Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 86–92

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Effect of the traditional Chinese medicine tongxinluo on endothelial dysfunction rats studied by using urinary metabonomics based on liquid chromatography–mass spectrometry

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ARTICLE INFO

Article history:
Received 14 January 2011
Received in revised form 16 April 2011
Accepted 19 April 2011
Available online 27 April 2011

Keywords:
Metabonomics
Urine
Endothelial dysfunction
LC–MS
Tongxinluo
Simvastatin

ABSTRACT

A urinary metabonomic method based on ultra-fast liquid chromatography coupled with ion trap-time of flight mass spectrometry (UFLC/MS-IT-TOF) was employed to study the preventive efficacy and the metabolic changes caused by simavastatin and the traditional Chinese medicine tongxinluo in endothelial dysfunction rats. Principal component analysis (PCA) was applied to study metabolic patterns of endothelial dysfunction rats and healthy control rats. 1-Methyladenosine, indoxyl sulfate, hippuric acid, riboflavin, coproporphyrin, and p-cresol glucuronide were identified as potential biomarkers, indicating that pathways of adenine, tryptophan, phenylalanine, riboflavin and porphyrin metabolism were disturbed in endothelial dysfunction rats. Applications of simvastatin and tongxinluo to endothelial dysfunction rats improved endothelial function according to the results of histopathology and measurements of endothelin-1 and nitric oxide. Metabonomic studies suggested that tongxinluo prevents endothelial dysfunction by regulating multiple metabolic pathways to their normal state, whereas simvastatin only altered selected metabolic pathways. This research demonstrated that metabonomics is a powerful and promising tool for disease investigation and the efficacy evaluation of complex traditional Chinese medicines.

1. Introduction

As a systemic pathological state, endothelial dysfunction is broadly characterized as reduced vasodilation, a proinflammarory and prothrombic state of the endothelium [1]. A commonly accepted mechanism of endothelial dysfunction is that it is related to the increased production of reactive oxygen species (ROS) in aerobic cells. ROS include hydroxyl radical ($HO^{\bullet}$), superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$), which produces unpaired electrons as well as free radicals [2]. The alteration of endothelial function plays a pivotal role in the development and progression of cardiovascular diseases such as hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure. Furthermore, endothelial dysfunction is usually considered as a marker and the early stage of atherosclerosis [3,4].

Endothelial dysfunction is a complex pathological process involving numerous metabolic pathways. A number of studies of endothelium-related metabolism have been reported [5,6]. Moreover, an integral approach that can meet the need for offering systemic views of metabolic alteration of endothelial dysfunction is still needed. As an important part of systems biology, metabonomics is developing rapidly, and it has been used for studies of disease diagnosis, drug toxicity, and pathophysiology [7–9]. Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) combined with gas chromatography (GC–MS), liquid chromatography (LC–MS), and capillary electrophoresis (CE–MS) are the most widely used analytical techniques. Among these, LC–MS has shown growing applications because of its excellent sensitivity and powerful separation.

Because of its general mildness in nature and its emphasis on maintaining balance in individuals, traditional Chinese medicine (TCM) has gained increasing attention from scientists and patients. However, evaluating the holistic efficacy of TCM remains a difficult task due to the mistiness of active compounds and the unknown synergistic actions of multiple components. The interaction between TCM and organism is still a black box. Metabonomics is an ideal tool to bridge TCM and molecular pharmacology [10],

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doi:10.1016/j.jpba.2011.04.020

partially opening the black box. Several published studies reported applications of metabonomics in evaluating the efficacies of TCM (e.g., berberine [11], Liu Wei Di Huang Wan [12], epimedium [13] and tongxinluo [14]). In our group's earlier work, atherosclerosis was studied by a LC–MS-based metabonomics approach [15]. Plasma and urinary metabolic profiling suggested abnormal metabolism of phenylalanine, tryptophan, bile acids and amino acids in atherosclerotic rats. Moreover, the efficacy of tongxinluo, a traditional Chinese medicine against cardiovascular disease, was investigated on depression-atherosclerosis rats. A similarity evaluation with 114 variables demonstrated that the metabolic pattern of tongxinluo-treated depression-atherosclerosis rats, which were subjected daily to atherosclerosis modeling plus restriction in skintight cages for 6 h, was more similar to control rats [14].

In this work, endothelial dysfunction, an early symptom of atherosclerosis, was investigated to discover disturbed pathways. The efficacy and mechanism of simvastatin and tongxinluo against endothelial dysfunction were compared.

2. Experimental

2.1. Chemicals

Formic acid of HPLC grade was purchased from Tedia (OH, USA). Acetonitrile of HPLC grade was purchased from Merck (Darmstadt, Germany). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Authentic standards, including 1-methyladenosine, coproporphyrin I dihydrochloride, hippuric acid, indoxyl sulfate potassium salt and riboflavin, were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Animal handling and sample collection

Male Wistar rats (200–250 g) supplied by an experimental animal center (Vital River Laboratories, Beijing, China) were housed five rats/cage. The room temperature was regulated at 20–23 °C with 40–60% humidity. A 12-h light/dark cycle was set. The rats were divided into four groups: a healthy control group (HCG, $n = 10$), a endothelial dysfunction group (EDG, $n = 10$) fed with a high methionine diet consisting of regular diet plus 3% methionine, a "endothelial dysfunction + simvastatin group" (EDSVTG, $n = 7$) fed with a high methionine diet and orally dosed with simvastatin by gavage, a "endothelial dysfunction + tongxinluo group" (EDTXLG, $n = 9$) fed with a high methionine diet and orally dosed with tongxinluo by gavage. Simvastatin (0.5 mg/mL) and tongxinluo crude drug (0.12 g/mL) were dissolved in saline water, and the dosages were 5 mg/kg/d and 1.2 g/kg/d, respectively. The HCG and EDG rats were orally administrated with an equivalent volume of 0.1% CMC-Na. All rats were kept for 6 weeks. On the last day, 24-h urine samples were collected and stored at −80 °C pending sample preparation and LC–MS analysis.

2.3. Histopathology and measurements of endothelin-1 and NO

After the urine collection, rats were narcotized by 10% chloral hydrate. The thoracic aortas were harvested and fixed in 4% paraformaldehyde and then embedded in paraffin wax. Then, 400–500-μm slices were acquired and stained with hematoxylin–eosin (HE) pending examination with light microscopy.

The levels of endothelin-1 (ET-1) in plasma and nitric oxide (NO) in serum were determined by radioimmunoassay (Iodine [^{125}I] Endothelin Radioimmunoassay Kit, The General Hospital of the People's Liberation Army, Beijing, China) and nitrate reductase method (Nitric Oxide Detection Kit, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively.

2.4. Sample preparation and LC–MS analysis

Urine samples were thawed at room temperature and then centrifuged at 13,000 rpm (Biofuge Stratos, Thermo Scientific, USA) for 10 min at 4 °C. A 300 μL aliquot of supernatant was transferred into 900 μL distilled water in Eppendorf tubes. After vortexing, the solution was filtered through 0.22-μm nylon filter film. In a typical LC–MS analysis, a 4 μL aliquot of filtered urine was injected into a Shimpack XR ODS column (50 mm × 2.0 mm × 2.2 μm, Shimadzu, Japan) using a Shimadzu UFLC/MS-IT-TOF (Shimadzu, Japan). The column and sample glass vials were maintained at 35 °C and 4 °C, respectively. The gradient duration was 26 min at a flow rate of 0.3 mL min^{-1} with the mobile phase containing: (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. From 0 to 18 min, mobile phase B increased linearly from 2% to 20% and then increased to 60% in the next 1 min. Then, mobile phase B was linearly increased to 98% in 0.5 min and kept at 98% for 1.5 min. At 21.1 min, the percent of phase B was adjusted to 2% for re-equilibration for 4.9 min. Positive ionization mode and negative ionization mode mass spectra were obtained simultaneously on a full scan operation with scan range of 100–1000 *m/z* by switching the interface voltage between 4.5 kV and −3.5 kV in each 0.1 s. The flow rate of the nebulizing gas (N_2) was 1.5 L min^{-1}. The curved desorption line and heat block temperature were both 220 °C, and the microchannel plate detector voltage was set to 1.75 kV. Ultra-high purity argon was employed as both the ion cooling gas and the collision gas.

2.5. Data pretreatment

The raw LC–MS data were processed by Profiling Solution (Shimadzu, Japan) for peak resolution and alignment. The primary parameters were as follows: width (5 s), slope (2000 min^{-1}), ion *m/z* tolerance (25 mDa), ion RT tolerance (0.3 min), and ion intensity threshold (10,000 counts). Other parameters were set as default. A matrix consisting of matched ion features with retention time, *m/z* value and corresponding intensities was generated and then was exported to an Excel table. Before principal component analysis (PCA), the individual positive (or negative) ion intensity was normalized to the sum of positive (or negative) ions intensities within each chromatogram to dispel the influence of concentration of urine and MS response shift in the long duration of LC–MS analysis. The total peak area of either ion mode in each chromatogram was set to 10,000. Ions with less than 30% relative standard deviation (RSD) in QC samples [16,17] were retained for further PCA modeling, as they were considered stable enough for prolonged LC–MS analysis,.

3. Results and discussion

3.1. Pathological study

The pathological results are shown in Fig. 1. Typical pathological characteristics of endothelial dysfunction, such as swollen endothelial cells and thickened endomembrane, in addition the infiltration of inflammatory cells, are observed in EDG rats (Fig. 1B). In EDTXLG and EDSVTG rats, pathological characteristics of endothelial dysfunction were still observed; however, these symptoms were obviously alleviated (Fig. 1C and D). The above phenomena indicated that the artificial modeling of endothe-

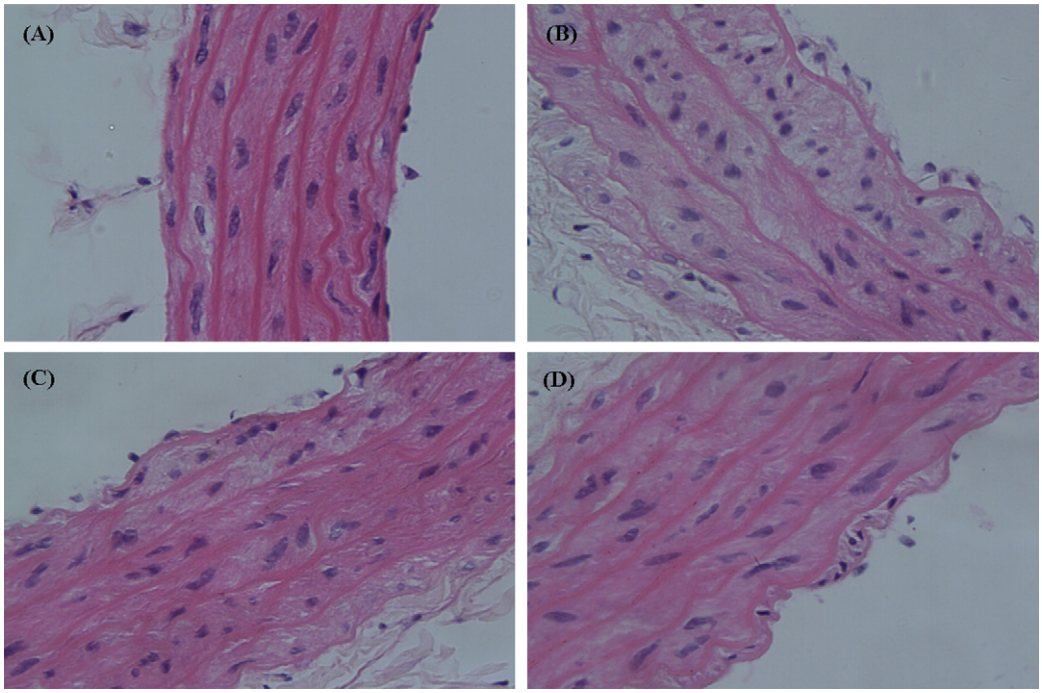

Fig. 1. HE staining of thoracic aorta in (A) HCG, (B) EDG, (C) EDTXLG and (D) EDSVTG rats. Magnification, 200×.

lial dysfunction in rats was successful and that the pathological condition was improved with administration of tongxinluo or simvastatin.

3.2. Changes of endothelin-1 and NO

A delicate balance between vasodilation and vasoconstriction is maintained by NO in a healthy individual. One process is controlled by prostacyclin and other vasodilators, and the other is controlled by and endothelin (especially endothelin-1) and other vasoconstrictors. Endothelial dysfunction may result when this delicate homeostasis is disturbed, and the over-expression of endothelin, as well as a reduced level and bioactivity of NO in blood, may occur [18]. Compared to healthy rats, the levels of endothelin-1 and NO in endothelial dysfunction rats were up- and down-regulated, respectively (Table 1, $p < 0.05$, calculated by both Student's t-test and Wilcoxon test). In contrast, in tongxinluo- or simvastatin-treated endothelial dysfunction rats, the levels were returned to the normal values, at least in part. These results were in accordance with the outcome of the pathological study discussed above, indicating that impaired endothelial function in endothelial dysfunction rats was partially improved when they were treated with tongxinluo or simvastatin.

Table 1
Determination of endothelin-1 (ET-1) in rats' plasma and nitric oxide (NO) in rats' serum.

Group	ET-1 (pg mL^{-1})	NO (μmol L^{-1})
HCG	120.9 ± 17.2[a]	60.8 ± 17.6
EDG	175.6 ± 15.6*	35.0 ± 9.2*
EDTXLG	147.2 ± 14.6☆	53.3 ± 12.8☆
EDSVTG	140.1 ± 10.9☆[b]	53.2 ± 11.6*[c]

*$p < 0.05$ (compared with HCG); ☆$p < 0.05$ (compared with EDG); *$p < 0.05$ (compared with EDG). p-Values are calculated by both Student's t-test and Wilcoxon test.
[a] 9 rats were measured.
[b] 5 rats were measured.
[c] 6 rats were measured.

3.3. Metabonomic study

3.3.1. LC–MS analysis of metabolic profiling

Fig. 2 shows typical LC–MS total ion current (TIC) chromatograms of a urine sample in positive ionization mode (Fig. 2A) and negative ionization mode (Fig. 2B).

Elucidating the structures of potential metabolites has become the bottleneck in metabonomics study, particularly in LC–MS based metabonomic studies, due to the lack of databases of general applicability. In the process of biomarker identification, inconsistent fragmentation spectra are produced by different types of mass analyzers, further increasing difficulties of identification. Therefore, building a personalized LC–MS database with authentic standards is one practical way of identifying biomarkers. In this present study, 244 ions (see Supplementary material) above the limit of detection originating from 96

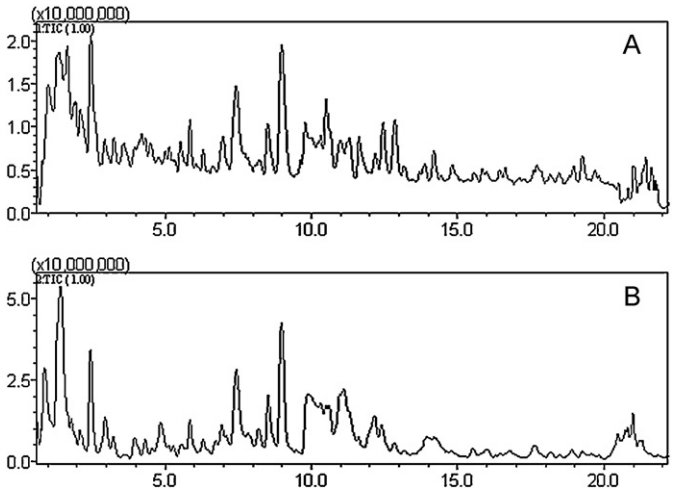

Fig. 2. Typical urinary total ion current (TIC) chromatograms of HCG rats in (A) ESI+ mode and (B) ESI− mode.

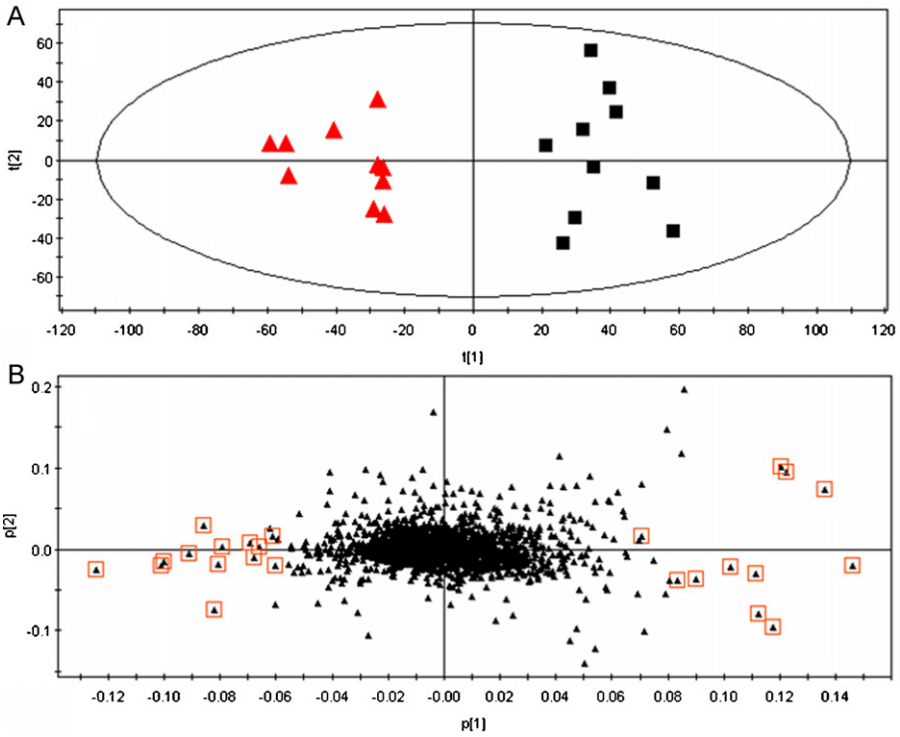

Fig. 3. (A) PCA score plot of urine metabolic profiling of HCG rats (■) and EDG rats (▲); (B) loading plot of PCA model, (□) potential biomarkers.

metabolites were annotated. Excepting N-methyl-2-pyridone-5-carboxamide (or N-methyl-4-pyridone-3-carboxamide), isovalerylglycine (or 2-methylbutyrylglycine), dihydroxyquinoline, phenylalanylhydroxyproline, p-cresol glucuronide and decenedioic acid, the identification of most of the molecular ions were unambiguously validated with authentic standards according to their corresponding retention times and mass fragmentation spectra. A few metabolites with very low abundances in rat urine or those with unavailable MS^2 fragmentation spectra were identified by meticulously comparing retention times and accurate molecular weights with authentic standards. The fragment ions and adduct ions in urine samples were annotated according to the identical retention times and accurate molecular weights of ions originated from authentic references. This list of annotated ions facilitates the identification of compounds in urine metabonomic studies, specifically for the interpretation of the structures of fragment ions and adduct ions.

3.3.2. *Metabonomic fingerprint analysis of endothelial dysfunction*

The raw LC–MS data from metabolic profiling were pretreated following the procedure described in Section 2.5. A modified "80% rule" [19] was employed to delete the missed values from the peak lists of metabolites. Further, the peaks with RSD higher than 30% were removed. Finally, 1651 ions, including ESI+ and ESI− ions, were obtained. To reflect the metabolic difference between HCG and EDG rats and the efficacies of tongxinluo and simvastatin against endothelial dysfunction, a non-supervised multivariate statistical analysis, PCA, was employed to construct the model. Pareto scaling and loading plot were applied to avoid chemical noise [20] and select potential biomarkers, respectively. The first and second principal components explained 30.8% and 12.6% of total variance, respectively (Fig. 3). The metabolic patterns of the rats subjected to endothelial dysfunction modeling deviated from healthy rats (Fig. 3A), indicating metabolic pathways were disturbed in EDG rats. PCA loading plot was employed to discover the differential metabolites (Fig. 3B). The ions marked with a red square contributed most to the classification of these two groups in the PCA model and were considered as potential biomarkers. Ions that eluted at or near the initial void (~1 min) were excluded from potential biomarkers because these ions might not be quantified accurately due to extremely high ion suppression. Twenty-one differential ions (from 18 potential biomarkers) are summarized in Table 2 with their corresponding retention time, *m/z*, ion mode, related metabolic pathways, as well as the ratio of the average ion intensity in endothelial dysfunction rats to the average ion intensity in healthy rats. Seven of 21 differential ions were identified by authentic standards, and another seven ions were deduced based on accurate molecular weights, MS^n and metabonomic databases such as Human Metabolome Database (HMDB), MassBank, Metlin, and Madison-Qingdao Metabonomics Consortium Database (MMCD). Because the number of variables was far higher than the number of samples in metabonomic study (1651 versus 20 here), nonparametric test was more appropriate than the Student's *t*-test when the significance of the difference of variables between two groups were tested. The *p*-values (Wilcoxon test) of differential ions were all found to be below 0.05 (Table 2).

Indoxyl sulfate, a metabolite of tryptophan, is a circulating uremic toxin stimulating glomerular sclerosis [21]. The reduced excretion of indoxyl sulfate in urine may indicate an elevated level in the serum of endothelial dysfunction rats. In an in vitro study, indoxyl sulfate induced endothelial dysfunction by inhibiting endothelial proliferation and migration [22]. It has been suggested that indoxyl sulfate also plays a role in oxidative stress by increasing NAD(P)H oxidase activity in endothelial cells and the production of reactive oxygen species (ROS), and by strongly decreasing the level of glutathione, one of the most active antioxidant systems in cells [23,24].

p-Cresol glucuronide, a glucuronide derivative of p-cresol that is also a uremic toxin like indoxyl sulfate, is a soluble derivative that excretes p-cresol in urine. p-Cresol inhibits the proliferation of endothelial cells, prevents the repair of wounded endothelium

Table 2
Potential biomarkers and related metabolic pathways.

No	t_R (min)	Ion (*m/z*)	ESI mode	Metabolites	Related pathway	Ratio[a,b]
1	1.38	282.1215	+	1-Methyladenosine[c]	Adenine metabolism	1.90
2	1.38	290.1247	+	Not identified	Unknown	0.24
3	2.00	153.067	+	2-PY or 4-PY[d]	Nicotinate and nicotinamide metabolism	0.70
4	3.32	244.1184	+	Not identified	Unknown	0.18
5	4.31	297.1458	+	Not identified	Unknown	0.28
6	5.73	317.1721	–	Isovalerylglycine or 2-methylbutyrylglycine dimer	Fatty acid oxidation	3.37
7	5.73	158.0831	–	Isovalerylglycine or 2-methylbutyrylglycine	Fatty acid oxidation	1.57
8	5.89	311.1622	+	Not identified	Unknown	0.37
9	5.90	324.0724	–	Not identified	Unknown	1.39
10	7.48	357.1092	–	Hippuric acid dimer[c]	Phenylalanine metabolism	0.51
11	7.49	180.0665	+	Hippuric acid[c]	Phenylalanine metabolism	0.60
12	8.68	308.1171	-	Not identified	Unknown	0.50
13	9.82	162.0564	+	Dihydroxyquinoline	Tryptophan metabolism	0.37
14	10.40	212.0019	–	Indoxyl sulfate[c]	Tryptophan metabolism	0.80
15	10.55	279.1357	+	Phenylalanylhydroxyproline	Proteolysis of collagen	0.37
16	10.72	377.1482	+	Riboflavin[c]	Riboflavin metabolism	1.34
17	11.18	567.1728	–	p-Cresol glucuronide dimer	Toluene and xylene degradation, trinitrotoluene degradation	2.31
18	13.55	265.073	–	Not identified	Unknown	11.63
19	17.65	199.0983	–	Decenedioic acid	Fatty acid oxidation	2.39
20	21.18	328.1418	+	Coproporphyrin doubly charged[c]	Porphyrin metabolism	4.19
21	21.18	655.2803	+	Coproporphyrin[c]	Porphyrin metabolism	3.17

[a] The ratio of average ion intensity in the EDG to HCG.
[b] Verified by Wilcoxon test, p-value < 0.05.
[c] Identified by retention time and MS2 spectrum of authentic standard.
[d] N-methyl-2-pyridone-5-carboxamide or N-methyl-4-pyridone-3-carboxamide.

[22], and hampers the response of endothelial cells to inflammatory cytokines [25,26].

Phenylalanylhydroxyproline is the product of the proteolytic breakdown of collagen. Normal endothelial cells synthesize basement membrane collagen [27]. A reduction of phenylalanylhydroxyproline in the urine may indicate a decrease in the synthesis of collagen and thus may be a sign of impaired endothelial cells.

Increased levels of coproporphyrin, a porphyrin metabolite arising from heme synthesis, indicate abnormalities of porphyrin metabolism. Heme is a prosthetic group of hematoglobin, myoglobin and catalase. A published study showed that heme can protect the endothelial cells of human umbilicus vein against oxidative stress [28]. Heme oxygenase (HO) metabolizes heme to form carbon monoxide (CO), which inhibits nitric oxide synthase and promotes arteriolar endothelial dysfunction [29].

3.3.3. *Effect of tongxinluo and simvastatin on endothelial dysfunction*

Tongxinluo consists of 12 herbs and insects and is a traditional Chinese formula widely used to treat cardiovascular diseases in China. It has been thought to have clinical benefits by reducing the occurrence of acute myocardial infarction and complications of heart surgery. Herein, tongxinluo was administrated to investigate the effect of protecting blood vessels against endothelial dysfunction in rats. Simvastatin is a powerful lipid-lowering drug belonging to the "statins" class and is widely used in controlling hypercholesterolemia and preventing cardiovascular disease. Furthermore, simvastatin seems to protect against endothelial dysfunction by improving endothelial function [30]. In this study, as a representative Western medicine, simvastatin was selected as a positive control for tongxinluo.

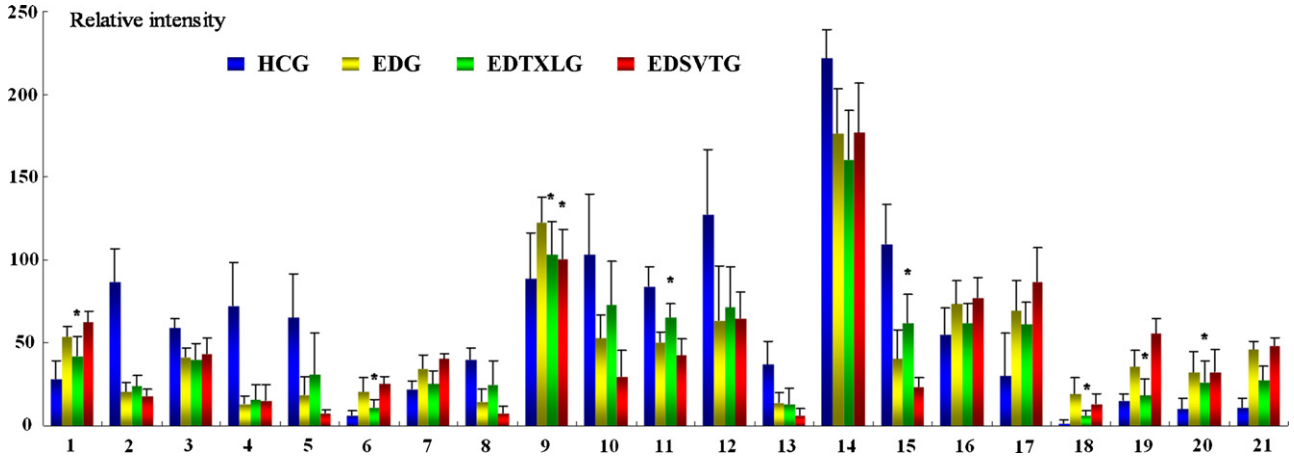

Fig. 4. Comparison of differential ions in urine of HCG, EDG, EDTXLG, EDSVTG rats. Peak no. in abscissa represent metabolites described in Table 2. *p-Value < 0.05 compared with EDG rats (Wilcoxon test).

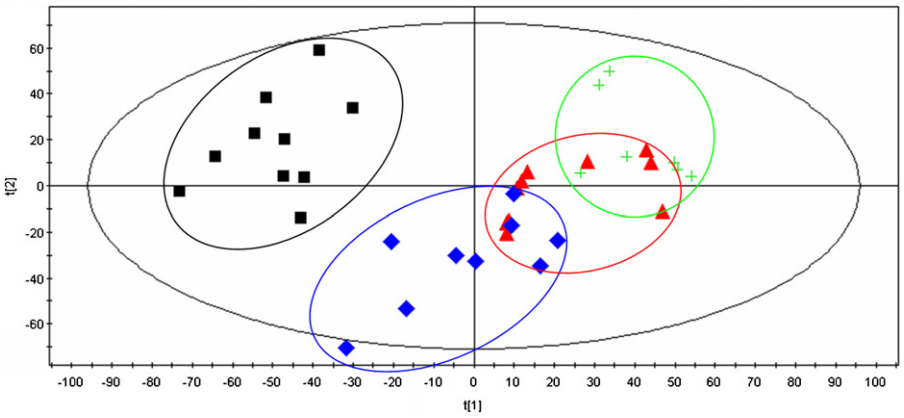

Fig. 5. Score plot of PCA performed on samples from 4 groups: HCG (■), EDG (▲), EDTXLG (♦), EDSVTG (+).

The concentrations of 21 differential ions in HCG, EDG, EDTXLG, EDSVTG rats are graphed in Fig. 4. In EDTXLG rats, the levels of 18 out of 21 ions were regulated to the corresponding levels in HCG rats, whereas 8 of them (marked with asterisks), including 1-methyladenosine, isovalerylglycine (or 2-methylbutyrylglycine), hippuric acid, phenylalanylhydroxyproline, decenedioic acid, coproporphyrin, showed a significant difference compared with endothelial dysfunction rats. This finding suggested that the disturbed pathwaysin EDG rats, such as fatty acid oxidation, proteolysis of collagen, adenine, phenylalanine and porphyrin metabolism, were regulated by tongxinluo. In contrast, 6 out of 21 differential ions showed the same trend in EDSVTG rats, and only one metabolite's *p*-value was less than 0.05 compared to EDG rats. These interesting results indicate that the mechanism by which tongxinluo protects against endothelial dysfunction differs significantly from that of simvastatin. Tongxinluo works by adjusting multiple metabolic pathways to the normal state, whereas simvastatin only changes a few metabolic pathways.

The global metabolic profilings of rats from four groups described above were subjected to PCA (Fig. 5) to compare the metabolic effects of tongxinluo and simvastatin on endothelial dysfunction. The distance of the metabolic pattern between EDTXLG and HCG was much shorter than that between EDSVTG and HCG, which may mean that the metabolic pattern of EDTXLG rats was more similar to healthy rats than to EDSVTG rats. This outcome was consistent with the comparison result of 21 differential ions, which may also be due to the differences of the working mechanisms of tongxinluo and simvastatin. Because it usually consists of a complex mixture of compounds, TCM fights diseases by acting with multiple organism targets.

4. Conclusions

In this study, a urinary metabonomic approach, combined with a pathological study and measurements of endothelin-1 and NO, was employed to discover metabolic disturbances of endothelial dysfunction and to evaluate preventive efficacies of simvastatin and tongxinluo against endothelial dysfunction in rats. Eighteen potential biomarkers were discovered, 11 of which were structurally identified by authentic standards or MS^n analysis. These metabolites demonstrated that abnormal metabolism occurred in the pathways of adenine, tryptophan, phenylalanine, riboflavin, and porphyrin in endothelial dysfunction rats. The pathological study and measurements of endothelin-1 and NO showed preventive efficacies of simvastatin and tongxinluo against endothelial dysfunction. Compared with the alterations of endothelial dysfunction-related metabolites, most of them were reset to a healthier level after tongxinluo administration. Principal components analysis showed that tongxinluo exhibited preventive efficacy against endothelial dysfunction by adjusting multiple metabolic pathways to their normal state, whereas simvastatin changed only selected pathways. This study gave new insight into the changes that occur in endothelial dysfunction rats and into the holistic efficacy evaluation of Chinese medicine through a metabonomic perspective.

Acknowledgements

This study has been supported by the National Basic Research Program of China (Nos. 2007CB914701, 2005CB523301), International cooperation project (No. 2009DFA41250) from State Ministry of Science & Technology of China and New drug R & D Project (No. 2009ZX09313-003) from the Ministry of Health, the National Natural Science Foundation of China (Nos. 20835006, 90709004).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.04.020.

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