Effects of Huatan Jiangzhuo decoction on diet-induced hyperlipidemia and gene expressions in rats

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[ABSTRACT] Huatan Jiangzhuo decoction (HJD) is a combination of six traditional Chinese medicines that were used for lipid metabolism-related disorders, but its efficacy and underlying mechanisms have not been explored by modern research strategies. This study aimed to investigate the therapeutic role of HJD in determining the transcriptome level. Hyperlipidemia model was established by feeding Sprague–Dawley rats with high-fat diet. Differentially expressed genes (DEGs) were detected by high-through transcriptome sequencing, followed by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The total cholesterol (TC) and triglyceride (TG) levels in hyperlipidemia model rats were significantly increased, whereas high-density lipoprotein (HDL) concentration decreased when compared to normal rats, and HJD significantly downregulated TC concentrations and liver coefficient in the hyperlipidemia rats. Histology staining showed that HJD greatly recovered the lipid accumulation in rat hepatic stellate cells and aortic arch vascular wall thickness of hyperlipidemia rats. One thousand nine hundred and thirty-six DEGs were identified in the HJD-treated hyperlipidemia rats, which were associated with various biological processes and signaling pathways such as peroxisome proliferator-activated receptors, AMP-activated Protein Kinase, and insulin signaling pathways. Quantitative reverse transcription–polymerase chain reaction further confirmed the downregulated expression of cholesterol 7α-hydroxylase (CYP7A1), liver orphan receptor (LXRα), peroxisome proliferator-activated receptor gamma (PPARγ), and Sterol Response Element-Binding Protein 1c (SREBP1c) genes in hyperlipidemia rats treated with HJD. Our data first elucidated the gene expression profile of high-fat diet-induced hyperlipidemia in rats after HJD treatment, and lipid metabolism-related genes (CYP7A1, LXRα, PPARγ, and SREBP1c) may be potentially biomarkers for HJD-alleviated hyperlipidemia.

[KEY WORDS] Hyperlipidemia; Huatan Jiangzhuo decoction; High-fat diet; Fatty liver; Next-generation sequencing


Introduction

Hyperlipidemia is a common type of dyslipidemia featured by abnormally increased lipid or lipoprotein levels in blood [1]. Lipoproteins such as low-density lipoprotein (LDL) and high-density lipoprotein (HDL) function as key emulsifiers and transporters of lipid molecules such as phospholipid, cholesterol, and triglyceride (TG) [2]. Hyperlipidemia is characterized chiefly by the elevation of TG, total cholesterol (TC), and LDL levels, or reduction in HDL content and is still the major cause of atherosclerosis and coronary artery disease, which is still the leading cause of disease-associated mortality worldwide [1,3]. More importantly, hyperlipidemia is also closely associated with nonalcoholic fatty liver disease (NAFLD), which reportedly affects 10%–24% of the global population, and NAFLD is also responsible for 42%–90% cases with asymptomatic elevation of aminotransferases [4]. Also, hyperlipidemia was listed as a causative factor of acute pancreatitis [5]. However, poor understanding of hyperlipidemia pathogenesis has created challenges for clinical management.

Great efforts have been made for the treatment of hyperlipidemia during the past decades. For instance, fibrates, the agonists of peroxisome proliferator-activated receptor, was used to cope with elevated triglyceride level in hyperlipidemia patients [6]. Also, statins, inhibitors of HMG-CoA, have been applied for the treatment of hyperlipidemia patients by repressing LDL production [7-8]. As one extensively investigated statin, atorvastatin, in combination with ezetimibe, has recently been shown as a promising treatment modality for hyperlipidemia patients complicated with acute coron-
Natural plant products, especially those being tested in traditional medicine, appear to be reliable sources of novel drug development. Curcumin was previously shown to exert anti-cancer, anti-inflammatory, and antioxidant effects [13]. Recent studies demonstrated that curcumin could also effectively reduce lipid level in various hyperlipidemia animal models [13-15]. In addition, the combined extracts from several medicinal plants, which were used as traditional Chinese medicine formulae, could also be promising candidates for hyperlipidemia treatment. For instance, the Xuefu Zhuyu decoction, which has been applied for the treatment of cardiovascular disease in Chinese medicine for centuries, ameliorated the high-fat diet-induced hyperlipidemia symptoms in rats by regulating ketone body and glycoprotein metabolism, glutathione biosynthesis, and other metabolic pathways [16]. The Huanglian Jiedu decoction, another Chinese medicine formula, significantly lowered the TC and LDL levels in rats with high-fat diet-induced hyperlipidemia, and the HDL level was then greatly elevated on the contrary [17]. Reduction in lipid in rat blood by the Huanglian Jiedu decoction was at least partially mediated by the altered expression of low-density lipoprotein receptors and peroxisome proliferator-activated receptors (PPARs) in rat livers [17]. These foundational research studies revealed great potential of traditional Chinese medicine for the management of hyperlipidemia and related disorders.

Huatang Jiangzhuo decoction (HJD) is composed of several Chinese herbal medicinals including Atractylodis Rhizoma, Atractylodis Macrocephalae Rhizoma, Citri Reticulatae Pericarpium, Pinelliae Rhizoma, Poria, and Alisma Rhizoma. HJD has also been applied in traditional Chinese medicine for treating angina pectoris, fat diabetes, and hyperlipidemia, but the pharmaceutical mechanisms remained unclear. In the study, rat hyperlipidemia model was established to investigate the gene expression alteration induced by HJD, which might provide new insights into the underlying molecular mechanisms of its lipid-lowering effect.

**Material and Methods**

**Animals and reagents**

Sixty 6-week-old male Sprague–Dawley rats (200 ± 20 g) were purchased from the Experimental Animal Science and Technology Development Co., Ltd., affiliated to the Southern Medical University (Guangzhou, China; License number: SCXK 2011-0015). All the experimental rats were maintained in S-Phase Fraction grade barrier system at the Experimental Animal Center of Guangzhou University of Chinese Medicine at constant temperature of 21–25 °C, steady humidity of 50%–70% and a 12-h light/dark cycle. They were allowed to consume the food and drink water freely. All animal experiment procedures were performed strictly according to the animal usage guidelines by the Animal Ethics Committee of Guangzhou University of Chinese Medicine.

**Reagent preparation**

The herbal medicines (the medicine inspection reports were supported in Supplemental Table 1) used for the preparation of HJD were purchased from the Guangzhou Zisun Chinese Medicine Co., Ltd. and Yulin Chinaherborn Pharmaceutical Co., Ltd.. A mixture of Atractylodis Rhizoma (10 g), Atractylodis Macrocephalae Rhizoma (15 g), Citri Reticulatae Pericarpium (10 g), Pinelliae Rhizoma (10 g), Poria (15 g), and Alisma Rhizoma (10 g) was pre-immersed in distilled water for 20 min. For the first time, mixed medicine was boiled in ddH2O (1 : 10, V/V) for 1 h; for the second time, mixed medicine was boiled in ddH2O (1 : 5, V/V) for 30 min. Twice the filtrate was combined and concentrated to crude drug (2.52 g mL⁻¹, 28 mL), main chemicals of crude drug detected by UPLC/MS was shown in Supplemental Table 2). The drug was stored at 4 °C before use. Atorvastatin (20 mg/pill) used as control was obtained from Pfizer Pharmaceuticals.

**Model establishment**

Experimental rats acclimatized for 1 week were randomly divided into six groups, with ten rats in each group, and five of the six groups were used for the establishment of hyperlipidemia models. The residual group, as the normal control group, was maintained under normal conditions and fed with 150 g normal food each day. The five groups for model establishment were fed with high-fat diet (150 g·d⁻¹) with free access to drinking water. The high-fat diets were provided by the Guangdong Medical Laboratory Animal Center, consisting of sucrose (20%), lard (15%), cholesterol (1.2%), sodium cholate (0.2%), casein (10%), calcium hydrogen phosphate (0.6%), stone powder (0.4%), premixed feed (0.4%), and base feed (52.2%), with a fat percentage of 18.5%. The body weights of each rat were daily recorded. The model establishment by feeding with high-fat diet lasted for 30 d. After fasting for 12 h, the indexes of blood lipid including total cholesterol (TC), triglyceride (TG), low-density lipoproteins (LDL), and high-density lipoprotein (HDL) were detected for the evaluation of model establishment, using a blood lipid detection kit provided by the Nanjing Jiancheng Biotechnology Company as described by the manufacturer.

**Drug dose calculation**

The dose of HJD (a clinical empirical prescription) was calculated by the dose of human body. Calculation method of drug concentration: An ordinary adult dose contains 140 g crude drug and the crude drug concentration 140 g/70 kg = 2 g kg⁻¹. According to the internationally recognized dose
conversion formula for human and rat (the equivalent dose ratio of body surface area of human and rat was 0.018), we calculated the rat dose (2 g·kg⁻¹ × 70 kg × 0.018/0.2 kg = 12.6 g·kg⁻¹), which was considered as the medium dose (One dose per day). Gavage once a day in the morning: The dose was as follows: (1) low-dose = body weight (kg) × 6.3 g·kg⁻¹; (2) medium dose = body weight (kg) × 12.6 g·kg⁻¹; (3) high-dose = body weight (kg) × 25.2 g·kg⁻¹.

**Drug administration and efficacy evaluation**

After model establishment, the five groups, with ten rats in each group, were randomly designated as the model group, atorvastatin group, HJD-low dosage group, HJD-medium dosage group, and HJD-high dosage group. The atorvastatin group was then treated with 1.8 mg·kg⁻¹ atorvastatin each day by intragastrical administration for 30 d, and three HJD groups were daily given 6.3, 12.6, and 25.2 g·kg⁻¹ extracts for 30 d, respectively. The normal group and the model group were given the same volume of drinking water. After fasting for 12 h, the rats were sacrificed, body weight and length were measured, and the Lee’s indexes were calculated using the following formula: Lee’s index = [weight (g) × 1000/body length (cm)]¹⁰/³ (1/3). The TC, TG, LDL, and HDL levels in rat blood were detected again, and liver coefficients were then calculated.

**Hematoxylin and Eosin (HE) staining**

Freshly collected rat liver tissues were fixed with 4% polyoxymethylene for 24 h, dehydrated using gradient ethanol, cleared by xylene, embedded with paraffin, and sliced into 4 μm sections. Tissue slides were then treated with xylene and usual gradient ethanol for dehydration, incubated with hematoxylin for 5 min, stained with eosin solution for 1–3 min, dehydrated again with gradient ethanol and xylene, and sealed with neutral gum. The nucleus in blue and cytoplasm in red of liver cells were observed using a microscope.

**Transcriptome sequencing**

The gene expression profile analysis by RNA sequencing was performed as previously described with minor modifications. Briefly, all RNA samples were extracted from fresh liver tissues of rats after the specified model establishment and drug administration using Trizol solution (Thermo Fisher Scientific) following the instructions of the manufacturer. RNA quality control was performed using the OD₂₅₀/OD₂₈₀ ratio, concentration and absorption peak form detected by Nanodrop instrument and the RNA integrity evaluated by the Agilent 2100 instrument. The mRNA samples were then isolated using Oligo (dT) magnetic beads, followed by RNA fragmentation, reverse transcription using random primers, terminal repair, and adaptor ligation, and the cDNA library was finally constructed by polymerase chain reaction (PCR) amplification. The quality of cDNA library was checked by quantitation with Qubit 2.0, insert size detected with Agilent 2100, and the library concentration (> 2 nmol·L⁻¹) by quantitative PCR method. All samples were sequenced using a HiSeq 2500 sequencing system (Illumina), and raw data with a Q30 base percentage of over 86.09% were obtained, which were subjected to filtering by removing reads with adaptor sequencing and these low-quality reads. The obtained clean reads were blasted and mapped against the reference genome database (Species: *Rattus norvegicus*; Version: Rnor 6.0) using the TopHat2 software. The Map rates of different samples against reference database were found to be between 73.76% and 83.81%. The mapped values of Fragments Per Kilobase per Million (FPKM) were calculated and used for evaluation of gene expression levels. The expression differences were further analyzed using the DESeq software for characterization of differentially expressed genes (DEGs), which were defined by a fold change (FC) of ≥ 2 and a false-discovery rate of < 0.05.

**GO and KEGG pathway annotation**

The analyses of gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGGs) pathways with significantly enriched differentially expressed genes (DEGs) were performed as described previously. Briefly, the significantly DEGs identified by RNA sequencing among groups were subjected to the characterization of the GO terms with the most significant enrichment by comparison with reference genome database (http://www.geneontology.org/). The GO terms of biological processes, cellular components, and molecular functions with enrichment of highest number of DEGs were separately listed. Similarly, the KEGG pathways with significantly enriched differential genes identified by RNA sequencing were then carried out, in comparison with the whole rat genome.

**Quantitative RT–PCR**

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was used to validate the expression levels of DEGs identified by RNA sequencing. All RNA samples were extracted from freshly collected rat liver tissues using Trizol solution (TAKARA) following the instructions of the manufacturer. Approximately 1 mg of RNA samples was used for the synthesis of cDNA with Bestar qPCR RT Kit (DBI Bioscience), according to the manufacturer’s instructions. The quantitation of mRNA level by real-time PCR was then performed with a DBI Bestar® SybrGreen qPCR master Mix kit (DBI Bioscience) using the following procedures: 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 15 s, for 40 cycles on an Agilent Stratagene fluorescence quantitation PCR system (M × 3000P). The glyceraldehyde-3-phosphate dehydrogenase was applied as the internal control for relative quantitation, and at least three biological and three technical replicates were performed. The sequences of primers used in this study are listed in Table 1.

**Statistical analysis**

Data of this study were analyzed using the SPSS 20.0 software package. The normality test was performed on all data presented as mean ± standard deviation. The one-way ANOVA followed by Bonferroni post-tests was used to analyze the significance of difference in measurement data with normal distribution, and nonparametric rank sum test was used for the analysis of skewness measurement data. Signific-
Table 1  Primers sequencing used for mRNA quantitation

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Sequence (5’-3’)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH F</td>
<td>CCTCGTCTCATAGACAAAGTAGGT</td>
<td>169</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>GGTTAGAGTCATGGAACATG</td>
<td></td>
</tr>
<tr>
<td>CYP7A1 F</td>
<td>TAGGTTCTCATAGACAGCCTC</td>
<td>91</td>
</tr>
<tr>
<td>CYP7A1 R</td>
<td>GTTGGTGGTGAGATGAGC</td>
<td></td>
</tr>
<tr>
<td>LXRα F</td>
<td>CCTACAGAATCTCGTCCACAGA</td>
<td>171</td>
</tr>
<tr>
<td>LXRα R</td>
<td>GTAGGCTGCTCCCTTGATGA</td>
<td></td>
</tr>
<tr>
<td>PPARγ F</td>
<td>CCTCCCTGTATAAATAGGG</td>
<td>124</td>
</tr>
<tr>
<td>PPARγ R</td>
<td>ACAGCAAACTCAAATCTAGGC</td>
<td></td>
</tr>
<tr>
<td>SREBP1-c F</td>
<td>CTTGAGGCGATGATGACGAC</td>
<td>122</td>
</tr>
<tr>
<td>SREBP1-c R</td>
<td>CTCCAGACGGTCAGAACA</td>
<td></td>
</tr>
</tbody>
</table>

F: forward primer; R: reverse primer

Results

Inhibition of hyperlipidemia by HJD in rat model

For research on the therapeutic effects of HJD on hyperlipidemia, the hyperlipidemia rat models were established by feeding high-fat diet as described in the Materials and Methods section. We observed that the TC and TG levels in blood samples of the model rats were significantly increased, whereas the HDL cholesterol (HDL-C) concentrations of the normal rats (Fig. 1A). However, the administration of both atorvastatin and HJD in hyperlipidemia model rats was markedly increased compared with the normal rats (Fig. 1B). Moreover, we measured the liver coefficient of experimental rats and found that the liver coefficient of the rats treated with high-dosage HJD was remarkably downregulated in comparison with the model group (Table 3). Compared with the model group, the liver weight of rats treated with high-dosage HJD was also significantly decreased. For a more direct demonstration of HJD efficacy, the rat liver tissues were HE stained. We observed significantly shrunken nucleus and fat accumulation in hepatic stellate cells of liver tissues collected from hyperlipidemia model rats, showing a drastic contrast to the normal rats (Fig. 1A). Also, the thickness of the vascular wall in the aortic arch of the hyperlipidemia model rats was markedly increased compared with the normal rats (Fig. 1B). However, the administration of both at-

Table 2  Blood lipids in hyperlipidemia models treated with HJD (mean ± SD, n = 10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC (mmol·L⁻¹)</th>
<th>TG (mmol·L⁻¹)</th>
<th>LDL-C (mmol·L⁻¹)</th>
<th>HDL-C (mmol·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.67 ± 0.52</td>
<td>1.06 ± 0.34</td>
<td>1.35 ± 0.24</td>
<td>0.80 ± 0.20</td>
</tr>
<tr>
<td>Model</td>
<td>2.82 ± 0.17*</td>
<td>1.89 ± 0.23*</td>
<td>2.47 ± 0.36*</td>
<td>0.71 ± 0.21</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>2.30 ± 0.27*</td>
<td>1.34 ± 0.23*</td>
<td>1.86 ± 0.27*</td>
<td>0.63 ± 0.27</td>
</tr>
<tr>
<td>HJD (L)</td>
<td>2.72 ± 0.31</td>
<td>1.38 ± 0.30</td>
<td>2.26 ± 0.29</td>
<td>0.52 ± 0.19</td>
</tr>
<tr>
<td>HJD (M)</td>
<td>2.35 ± 0.13*</td>
<td>1.14 ± 0.28</td>
<td>1.66 ± 0.24*</td>
<td>0.71 ± 0.12</td>
</tr>
<tr>
<td>HJD (H)</td>
<td>2.29 ± 0.15*</td>
<td>1.16 ± 0.31</td>
<td>1.52 ± 0.29*</td>
<td>0.83 ± 0.27</td>
</tr>
</tbody>
</table>

TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoproteins cholesterol; HDL-C: high-density lipoprotein cholesterol; HJD: Huatan Jiangzhuo decoction; L, M and H indicate low, medium and high dosages respectively. *P < 0.05 vs normal; †P < 0.05 vs model

Table 3  Liver coefficient and Lee’s index of hyperlipidemia rats treated with HJD (mean ± SD, n = 10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Liver coefficient</th>
<th>Body length (cm)</th>
<th>Lee’s index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>429.45 ± 26.10</td>
<td>12.30 (2.55)</td>
<td>0.028 (0.007)</td>
<td>24.45 ± 0.93</td>
<td>25.99 ± 0.47</td>
</tr>
<tr>
<td>Model</td>
<td>507.27 ± 23.80*</td>
<td>24.50 (5.45)*</td>
<td>0.047 (0.010)*</td>
<td>25.77 ± 0.79*</td>
<td>27.00 ± 0.52*</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>514.52 ± 40.83</td>
<td>23.50 (8.30)</td>
<td>0.047 (0.012)</td>
<td>26.06 ± 0.60</td>
<td>26.89 ± 0.61</td>
</tr>
<tr>
<td>HJD (L)</td>
<td>476.75 ± 47.82</td>
<td>24.60 (7.30)</td>
<td>0.051 (0.009)</td>
<td>25.09 ± 0.91</td>
<td>26.88 ± 0.88</td>
</tr>
<tr>
<td>HJD (M)</td>
<td>482.90 ± 30.97</td>
<td>22.15 (4.45)</td>
<td>0.046 (0.005)</td>
<td>24.75 ± 0.75*</td>
<td>26.65 ± 0.76</td>
</tr>
<tr>
<td>HJD (H)</td>
<td>497.73 ± 41.63</td>
<td>17.60 (4.15)*</td>
<td>0.038 (0.007)*</td>
<td>25.71 ± 0.94</td>
<td>26.83 ± 0.61</td>
</tr>
</tbody>
</table>

HJD: Huatan Jiangzhuo decoction; L, M and H indicate low, medium and high dosages respectively. *P < 0.05 vs normal; †P < 0.05 vs model
orvastatin and HJD with high dosage has effectively recovered the change of liver cell and aortic arch morphology in model rats with high-fat diet-induced hyperlipidemia. These physiological and morphological indexes clearly showed that HJD, especially administrated at high dosage, could effectively ameliorate the hyperlipidemia symptom induced by high-fat diet in rats.

Transcriptome changes induced by HJD in rat liver

To investigate the molecular mechanism underlying effects of HJD on hyperlipidemia, total mRNA samples were extracted from fresh liver tissues of the normal group, the model group, and the HJD group (model rats treated with high-dosage HJD). The gene expressional profile differences among groups were then analyzed by RNA sequencing for characterization of significantly differentially expression gene (DEG) (Supplemental Table 4). The DEGs among the normal group and the model group, the atorvastatin group and the model group, and also among the HJD group and the model group were intensively analyzed (Fig. 2A). As shown in Fig. 2, a total of 2502 DEGs were detected between the model group and the normal group, containing 1704 upregulated genes and 798 downregulated genes. Only four downregulated genes were characterized between the atorvastatin group and the model group. As many as 1936 DEGs were characterized in the liver tissues of rats treated with HJD in comparison with the model group (Table 4). The numbers of up- and downregulated genes induced by HJD administration were 1088 and 848, respectively. The distribution of DEGs was shown in volcano plots, also showing the great differences of gene expression among groups (Fig. 2A). Furthermore, the expression pattern of DEGs was demonstrated by heat maps, which revealed high variability of gene expression patterns in rat livers after the treatment of HJD (Fig. 2B). The great number and high variability of DEGs between the hyperlipidemia model rats and those treated with HJD indicated that HJD could result in a significant alteration of gene expression in rat livers, which might be the potential mechanisms of its therapeutic effects on hyperlipidemia.

**GO annotation of DEGs**

For a better view of the major biological events that were involved in the therapeutic effects of HJD on hyperlipidemia, the DEGs were categorized by gene GO annotation in terms of biological processes, cellular components, and molecular functions. By biological processes, we found that a majority of the DEGs among the normal and model groups were associated with cellular processes, single-organism processes, biological regulation, metabolic processes, response to stimulus, followed with multiple organismal processes and developmental processes, etc., indicating that the anti-hyperlipidemia effects of HJD were achieved by complex mechanisms (Fig. 3A). Similarly, those DEGs in rat liver treated with HJD were also related to such biological processes (Fig. 3B). Moreover, the DEGs were distributed into multiple subcellular components such as membrane, macromolecular complex, extracellular region, membrane-enclosed lumen, synapse, collagen trimer, and nucleoid (Fig. 3A), and it was also true of these DEGs between the HJD group and the hyperlipidemia model group, showing the prevalent regulatory effects of HJD in various subcellular organelles of rat hepatic cells (Fig. 3B). In addition, the molecular functions such as binding, catalytic activity, molecular transducer activity, receptor activity, and nucleic-acid-binding transcription factor activity of those differentially expressed among the model group and the normal group were also identical to those of DEGs among the HJD group and the model group. We observed that the biological processes, cel-
The diversity of biological processes, cellular components, and molecular functions of these DEGs preliminarily disclosed the great strengths of HJD in regulating cellular and molecular events, also suggesting that the anti-hyperlipidemia roles of HJD might be mediated by the synergetic act of various cellular signaling pathways in rat livers.

**KEGG pathways involved in hyperlipidemia treatment by HJD**

To characterize the major biological processes and signaling transduction pathways involved in the anti-hyperlipidemia roles of HJD, we subsequently performed a KEGG pathway analysis of DEGs identified by RNA sequencing. We found that these DEGs among the model group and the normal control group were significantly enriched in the multiple pathways such as chemokine signaling pathway, osteoclast differentiation, Leishmaniasis pathology, platelet activation, leukocyte trans-endothelial migration, fatty acid degradation, toxoplasmosis, FC gamma receptor (FcR)-mediated phagocytosis, cytokine-cytokine receptor interaction, focal adhesion, malaria pathology, steroid biosynthesis, nucleotide-
Fig. 3 Major biological processes and cellular components of differential genes (A) Categorization of differentially expressed genes among the model group and the normal group by the GO biological processes, cellular components and molecular functions. (B) GO biological processes, cellular components and molecular functions of differentially expressed genes among the HJD (25.2 g·kg⁻¹) group and the model group. All differentially expressed genes were categorized according to their GO annotations. The GO terms of biological processes, cellular component and molecular functions are shown on X-axis, and the percentages and numbers of genes are shown on the left-hand side and right-hand side Y-axis respectively. HJD: Huatan Jiangzhuo decoction; DE gene: differentially expressed gene.

oligomerization domain (NOD)-like receptor signaling pathway, Toll-like receptor signaling pathway, PPAR signaling pathway, nuclear factor (NF)-kappa B signaling pathway, etc. (Fig. 4A). By analysis of DEGs among the HJD-treated rats and the hyperlipidemia model rats, the following KEGG pathways were identified: ribosome biogenesis in eukaryotes, arginine and proline metabolism, RNA transport, glutathione metabolism, and pyrimidine metabolism.

To further disclose the roles of biological pathways associated with glycolipid metabolism and inflammatory factors in the rats treated with HJD, the DEGs associated with these related pathways were analyzed (supplemental Tables 5 and 6). We identified a great number of DEGs in rat livers with or without HJD treatment, which are closely associated with
Various biological processes and signaling pathways. For instance, phosphofructokinase (PFK), 6-phosphofructo-2-kinase (PFKFB3), Ca\(^{2+}\)/Calmodulin-dependent protein kinase 2 (CaMKK2), and sterol response element-binding protein 1 (SREBP-1) are involved in metabolic processes. Figure 4 shows the enrichment of differentially expressed genes in KEGG pathways. The percentages of annotated genes in each pathway are shown, with higher percentages indicating more enriched pathways. A common finding is the enrichment of metabolic pathways, including carbon metabolism, androgen metabolism, and amino acid metabolism. This suggests that the study focuses on the biological processes and pathways that are significantly altered under the experimental conditions.
protein 1c (SREBP1c) genes, which are key members of the AMP-activated protein kinase (AMPK) signaling pathway, were all significantly up-regulated in liver tissues of hyperlipidemia rats, compared with the normal control rats (Supplemental Table 5). Also, a group of AMPK pathway genes were found to be differentially expressed in livers of model rats treated with HJD in comparison with the model group, including PFKFB3, phosphoenolpyruvate carboxy kinase, PFK, stearoyl-CoA desaturase, phosphoinositide-3-kinase regulatory subunits, and peroxisome proliferator-activated receptor gamma (PPARγ) (Supplemental Table 6). Other KEGG pathways with significant enrichment of DEGs induced by HJD include the mitogen-activated protein kinase pathway, the PPAR pathway, the phosphatidylinositol-3 kinase (PI3K)-Akt signaling pathway, the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, the NF-kappa B pathway, and so on. The identification of various biological processes and signaling pathways here suggested that HJD treatment could induce great alteration of intracellular signaling pathways in rat liver, which mediated the therapeutic effects of HJD for hyperlipidemia.

**Validation of gene expression regulated by HJD**

According to the above-mentioned RNA sequencing, we randomly selected four key genes involved in lipid metabolism. The four key genes include cholesterol 7-a-hydroxylase gene (CYP7A1), Liver Orphan Receptor (LXRα), PPARγ, and SREBP1c genes. The results from RNA sequencing indicated that LXRα and PPARγ were downregulated in model rat livers treated with HJD, and the expression of CYP7A1 and SREBP1c was upregulated in model rats compared with the normal rats (Supplemental Tables 5 and 6). As a validation of the transcriptome assay results, the expression levels of these four genes among different groups of rats were further checked by quantitative RT-PCR. By real-time PCR, we found that the mRNA levels of CYP7A1 (Fig. 5A) and SREBP1c were upregulated in hyperlipidemia model rats compared with the normal group, which is consistent with the RNA sequencing results. Also, expression levels of all these four genes in hyperlipidemia model rats treated with HJD exhibited different extents of decrease compared with the hyperlipidemia model rats in the quantitative RT-PCR assay (Figs. 5A−5D). The consistency of these representative gene expression levels among large-scale RNA sequencing and individual gene analysis by quantitative RT-PCR assay indicated high reliability of these DEGs induced by HJD treatment of hyperlipidemia rat model.

![Fig. 5](image-url) 

**Fig. 5** Differentially expressed gene validation by quantitative RT-PCR in rat livers. The mRNA levels of CYP7A1 (A), LXRα (B), PPARγ (C) and SREBP1c (D) were quantitatively determined by real-time RT-PCR, using total mRNA samples collected from liver tissues of the normal group, the hyperlipidemia model group, the atorvastatin group and the HJD (25.2 g·kg$^{-1}$) group. Three individual rats from each group were used for the quantitative analysis. *P < 0.05 vs normal; **P < 0.05, ***P < 0.01 vs model. HJD: Huatan Jiangzhuo decoction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
Discussion

Hyperlipidemia, clinically featured by abnormally increased blood lipid or lipoprotein levels, is caused by a complex interplay of genetic and environmental factors, and the therapeutic effects of currently available anti-hyperlipidemia drugs have been greatly hampered by various adverse effects [25-26]. Traditional herbal medicines are promising alternatives for the treatment of hyperlipidemia because of the long application and relatively low incidence of severe adverse effects, but the pharmaceutical mechanisms still need further investigation using modern research techniques, which would provide necessary information for its efficacy and safe evaluation. Rat models have been widely used for the study of lipid metabolism, hyperlipidemia, and other metabolic disorders mainly due to their low cost, short period for model establishment, easy feed and management, strong resistance, and also their relatively big body sizes that meet the requirements for large-scale study in recent decades [21-22]. On the other hand, the rapid progress in next-generation sequencing technology has given great impetus to the study of molecular mechanisms and genetic factors associated with continuously increasing incidences of dyslipidemia diseases [23-24]. The decreased cost of high-throughput new sequencing systems also showed potential applicability in clinical diagnosis and treatment of dyslipidemia, especially monogenic dyslipidemia, caused by known causative gene mutations [25]. Thus, the combination of rat hyperlipidemia model with large-scale gene expression file analysis by high-through DNA sequencing technique should be a reasonable design for both pathology study and therapeutic efficacy evaluation.

Using hyperlipidemia rat model, here for the first time, HJD significantly ameliorated the hyperlipidemia symptoms was shown. The effects of HJD on hyperlipidemia in rat models here are also consistent with the previous investigations, showing the lipid-regulating roles of individual herbal medicines [26-29]. For instance, the extract and fractions of Citri Unshii Pericarpium could induce the decrease in plasma triglyceride, total cholesterol, and LDL cholesterol in hyperlipidemic rats, showing its therapeutic effects on high cholesterol diet-induced hyperlipidemia and other circulatory disorders [30]. Similarly, Poria cocos, as another constituents of the HJD, was also recently shown to produce significant improvements in hyperlipidemia and lipid metabolism in rats, as indicated by the great changes of seven major fatty acid components in rat plasma [29]. In the present study, the effective improvement of hyperlipidemia symptoms further validated the therapeutic roles of these herbal medicines and revealed the great value of combined administration of multiple plant-derived agents for the treatment of complex metabolic disorders. HJD has the clinical experience in the treatment of hyperlipidemia, while the specific mechanism is not very clear. Large numbers of DEGs were identified in hyperlipidemia rats treated with HJD in the HJD-treated group by our next-generation sequencing. By GO and KEGG annotation, these DEGs could be grouped into various biological processes and signaling pathways including glycolipid metabolism and inflammatory factors, showing the pleiotropic effects of HJD in regulating cellular functions. For instance, genes of the PPAR signaling pathway were downregulated by HJD treatment, including the CYP7A1, LXRa, and PPARγ. Also, SREBP1c and PPARγ in the AMPK signaling pathway were differentially expressed in hyperlipidemia rats treated with HJD. The involvement of these signaling pathways in hyperlipidemia pathology has already been extensively evidenced in previous reports [16-22]. Our discovery further validated its critical role in hyperlipidemia progression and also showed great potential of HJD in modulating normal functions of these key signaling pathways.

CYP7A1 is a cytochrome P450 (P450) enzyme in liver tissues that functions as an important regulator of lipid and bile salt homeostasis by catalyzing the biosynthesis of bile acids from cholesterol and is thus closely associated with lipid metabolism and hyperlipidemia [33-34]. LXRa acts as a nuclear hormone receptor associated with dietary fat absorption and metabolism and could regulate expression of CYP7A1 gene in hepatic cells [35-36]. As for PPARγ, it is also a well-known nuclear receptor that modulates lipid metabolism, cellular differentiation, and tumorigenesis, and its association with hyperlipidemia should be attributed to its regulation of genes involved in fatty acid oxidation and binding of lipids to nuclear receptors [17]. The function of PPARγ is notably susceptible to regulation by natural bioactive ligands from plant material [37-39]. Also, the SREBP1c gene is a key member of the sterol regulatory element-binding protein and regulates lipogenesis and lipid homeostasis in liver tissues, which mediate the alleviation of hyperlipidemia and lipid accumulation [40]. Therefore, CYP7A1, LXRa, PPARγ, and SREBP1c were closely related to the lipid metabolism. In our study, according to the gene expression profile, these four genes that were significantly downregulated in hyperlipidemia rat livers by HJD have been verified further, while the expressions of four genes were not statistically significant in the positive control (atorvastatin) group. Therefore, the importance and specificity of CYP7A1, LXRa, PPARγ, and SREBP1c genes for hyperlipidemia in rats after the HJD treatment were demonstrated. Besides, significant changes of these critical genes associated with lipid metabolism and hyperlipidemia pathogenesis supported the reliability of the gene expression profile alteration by HJD treatment, which also suggested the necessity of further investigation of other DEGs identified in this study.

In summary, a hyperlipidemia rat model by feeding high-fat diet was established, in which the administration of HJD greatly alleviated the high-lipid symptom in liver blood, showing the potential of HJD as a reliable treatment for hyperlipidemia. Besides, by next-generation sequencing, a large number of genes were identified as differentially expressed in hyperlipidemia rat livers treated with HJD, which covers
pleiotropic biological processes and signaling pathways. Further investigation of these genes might bring about new perspectives on hyperlipidemia prevention and treatment.

Our study also confirmed that lipid metabolism-related genes (CYP7A1, LXRs, PPARγ, and SREBP1c) were downregulated dramatically in HJD-treated hyperlipidemia, suggesting that they might be potential biomarkers for HJD-alleviated hyperlipidemia. However, our current research has limitations. For example, the detailed chemical components of HJD responsible for the treatment of hyperlipidemia deserve further investigations; the functions and mechanisms of CYP7A1, LXRs, PPARγ, and SREBP1c will be necessarily explored in the future.

Supplementary Materials

Supplementary materials are available as Supporting Information, and can be requested by sending E-mail to the corresponding author.

References


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