Application of GC/MS-based metabonomic profiling in studying the therapeutic effects of Huangbai–Zhimu herb-pair (HZ) extract on streptozotocin-induced type 2 diabetes in mice

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Abstract

A protocol for metabolic profiling of mice urine was developed based on gas chromatograph–mass spectrometer (GC–MS) to explore metabolic state directly. The intra-day, inter-day, repeatability, and stability RSD for most endogenous compounds were less than 3%. Type 2 diabetic mellitus (T2DM) mice model was induced by high calorie diet combined with streptozocin. Urine from the control, T2DM and Huangbai–Zhimu herb-pair (HZ) treatment mice were enrolled in the subsequent study to show the usefulness of the method. OPLS-DA scores plots demonstrate that the cluster of T2DM mice is separated from that of control mice, while HZ-T2DM mice are located close to control mice, indicating that metabolic profiles of these HZ-T2DM mice are placed toward those of control group. The results illustrate that HZ treatment could lower the level of $\text{d}$-glucose, hexadecanoic acid, octadecanoic acid, propanoic acid, 3-hydroxybutyric acid, and 2,3-dihydroxybutanoic acid in urine of DM mice, meanwhile the results show that HZ treatment could ameliorate T2DM symptoms by intervening the fatty acid metabolism, starch and sucrose metabolism, and glyoxylate and dicarboxylate metabolism. This preliminary application indicated that the method is suitable and reliable for urine metabolic profiling. This study might explain the metabolic effects of T2DM and the mechanisms of action of HZ against T2DM.

1. Introduction

Diabetes is a group of metabolic diseases characterized by hyperglycemia, which leading to various tissues and organ dysfunction, especially the eyes, kidneys, heart, blood vessels, skeletal and nerve. More than 90% of diabetes mellitus (DM) are type 2 diabetes mellitus (T2DM), and its morbidity is rising year by year. However modern medicine is still struggling to bring effective treatment [1].

Huangbai–Zhimu herb-pair (HZ) is a well-known Traditional Chinese Medicine (TCM) formula originated from Zishenwan which was recorded in Lanshimizang written by Li Donghuan in the Jin Dynasty. It was composed of Huangbai (the dried bark of Phelodendron chinense Schneid.) and Zhimu (the dried rhizome of Anemarrhena asphodeloides Bge.). Previous studies have indicated that there are several alkaloids and steroidal saponins in the extract of HZ. And the active components include, magnoflorine, menisperine, palmatine, berberine, timosaponin N or timosaponin E1, timosaponin D, timosaponin BII, anemarsaponin C or xilingsaponin B, timosaponin BII and timosaponin AIII [2]. In ancient clinical applications, HZ has been shown to be effective in treating seminal emission, eczema with itching, febrile diseases with high fever, diabetes due to internal heat and constipation [3,4]. Since then, HZ has been widespread used to treat the diabetes patients in clinics in China, however the specific mechanism of action remains unclear.
TCM such as HZ, composed of complex components, and has multiple roles when applied in living systems [5]. In spite of the complexity of the constituents of TCM, it is difficult to study the mechanism of action by using traditional analytical methods. On the other hand, metabonomics is a method used to quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification, and now it provides new insights into the effects of diet, drugs and diseases [6,7]. Previous researches have shown that metabonomics has been applied widely and successfully to disease diagnosis [8–11], pharmaceutical research [12], biomarker identification, evaluation of drug efficacy and toxicity [6–13], and other related topics. Thus, metabonomics can monitor metabolic responses in complex biological systems such as TCM. Metabolite profiling of biological samples combined GC/MS has been recognized to be one of the most useful metabonomic approaches in disease diagnosis and biomarker identification. This approach also gets the advantages of sensitivity and specificity as well as less time-consuming.

In this study, male C57BL/6j mice were grouped into three groups: control (C), diabetic model (DM), and HZ-treated DM (HZ-DM). We use high fat diet plus streptozotocin (STZ)-induced mice as the rodent model for type 2 diabetes. Then, we employed GC/MS-based metabonomics to analyze the changes of metabolic profiles in the urine specimen of each three groups. We attempt to identify potential biomarkers related to type 2 diabetes and then to explore the therapeutic mechanism of HZ.

2. Experimental

2.1. Chemicals and reagents

HZ was gathered from the regions of Anguo City in Hebei Province, China. The plants were identified to be the dried rhizome of Anemarrhena asphodeloides Bge and dried rhizome of Phellodendron amurense by Associate Professor Tianxiang Li. Rhizona Anemarrhena and Cortex Phellodendri were reflux extracted with 50% ethanol, for three times, 1 h for each time. The filtered solutions were combined and concentrated by rotary evaporation with 50% ethanol, for three times, 1 h for each time. The dosage for mice was 0.1 mL extracts per 10 g body weight. The mice were fed on the high-fat diet (HFD) [14,15], in which 60% of kilocalorie are from fat. After 3 weeks of HFD feeding, the mice were intraperitoneal injected once with low-dose STZ (100 mg kg $^{-1}$ body weight, STZ was dissolved in 0.1 M sodium citrate buffer, pH 4.5; Sigma–Aldrich, St. Louis, MO, USA) to induce partial insulin deficiency. Three weeks after STZ injection, animals with comparable degrees of hyperglycemia and body weight were randomly divided to model and treatment groups termed as DM and HZ-DM group respectively. The normal diet-fed mice injected once with sodium citrate buffer were used as non-diabetic termed as a control group. All mice were treated by intragastric administration with normal saline or HZ (2.6 g kg $^{-1}$) for six weeks. Fasting glucose levels and body weight were monitored weekly and urine samples were collected from mice for 24 h in individual urine collection cages. The urine samples were collected over ice to 0.1 mL of 1% sodium azide solution and then centrifuged for 10 min at 4 °C. The supernatant was stored at −80 °C until measurement.

2.3. Urine sample preparation

The urine samples were thawed at room temperature. 200 μL urine sample was transferred to a 1.5-mL sample vial and then was diluted with purified water in a ratio of 1:1 (v/v). 10 μL urease (0.8 mg/10 μL) and 50 μL heptadecanoic acid (1 mg/mL) was added to each sample vial, the samples incubated at 37 °C for 20 min and then mixed with 500 μL acetonitrile. The samples were then vortexed for 30 s and centrifuged at 10,000 rpm for 10 min to remove any particulates. 500 μL supernatant was transferred to a 1.5-mL sample vial, dried by nitrogen, mixed with 50 μL of methoxyamine pyridine solution (15 mg/mL) and incubated at 70 °C for 1 h. Then 70 μL of derivatization reagent (BSFTA + TMCS) was added and incubated at 70 °C for 1 h. Finally, 70 μL of n-heptane was added to each sample vial, and the solution was vortexed for 30 s. Afterwards, the samples were centrifuged at 10,000 rpm for 5 min. The supernatant was transferred into a 0.5-mL sample vial for GC–MS analysis.

2.4. GC–MS analysis

A 1 μL sample was injected into the GC–MS (QP2010, SHIMADZU JAPAN) system for analysis. A Rtx-5MS capillary column (Agilent 0.25 mm × 30 m × 0.25 μm) and FID detector was utilized. The temperature of the column was kept at 70 °C for 5 min, then raised to 160 °C at 15 °C/min, maintained for 1 min, and then raised to 210 °C at 5 °C/min, maintained for 5 min, and then raised to 300 °C at 15 °C/min, maintained 5 min. A total of 38 min. Mass spectrometric conditions: EI ionization; electron energy 70 eV scan 60–600 m/z; full scan mode; the voltage is 0.75–0.8 V; solvents cutting is 5.0 min. The temperature of the vaporizing chamber and the injection port was 200 °C and 70 °C respectively. High purity helium was used as the carrier gas at a flow rate of 1 mL/min. The volume of injection was 1 μL, no streaming. Mass spectra were obtained from m/z 60 to 600, and the acceleration voltage was turned on after a solvent delay of 5 min.

2.5. Data analysis

The basic statistics including the body weight and blood glucose were performed by SPSS 17.0 version. Data is shown as mean ± standard deviations (SD). The significance $P < 0.05$ was computed by using an independent t test between two groups. Detected peaks were aligned using hand integral methods. Peaks were taken into account only when they were consistently detected in at least 80% of the samples. All the detected peaks were identified by comparing the MS spectra with those available in the NIST mass spectral library (Wiley Registry, 2008 edition) and customized
reference mass spectral libraries [16]. Only those compounds with a matching probability of more than 80% were examined the retention time and m/z data pairs were used as the identifier for each peak within each sample.

Statistical analysis was performed using multivariate statistic combined with univariate statistics. Normalized data were exported to SIMCA-P+ (Version 12.0, Umetrics, Sweden) to perform orthogonal partial least squares discriminant analysis (OPLS-DA) and a model was built to identify variables that accounted for the differentiation of Control group, T2DM group and HZ-DM group [9].

3. Results and discussion

3.1. Body weight and blood glucose

Body fluid parameters determined by biochemical analysis are set out in Table 1. As expected, T2DM mice were hyperglycaemic in comparison with control mice. Body weights of mice were not any significant change. In addition, body weights and blood glucose of HZ treatment mice had the significant change comparison with T2DM mice.

3.2. Metabonomic analysis

Typical GC/MS TIC chromatograms of urine samples from the control, T2DM, HZ-T2DM groups are presented in Fig. 1. Within one TIC chromatogram, over 25 signals were usually detected in a single specimen and some of these peaks were not investigated further as they were not consistently found in other sets of samples, with a much lower concentration, or of poor spectral quality to be confirmed as metabolites. As shown in Table 2, a total of 25endogenous metabolites such as amino acids, organic acids, carbohydrates and fatty acids were detected sequentially and these compounds are involved in many biochemical processes in biosystems, such as energy metabolism and lipid metabolism.

3.3. Validation of assay method

3.3.1. Precision

Twenty endogenous compounds were selected randomly to examine the precision of the method while the heptadecanoic acid as the internal standard. The inter-day precision was determined in five replicates, and replicates were processed independently on five separate days, and the intra-day precision was determined in six replicates on the same day. The precision of the analysis was determined as the relative standard deviation (RSD) of the peak area for the same twenty compounds. RSD values of the twenty compounds were less than 3%, as shown in Fig. 2A.

3.3.2. Reproducibility

The repeatability of the analysis was calculated as the relative standard deviation (RSD) of the peak area for each metabolite. RSD values of the twenty compounds were less than 3%, as shown in Fig. 2B.

3.3.3. System stability in the day

To evaluate the deposited stability, one extracted sample was kept in sample manager on five consecutive days at 4 °C and analyzed every day. System stability in the day of the analysis was calculated as the relative standard deviation (RSD) of the peak area for the same twenty compounds. RSD values of the twenty compounds were less than 3%, implying good stability, as shown in Fig. 2B.

3.4. OPLS-DA study

For the metabolic profile, the OPLS-DA model demonstrated satisfactory modeling and predictive abilities using one predictive component and five orthogonal components ($R^2Y=0.999, Q^2=0.844$). Distinct separation trends were achieved between the metabolite profiles of the two groups, as shown in Fig. 3A. However, a separate OPLS-DA model using one predictive component and five orthogonal components ($R^2Y=0.538, Q^2=0.320$) can discriminate the T2DM and HZ-T2DM group, the separation trends could be observed between the T2DM group and control group as displayed in Fig. 3C.

The consequences of the therapeutic effect were almost in accordance with the variation of blood glucose. Compared with control mice, blood glucose values of T2DM mice were significantly elevated, besides, the blood glucose values were meliorated in HZ-T2DM mice although they did not reach the levels of control mice. The results of metabolomic analysis are supported by those of clinical indicators, both indicating different therapeutic effects of HZ treatment on T2DM mice, it will reveal the hypoglycemic mechanism of HZ due to the plots separated clearly among the three groups.

![A GC/MS chromatograph of urine samples obtained from the control mice (A), T2DM mice (B) and HZ-T2DM mice (C), respectively. Some typical metabolites are labeled. 1: propanoic acid; 2: 3-hydroxybutyric acid; 3: pentanoic acid; 4: oxalic acid; 5: 2,3-dihydroxybutanoic acid; 6: l-threonic acid; 7: pentitol; 8: xylonic acid; 9: mannonic acid; 10: d-glucose; 11: d-galactose; 12: hexadecanoic acid; 13: myo-inositol; 14: octadecanoic acid.](image)

![Fig. 1](image)
3.5. Target analyses of highlighted metabolites

To identify which variables were accountable for such momentous separation, variable importance in the projection (VIP) statistics from OPLS-DA modeling was first used to pre-select variables. As shown in Fig. 3B, according to the criterion for VIP statistics (VIP > 1), a total of 12 variables were obtained, which mostly contributed to discriminate metabolic profiles between the two groups. The Wilcoxon test (P < 0.05) was used to investigate the differences of bio-molecules between T2DM group and control. Then, six metabolites were chosen as potential biomarkers to characterize the anti-diabetes mechanism of HZ treatment according to the criterion for the compound changing tendency of T2DM and HZ-T2DM must be opposite (Fig. 3D).

To quantitatively access the changes of the metabolite levels, the integral levels of the metabolites in different groups are comparable in Table 3. Box plots for typical metabolites in urine indicate diversities of individual metabolite levels in distinct groups (Fig. 4).

Compared with control mice, T2DM mice display increased levels of δ-glucose, hexadecanoic acid, octadecanoic acid, propanoic acid, 3-hydroxybutyric acid and 2,3-dihydroxybutanoic acid. Compared with those in the T2DM group, the levels of six metabolites in the HZ-T2DM group have the recovering tendency toward the control levels (Table 3).

3.6. Mechanism analysis of HZ treatment T2DM mic

In order to identify possible pathways affected by HZ treatment, biomarkers contributing to the separation of the control, T2DM, and HZ-T2DM were analyzed using MetPA. Metabolic pathway analysis with MetPA generated 13 networks (Table 4). The pathway impact value calculated from pathway topology analysis, and the threshold was set to 0.10. We filtered out 3 unique pathways as potential pathways (Fig. 5) to HZ treatment above this threshold. Results suggested that these pathways showed marked perturbations over the time-course of diabetes and could contribute to the development of diabetes. The detailed construction of the fatty acid metabolism (Fig. 6) was generated using the reference map by searching KEGG.

3.6.1. Starch and sucrose metabolism

Diabetic patients with absolute or relative deficiency of insulin, liver glycogen synthesis decrease, gluconeogenesis increased, can lead to elevating blood sugar. Abnormal glucose metabolism and lipid metabolism are an important mechanism in the pathogenesis of type 2 diabetes mellitus. Our results showed that compare with the control group, glucose increased significantly in the T2DM mice and we found a drop of glucose in HZ-T2DM group compared with the T2DM group, probably implying that HZ treatment affected the pathways related to starch and sucrose metabolism.

3.6.2. Fatty acid metabolism

Free fatty acids is consist of octadecanoic acid, oleic acid, linoleic acid, hexadecanoic acid, palmitic acid, lauric acid, myristic acid etc., they are the important intermediate products of lipid metabolism [17]. The fatty acids can provide energy for the liver, skeletal muscle, myocardium and brain, and can prevent the decomposition of fasting state protein, they can also promote beta cell secretion under the glucose load [18,19].

Our results showed that levels of octadecanoic acid and hexadecanoic acid were significantly increased in T2DM mice compared with those in control mice, this suggests that the existence of abnormal glucose tolerance in T2DM mice and probably implying that HZ treatment affected the pathways related to fatty acid metabolism. Type 2 diabetes mellitus patients with free fatty acid levels are often at a high value, lipid metabolism disorder may lead to the emergence and progress of diabetes mellitus, fatty acid levels are often at a high value, lipid metabolism disorder may lead to the emergence and progress of diabetes mellitus, fatty acid oxidation increases and competitive inhibition of glucose in the muscle, there will be the inactivation of pyruvate dehydrogenase, and make the intracellular blood glucose concentration increased [20,21]. The high level of free fatty acid in a long time will be increase insulin receptor substrate protein serine residue phosphorylation and the activity of insulin receptor substrate associated PI-3 kinase [22,23], thus, it will inhibit of insulin receptor substrate
function and increase the occurrence and development of type 2 diabetes mellitus [24].

3.6.3. Glyoxylate and dicarboxylate metabolism

3-hydroxybutyrate is also namely hydroxybutyric acid, when the utilization of glucose is bad in diabetes patients, the fatty acid was oxidation and decomposition, it will produce the ketone bodies, of which the most important is the hydroxybutyrate, our results showed that levels of 3-hydroxybutyrate were increased in T2DM mice compared with those in control mice, it revealed the diabetes mice maybe have fatty acid oxidation. Ketoacidosis is common in patients with type 2 diabetes. 2,3-Dihydroxy butanoic acid is a precursor of beta hydroxy butyric acid. Beta hydroxy butyric acid is the most important
Fig. 3. OPLS-DA scores plot (A,C) and loading plot (B,D) of urine samples from the mice. Key: control mice (■), T2DM mice (●) and HZ-T2DM mice (▲).

Table 3 Summary of GC–MS detected statistically significant changes in relative of metabolites in urine samples (n = 7).  

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Control Mean ± SD</th>
<th>T2DM Mean ± SD</th>
<th>HZ-T2DM Mean ± SD</th>
<th>P values T2DM vs Control</th>
<th>P values HZ-T2DM vs T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Glucose</td>
<td>41,765,612.71 ± 110,501,424.6</td>
<td>126,039,933.1 ± 123,362,570.5</td>
<td>104,762,084.1 ± 101,276,792.5</td>
<td>0.20</td>
<td>0.73</td>
</tr>
<tr>
<td>Hexadecanoic acid</td>
<td>8,311,665 ± 14,696,535.81</td>
<td>31,471,316.14 ± 23,842,793.31</td>
<td>29,450,679.59 ± 25,039,743.42</td>
<td>0.05</td>
<td>0.79</td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>4,494,209.286 ± 5,813,476.663</td>
<td>29,935,085.14 ± 37,986,643.69</td>
<td>58,803,357.43 ± 68,072,304.97</td>
<td>0.11</td>
<td>0.98</td>
</tr>
<tr>
<td>3-Hydroxybutyric acid</td>
<td>0 ± 0</td>
<td>93,631,330.57 ± 95,654,900.12</td>
<td>58,803,357.43 ± 68,072,304.97</td>
<td>0.02</td>
<td>0.45</td>
</tr>
<tr>
<td>Propanoic acid</td>
<td>29,658,324.71 ± 21,451,747.12</td>
<td>46,156,426.86 ± 38,367,182.55</td>
<td>15,143,180.71 ± 13,968,194.84</td>
<td>0.34</td>
<td>0.11</td>
</tr>
<tr>
<td>2,3-Dihydroxybutanoic acid</td>
<td>2,881,465.429 ± 3,215,014.857</td>
<td>9,700,718.143 ± 95,654,900.12</td>
<td>6,131,455.857 ± 68,072,304.97</td>
<td>0.02</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Table 4 Results from pathway analysis with MetPA.  

<table>
<thead>
<tr>
<th>No.</th>
<th>Pathway name</th>
<th>Total</th>
<th>Expected</th>
<th>Hits</th>
<th>Raw P</th>
<th>Holm P</th>
<th>FDR P</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fatty acid biosynthesis</td>
<td>49</td>
<td>0.12</td>
<td>2</td>
<td>5.78E-03</td>
<td>4.63E-01</td>
<td>4.63E-01</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>Synthesis and degradation of ketone bodies</td>
<td>6</td>
<td>0.01</td>
<td>1</td>
<td>1.49E-02</td>
<td>1.00E+00</td>
<td>5.95E-01</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>Fatty acid elongation in mitochondria</td>
<td>27</td>
<td>0.07</td>
<td>1</td>
<td>6.55E-02</td>
<td>1.00E+00</td>
<td>7.90E-01</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>Glycolysis or Gluconeogenesis</td>
<td>31</td>
<td>0.08</td>
<td>1</td>
<td>7.49E-02</td>
<td>1.00E+00</td>
<td>7.90E-01</td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td>Pentose phosphate pathway</td>
<td>32</td>
<td>0.08</td>
<td>1</td>
<td>7.72E-02</td>
<td>1.00E+00</td>
<td>7.90E-01</td>
<td>0.00</td>
</tr>
<tr>
<td>6</td>
<td>Propanoate metabolism</td>
<td>35</td>
<td>0.09</td>
<td>1</td>
<td>8.42E-02</td>
<td>1.00E+00</td>
<td>7.90E-01</td>
<td>0.00</td>
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<tr>
<td>7</td>
<td>Butanoate metabolism</td>
<td>40</td>
<td>0.10</td>
<td>1</td>
<td>9.58E-02</td>
<td>1.00E+00</td>
<td>7.90E-01</td>
<td>0.00</td>
</tr>
<tr>
<td>8</td>
<td>Galactose metabolism</td>
<td>41</td>
<td>0.10</td>
<td>1</td>
<td>9.80E-02</td>
<td>1.00E+00</td>
<td>7.90E-01</td>
<td>0.00</td>
</tr>
<tr>
<td>9</td>
<td>Nicotinate and nicotinamide metabolism</td>
<td>44</td>
<td>0.11</td>
<td>1</td>
<td>1.05E-01</td>
<td>1.00E+00</td>
<td>7.90E-01</td>
<td>0.00</td>
</tr>
<tr>
<td>10</td>
<td>Glyoxylate and dicarboxylate metabolism</td>
<td>50</td>
<td>0.12</td>
<td>1</td>
<td>1.18E-01</td>
<td>1.00E+00</td>
<td>7.90E-01</td>
<td>0.01</td>
</tr>
<tr>
<td>11</td>
<td>Starch and sucrose metabolism</td>
<td>50</td>
<td>0.12</td>
<td>1</td>
<td>1.18E-01</td>
<td>1.00E+00</td>
<td>7.90E-01</td>
<td>0.02</td>
</tr>
<tr>
<td>12</td>
<td>Fatty acid metabolism</td>
<td>50</td>
<td>0.12</td>
<td>1</td>
<td>1.18E-01</td>
<td>1.00E+00</td>
<td>7.90E-01</td>
<td>0.03</td>
</tr>
<tr>
<td>13</td>
<td>Amino sugar and nucleotide sugar metabolism</td>
<td>88</td>
<td>0.22</td>
<td>1</td>
<td>2.00E-01</td>
<td>1.00E+00</td>
<td>1.00E+00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Note: Total is the total number of compounds in the pathway; the Hits is the actually matched number from the user uploaded data; the Raw P is the original P value calculated from the enrichment analysis; the Holm P is the P value adjusted by Holm–Bonferroni method; the FDR P is the P value adjusted using false discovery rate; the Impact is the pathway impact value calculated from pathway topology analysis.
Fig. 4. Box plots of relative integral levels of metabolites in urine samples of different groups of mice.

Fig. 5. Summary of pathway analysis. 1: fatty acid metabolism; 2: starch and sucrose metabolism; 3: glyoxylate and dicarboxylate metabolism.
Fatty acid degradation

4. Conclusions

The findings in this study revealed the preventive and therapeutic efficacies of TCM (HZ) against type 2 diabetes by employing GC/MS-based metabolomic profiling combined with multivariate statistical analysis. Additionally, six potential biomarkers and three metabolic pathways identified in this study might help explain the metabolic effects of type 2 diabetes and the mechanisms of action of HZ against type 2 diabetes.

In this study, after taking the medicine (HZ), the treatment effect on diabetic is not obvious, the majority of biochemical parameters did not show significant changes, it might be because the shorter and less number of samples associated with treatment.

In the future, increasing the number of samples, and prolonging the time course of treatment will be better for evaluation of the therapeutic effect of HZ on type 2 diabetes.

Conflict of interest

The authors confirm that there is not any conflict of interests.

Authors’ contribution

Lili Song and Hongyue Liu equally contributed to this paper.

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References


