EXPERIMENTAL STUDY

Effect of Huanglianjiedu Tang on fever in rats induced by 2, 4-dinitrophenol

Shumin Liu, Na Wang, Pingping Chen, Xuzhao Li, Changfeng Liu

Abstract

OBJECTIVE: To study metabolic characteristics of fever in rats induced by 2, 4-dinitrophenol (DNP) and the effect of Huanglianjiedu Tang (HLJDT) on the fever.

METHODS: The urine samples were analyzed by ultra-performance liquid chromatography/electrospray ionization quadruple time-of-flight mass spectrometry (UPLC/ESI-Q-TOF-MS) at the positive ion mode scanning, and experimental data were analyzed by the principal component analysis.

RESULTS: Eight potential biomarkers indicating the occurrence and evolution of fever were determined according to ions in urine samples. Five of them were found increased, while the other three decreased. After HLJDT intervention, the increased five were reduced significantly in high dose group, compared with model group, while the decreased three had no obvious change. Five of the eight biomakers were identified with formyl-5-hydroxykynurenamine, gentisic acid, aminoadipic acid, phenylacetic acid, L-phenylalanyl-L-hydroxyproline on the basis of MS/MS. These biomarkers are associated with the metabolism of 5-hydroxytryptamine, tyrosine, lysine, phenylalanine and collagen protein, respectively.

CONCLUSION: HLJDT had significant effect on DNP-induced fever in rats. The effect was performed possibly by acting on 5-hydroxytryptamine in hypothalamus and some amino acid metabolism. These results suggested that HLJDT relieved fever by acting on multi-targets.

INTRODUCTION

Fever is one of important clinical manifestations at the process of many diseases. The high body temperature would induce internal environment disordered, and would accelerate the change of cell responses and metabolism in vivo, and the metabonomics and metabolomic networks would change accordingly. However, so far, there was no report on the total changes of endogenous metabolites caused by fever. Moreover, the treatment of fever mostly uses chemical drugs. Huanglianjiedu Tang (HLJDT), a preparation of Traditional Chinese Medicine (TCM), was made from a classical formula which has the effect of detoxification and heat dissipation. This formula came from Handbook of Prescription for Emergency. It consists of Huanglian (Rhizoma Coptidis) (HL), Huangqin (Radix Scutellariae Baicalensis) (HQ), and others. It is considered as a strong formula which has the effect of detoxification and heat dissipation.
(HQ), Huangbai (*Cortex Phellodendri Amurensis*) (HB) and Zhizi (*Fructus Gardeniae*) (ZZ). In terms of TCM theory, HL is the principal medicinal which can purge the heart and middle burner of the fire; HQ is the ministerial one which can clean heat in the lung and eliminate fire in the upper burner; HB is used to purge the lower burner of the fire and ZZ purges the fire in triple burners. In China, this formula is widely used for the treatment of fever. There have been many reports on the pharmacological effect of HLJDT on inflammation, gastrointestinal disorders, diabetes, vasodilation, acute liver injury and Alzheimer disease and other cardiovascular diseases.

Metabonomics can help us to understand disease processes and metabolic changes by means of analyzing changes of metabolites caused by pathological factors. This is very helpful to find new biomarkers related to disease, which can be used for clinical diagnosis and studying the targets and mechanisms of drugs actions.

In our study, the rats with fever induced by 2, 4-dinitrophenol (DNP) were orally administrated with HLJDT. The urine samples were collected and analyzed by means of the technology of metabonomics. The biological characteristics of fever at overall endogenous changes were explored, and the intervention effects, action targets and mechanisms of HLJDT were studied.

### METHODS

**Chemicals and reagents**

HLJDT recorded in "Handbook of Prescription for Emergency" consists of 9.0 g of HL, 6.0 g of HQ, 6.0 g of HB and 9.0 g of ZZ. These crude herbal medicines were purchased from the Heilongjiang Province Drug Company (Harbin, China) and authenticated by associate professor Lianzhi Wang of the Institute of Traditional Chinese Medicine, Heilongjiang University of Chinese Medicine.

The Tang was prepared according to the method specified in "Handbook of Prescription for Emergency". Briefly, the mixture of 9.0 g of HL, 6.0 g of HQ, 6.0 g of HB, and 9.0 g of ZZ was boiled in water (300 mL) for 1 h, followed by boiled in fresh water (240 mL) for 1 h again, then water extractions were combined and concentrated in vacuum, and then lyophilized.

DNP was purchased from the chemical company of Eastern China Normal University (Shanghai, China). The DNP was dissolved in pyrogen-free 0.9% sodium chloride solution at a concentration of 2.5 mg/mL. The urine of animals acclimated to individual stainless-steel metabolism cages (Suzhou Fengshi Laboratory Animal Equipment Co., Ltd., Suzhou, China) for 5 days, the rectal temperature of rats was measured at 8:00 and 18:00 for 3 days by a digital thermometer (MC-612, OMRON, China). Rats with a rectal temperature of 38.0°C-39.5°C were used for the experiment. The approval of the experiment was governed by the Legislation on the Protection of Animals Used for Experiment Purposes (Directive 86/609/EEC).

**Preparation of the test samples**

Forty rats were randomly divided into five groups including control group (CG), DNP group (DG), high dose HLJDT group (HG), moderate dose HLJDT group (MG) and low dose HLJDT group (LG). The HG, MG and LG rats were orally administrated with HLJDT at a dose of 24, 12, and 3 g/kg (crude medicine), respectively. Forty min later, the DG, HG, MG and LG rats were injected with a DNP solution (subcutaneous injection, 10 mL/kg) and CG rats were injected with equal-volume sodium chloride. The urine of the rats was collected respectively during 0-1 h, 1-3 h, 3-5 h, 5-7 h, 7-13 h and 13-22 h. The urine samples were centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant was filtered through a 0.22 µm filter membrane for UPLC/MS analysis.

**UPLC-ESI-MS conditions**

The UPLC/MS analysis was carried out using a Waters ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) coupled with a Waters Micromass Q-tof micro Mass Spectrometer (Manchester, UK). The data were obtained using MassLynx 4.1 workstation (Waters Corporation, Milford, MA, USA), and the MarkerLynx Application Manager (Waters Corporation, Milford, MA, USA) was used for the peak detection and principal component analysis (PCA). For the reversed-phase UPLC analysis, the ACQUITY UPLC BEH C18 column (50.0 mm x 2.1 mm i.d., 1.7 µm particles, Waters Corporation, Milford, MA, USA) was used. The column temperature was maintained at 40°C. The flow rate of the mobile phase was 0.4 mL/min. The injection volume was fixed at 2 µL. Mobile
phase A consisted of water and formic acid (100:0.1, v/v), while mobile phase B consisted of acetonitrile and formic acid (100:0.1, v/v). The column was eluted with a linear gradient in (Table 1). The eluate was directed to the mass spectrometer without splitting.

<table>
<thead>
<tr>
<th>Table 1 UPLC gradient elution program</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time (min)</strong></td>
</tr>
<tr>
<td>Initial</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>14</td>
</tr>
</tbody>
</table>

Note: ACN: acetonitrile.

An electrospray ionization (ESI) interface was used, and the profile data for positive ions from m/z 50-1000 were recorded. The source temperature was set at 110 ℃ with a cone gas flow of 100 L/h, a desolvation gas temperature and flow were set at 300 ℃ and 600 L/h, respectively. The capillary voltage (3.2 kV) and the cone voltage (35 V) were set. A scan time of 0.4 s with an inter-scan delay of 0.1 s was used throughout, with collision energy of 4 eV. A lock-mass of leucine-enkephalin at a concentration of 0.2 mg/µL, in acetonitrile (0.1% formic acid): H2O (0.1% formic acid) (50:50, v/v) for positive ion mode ([M+H]+=556.2771) was employed via a lock spray interface. Data were collected in centroid mode, the lock spray frequency was set at 5 s and the lock mass data was averaged over 10 scans for correction. The mass spectrometric data was collected in full scan mode from m/z 50 to 1000 from 0 to 12 min in positive ion mode.

Data processing and analysis

Integrated mass spectrometry data from the UPLC-TOF/MS system were processed to generate a multivariate data matrix with MarkerLynx 4.1 (Waters Corporation, Milford, MA, USA). Data were analyzed further with principal component analysis (PCA), then the score plot that reflects straggling extent of inter-group, the loading plot that reflects contribution extent of ions, and the trend plot that reflects ions variation of each group were generated to accomplish the statistical analysis of experimental data.

RESULTS

Validation of UPLC conditions

The flow rate into the electrospray ion source recommended was limited within 0.4 mL/min in order to reach the best ionization efficiency and avoid the ion suppression which may seriously influence the analytical sensitivity. Considering sensitivity and resolution, the ultimate flow rate was optimized at 0.4 mL/min in this study. Furthermore, the effects of formic acid with different concentrations (0.01%, 0.1%, and 1%) were investigated; the optimal peak resolution and shape were obtained with 0.1% formic acid. The liquid chromatography column temperature was set at 40 ℃ to reduce the column pressure. The gradient elution was adopted to obtain better separation. For testing the ability of the UPLC system to differentiate, urine samples in the control rats, DNP-treated rats and HLJDT-treated rats were analyzed. Typical chromatograms of these samples were displayed in (Figure 1). Visual examination of these chromatograms showed distinct peaks among different samples. The total changing path of metabolites reflected a series of biology events of pathological course. The scores plots from DNP-treated rats and control rats were shown in (Figure 2). The in vivo metabolism of the rats were disturbed after injection of DNP and their location was away from control group at 1-3 h, the variation was maximal at 3-5 h, then they gradually came back to control group position at 7-13 h and finally coincided with control group at 13-22 h. This result indicated that the DNP-induced fever model was successfully created. The variation of metabolites was maximal at 3-5 h in DNP-treated group, therefore urine samples of 3-5 h periods in all groups were analyzed to investigate the intervention effects of HLJDT on fever induced by DNP. In Figure 3 presented were the PCA results from all groups’ samples of 3-5 h. It was clear that distinct clusters presented in PCA scores plot of urine samples from control rats, DNP-treated rats, and HLJDT-treated rat. The graph of scores plot of LG was near to that of DG and the graph of HG was away from that of DG. The result illustrated that HLJDT had significant intervention effects on fever in rats induced by DNP.

Detection of potential biomarkers

Because more peaks could be detected in the positive ion mode than in the negative ion mode, the positive ion mode was used in this study. The corresponding loadings plot indicated that the ions and their chromatographic retention times were attributable to the clustering observed in the scores plot. Eight dominant ions peaks were selected as potential biomarkers of the fever model according to the amount variation of ions in control group and DNP group. Among them, five ions [m/z 209.0916 (1.35 min), 155.0454 (1.92 min), 162.0220 (2.60 min), 137.0351 (3.25 min) and 288.0294 (5.19 min)] increased in DNP group compared with control group, inversely, three ions [271.1406 (0.72), 267.1355 (0.81 min) and 279.1406 (2.84 min)] decreased in DNP group compared with control group. The trend lines for eight ions were shown in Figure 4. These results demonstrated that eight corresponding compounds could be biomarkers, which might be related to the developing of fever in-
Figure 1 BPI chromatograms of rats urine samples using reversed-phase ultra-performance liquid chromatography/electrospray ionization quadruple time-of-flight mass spectrometry
A: control group; B: 2, 4-dinitrophenol model group; C: high dose group of Huanglianjiedu Tang. BPI: base peak ion.

Figure 2 Scores plot from PCA of reversed-phase UPLC/ESI-Q-TOF-MS data obtained from DNP-treated and control group urine samples
PCA: principal component analysis; UPLC/ESI-Q-TOF-MS: ultra-performance liquid chromatography-electrospray ionization quadruple time-of-flight mass spectrometry; DNP: dinitrophenol; 0: Control; 1: DNP-model 0-1 h; 2: 1-3 h; 3: 3-5 h; 4: 5-7 h; 5: 7-13 h; 6: 13-22 h.
Figure 3 Scores plot from PCA of reversed-phase UPLC/ESI-Q-TOF-MS data obtained from control, DNP-model and HLJDT-treated rats urinary samples collected during 3-5 h periods.

PCA: principal component analysis; UPLC/ESI-Q-TOF-MS: ultra-performance liquid chromatography-electrospray ionization quadruple time-of-flight mass spectrometry; DNP: dinitrophenol; HLJDT: Huanglianjiedu Tang. 0: Control group; 1: DNP-model group; 2: Huanglianjiedu Tang group (high dose group); 3: Huanglianjiedu Tang group (middle dose group); 4: Huanglianjiedu Tang group (low dose group).

Figure 5 Relative amount of eight ions in control, DNP-model and Huanglianjiedu Tang group urinary samples collected during 3-5 h periods.

DNP: dinitrophenol. Model group compared with control group, \(^{a}P<0.01\); high dose group compared with model group, \(^{b}P<0.05\).
Reduced by DNP.
Meanwhile, the amount of these eight ions was analyzed in HLJDT-treated groups. The results showed that five increased ions in DNP-treated group were reduced. However, the three decreased ions remained nearly unchanged in three HLJDT-treated groups (Figure 4). These results demonstrated that the effect of HLJDT on fever was significant, and intervened mainly by regulating five increased ions in induced-fever rats, which was consistent with the results of HLJDT-treated groups in scores plot.

**Identification of the compounds**
On the basis of determination, the biomarkers were scanned by tandem mass spectrometer according their retention time and exact mass. The structures were identified according to their main daughter ions and the splitting information which was provided by mass fragment software in Markerlynx. Five biomarkers peaks (209, 155, 162, 279, 137 m/z) were identified as formyl-5-hydroxykynurenamine, gentisic acid, aminoadipic acid, L-phenylalanyl-L-hydroxyproline, and phenylacetic acid respectively. The structure splitting scheme is shown in (Table 2).

**DISCUSSION**
This study compared the alteration of the final metabolites in urine samples of normal and fever rats induced by DNP by means of metabonomic methods. Eight characteristic biomarkers were found in the development of fever, and the structures of five biomarkers were identified on the basis of MS/MS. Among them, the amount of formyl-5-hydroxykynurenamine, gentisic acid, aminoadipic acid and phenylacetic acid showed higher in DNP than those in CP, while L-phenylalanine-L-hydroxyproline acid had lower amount in DNP than that in CP. Formyl-5-hydroxykynurenamine was a metabolite of 5-hydroxytryptamine which was monoamine transmitter of hypothalamus. The fact that the amount of formyl-5-hydroxykynurenamine became higher could show the amount of 5-hydroxytryptamine increased accordingly, which was consistent with literature reports. Therefore, we can infer that DNP could generate endogenous mediators in rats, furthermore, these mediators reacted with 5-hydroxytryptamine in hypothalamus, and finally made the rats generate heat.

Gentisic acid was a pivotal intermediate metabolite at a circulation in which glycosuric acid was transformed into fumarate and finally went into tricarboxylic acid cycle, and glycosuric acid was a metabolite of tyrosine, which indicated that the fever induced by DNP can lead to the abnormality of tyrosine metabolism. Aminoadipic acid was an intermediate metabolite at the degradation process of lysine, which indicated that the fever induced by DNP could cause the changes of lysine metabolism. Phenylacetic acid was produced by phenylalanine through a metabolic bypass, the basic process of which is phenylalanine → phenyllactic acid → phenylacetic acid → phenyl-acetylglutamine. These metabolites were excreted with sweat and urine, which indicated that the fever induced by DNP could lead to the disorder of metabolism of phenylalanine. The results of these experiments indicated that the metabolism of tyrosine, lysine and phenylalanine would speed up because of the DNP-induced fever.

<table>
<thead>
<tr>
<th>No</th>
<th>tR (min)</th>
<th>M+H (m/z)</th>
<th>MS/MS (m/z)</th>
<th>Actual-M (m/z)</th>
<th>Formula</th>
<th>Biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.35</td>
<td>209.09</td>
<td>108</td>
<td>208.08</td>
<td>C10H12N2O3</td>
<td>Formyl-5-hydroxykynurenamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>150</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>167</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>191</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>209</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>92</td>
<td>154.03</td>
<td>C7H6O4</td>
<td>Gentisic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>109</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>137</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>155</td>
<td>161.07</td>
<td>C11H14NO3</td>
<td>L-2-Aminoadipic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>116</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>162</td>
<td>278.13</td>
<td>C16H18N2O4</td>
<td>L-phenylalanyl-L-hydroxyproline</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>148</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>232</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>260</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>279</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>79</td>
<td>136.05</td>
<td>C8H8O2</td>
<td>Phenylacetic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>92</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>120</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>137</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: ESI: electrospray ionization.
L-hydroxyproline acid in rats reduced visibly, which indicated that the metabolism of collagen protein had slowed down. There was a lot of glycine in collagen protein, which was not only involved in synthesizing collagen but also was a transmitter to inhibit central nerve in brain cells and have sedative action. This was coincident with the dispirited and less autonomic activities syndromes after induction of fever. After the administration of HLJDT, the amount of formyl-5-hydroxykynurenamine, gentisic acid, aminoadenities syndromes after induction of fever.

*REFERENCES*

20. Li PF, Hao WL. Studies on antipyretic effect and its mech-


