxyribonic acid, sebacic acid, galacturonic acid, uroporphyrin IV, hippuric acid. Our results show that limonin can increase the expression of lecithin, 3-dehydroxycarnitine, riboflavin, decenedioic acid, glyoxylic acid, 4-hydroxyisovaleric acid, glycolic acid, indoxyl sulfate, indoleacrylic acid, topiramate, glycine, glucosamine 6-sulfate, to relieve inflammation problems to relieve the formation of NBP. In addition, glycine, serine and threonine metabolism, glycerophospholipid metabolism, glyoxylate and dicarboxylate metabolism, primary bile acid biosynthesis, and many other metabolisms were confirmed to have an impact on NBP. In our study, we highlight the metabolite change, and many other potential mRNAs, proteins, genes, enzymes and bioprocess closed to other pathways need future experiments to validate.

At present study, we discovered a significant perturbation of metabolic profile in the urine of NBP rats, which could be obviously reversed by limonin treatment. It is noteworthy that the changes of the most metabolites could be reversed according to limonin treatment, suggesting the possible pharmacological mechanisms of limonin. Most of them were reset to a normal state after limonin administration. Results of differential metabolite identification suggest that the development of NBP involves serious disorders of the metabolism of glycine, serine and threonine metabolism, glycerophospholipid metabolism, and many other metabolisms. Interestingly, it is important to note that the disturbed metabolic pathways are able to be reversed by limonin treatment. In other words, limonin might contribute to repair these metabolites involved pathways, could effectively regulate the metabolic disorders of NBP rats. These findings highlight the pharmacological characteristics of limonin. Furthermore, the further experiments should be taken fully into account a dose and time dependent manners of limonin. And the limitation of this study is that we only focused on the pharmacological mechanisms without the adverse drug reaction profiles and safety evaluation of limonin.

**Conclusion**

In this study, for the first time, we report a comprehensive analysis of metabolic patterns of the treatment of NBP with limonin, using UPLC-Q-TOF/MS-based urine metabolomics study protocol combined with multivariate statistical analysis. More importantly, 20 differential metabolites, 4 related pathways were discovered and associated with NBP. Particularly, glycine, serine and threonine metabolism, and glycerophospholipid metabolism were found as the most altered functional pathways associated with NBP. Compared with the alterations of NBP related
metabolites, most of them were reset to a healthier level after limonin administration. Our findings also show that limonin exhibited preventive efficacy against NBP by adjusting these multiple metabolic pathways to their normal state and may be mediated through protein, gene, enzyme, and bioprocess. Based on our findings, this makes these pathways possible therapeutic targets for advanced NBP. In conclusion, these results provide a better understanding of the pathogenesis of NBP and the action mechanism of limonin in NBP. Our results also prove that metabolomics is a powerful technology platform for studying the action mechanism of natural products and investigating disease pathogenesis.

Acknowledgments

This work was supported by grants from the Key Program of Natural Science Foundation of State (Grant No. 81430093, 81173500, 81373930, 81302905, 81202639), National Key Technology Research and Development Program of the Ministry of Science and Technology of China (Grant No. 2011BAI03B03, 2011BAI03B06, 2011BAI03B08), National Key Subject of Drug Innovation (Grant No. 2009ZX09502-005), Specialized Research Fund for the Doctoral Program of Higher Education (Grant No. 20122327120006), Fund Project of Heilongjiang Provincial Department of Education (Grant No. 12521498).

Competing financial interests

The authors declare no competing financial interests.

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Fig. 1. **Multivariate analysis of untargeted metabolomics data.** Three-dimensional PCA scores plot from the urine metabolomes of control group (Sham), NBP model group (n = 8 in each group) (A), each colored point represents a sample; loading plot of PLS-DA of NBP in negative mode (B); S-score plot constructed from the supervised OPLS analysis of urine sample in negative mode (C); panel D shows the VIP-score plots constructed from the supervised OPLS analysis of urine (ESI- mode). Ions with the highest abundance and correlation in the NBP group with respect to the controls are present on the upper far lower far left hand quadrant, whereas ions with the lowest abundance and correlation in the NBP group with respect to the control group are residing in the right hand quadrant.

Fig. 2. **MetaboAnalyst analysis of protective effects of limonin against NBP.** PCA score plots of urine samples in control group, model group, and limonin-treated group (A); PLS score plot of control group, model group, and limonin-treated group (n = 8 in each group) (B); Hierarchical cluster analysis of auto-scaled and log-transformed metabolome data depicting the time course of protective effects of limonin-treated group against NBP (C); Heat map visualization for the urine samples from in control group, model group, and limonin-treated group (D). The heatmaps were constructed based on the potential candidates of importance.

Fig. 3 **Significance changes of potential biomarker candidates and pathway Analysis.**

VIP ranking-plot of the metabolite marker candidates (A); Summary of metabolic pathways responded to NBP followed by interventions with limonin (B). Note, C, control group; M, model group; L, limonin-treated group.
Fig. 1. Multivariate analysis of untargeted metabolomics data. Three-dimensional PCA scores plot from the urine metabolomes of control group (Sham), NBP model group (n = 8 in each group) (A), each colored point represents a sample; loading plot of PLS-DA of NBP in negative mode (B); S-score plot constructed from the supervised OPLS analysis of urine sample in negative mode (C); panel D shows the VIP-score plots constructed from the supervised OPLS analysis of urine (ESI- mode). Ions with the highest abundance and correlation in the NBP group with respect to the controls are present on the upper far lower far left hand quadrant, whereas ions with the lowest abundance and correlation in the NBP group with respect to the control group are residing in the right hand quadrant.
Fig. 2. MetaboAnalyst analysis of protective effects of limonin against NBP. PCA score plots of urine samples in control group, model group, and limonin-treated group (A); PLS score plot of control group, model group, and limonin-treated group (n = 8 in each group) (B); Hierarchical cluster analysis of auto-scaled and log-transformed metabolome data depicting the time course of protective effects of limonin-treated group against NBP (C); Heat map visualization for the urine samples from in control group, model group, and limonin-treated group (D). The heatmaps were constructed based on the potential candidates of importance.
Fig. 3 Significance changes of potential biomarker candidates and pathway Analysis.

VIP ranking-plot of the metabolite marker candidates (A); Summary of metabolic pathways responded to NBP followed by interventions with limonin (B). Note, C, control group; M, model group; L, limonin-treated group.