Identification of potential biomarkers in cholestasis and the therapeutic effect of melatonin by metabolomics, multivariate data and pathway analyses

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Abstract. The present study investigated the anti-cholestatic effect of melatonin (MT) against α-naphthyl isothiocyanate (ANIT)-induced liver injury in rats and screened for potential biomarkers of cholestasis. Rats were administered ANIT by intraperitoneal injection and then sacrificed 36 h later. Serum biochemical parameters were measured and liver tissue samples were subjected to histological analysis. Active components in the serum were identified by gas chromatography-mass spectrometry, while biomarkers and biochemical pathways were identified by multivariate data analysis. The results revealed that the serum levels of alanine aminotransferase, aspartate aminotransferase, total bilirubin, direct bilirubin, γ-glutamyl transpeptidase, and alkaline phosphatase were reduced in rats with ANIT-induced cholestasis that were treated with MT. The histological observations indicated that MT had a protective effect against ANIT-induced hepatic tissue damage. Metabolomics analysis revealed that this effect was likely to be associated with the regulation of compounds related to MT synthesis and catabolism, and amino acid metabolism, including 5-aminopentanoate, 5-methoxytryptamine, L-tryptophan, threonine, glutathione, L-methionine, and indolelactate. In addition, principal component analysis demonstrated that the levels of these metabolites differed significantly between the MT and control groups, providing further evidence that they may be responsible for the effects induced by MT. These results provide an insight into the mechanisms underlying cholestasis development and highlight potential biomarkers for disease diagnosis.

Introduction

Cholestasis is characterized by a reduction in bile flow and bile acid accumulation (1), and a higher incidence of this condition is observed in hepatopathy. A previous study in Shanghai revealed that the total incidence of cholestasis was 10.26% among chronic liver disease patients (2). Cholestasis is classified as intrahepatic or extrahepatic (3), and mechanisms associated with the former type can be broadly classified as hepatocellular or obstructive. Plugging of interlobular bile ducts, portal expansion and bile duct proliferation, along with centrilobular cholate injury, are observed in the obstructive subtype (3,4). Cholestasis can be caused by pre-existing medical conditions including infections, drug treatments, and metabolic or genetic disorders (5); therefore, it is considered as a secondary disease (6). Without appropriate treatment, liver cells (such as portal myofibroblasts and hepatic stellate cells) are hyperactivated, leading to biliary fibrosis or even cirrhosis (7,8). To date, there are few therapeutic options available for the treatment of cholestasis (9).

Ursodeoxycholic acid (UDCA) is currently the only drug approved by the U.S. Food and Drug Administration for cholestasis treatment, which acts by relieving disease symptoms and restoring liver enzyme levels (10). However,
approximately one-third of patients present little or no response to UDCA therapy (11,12). Melatonin (MT) is a methoxyindole synthesized and secreted at night by the pineal gland under normal light/dark conditions (13,14). It is widely distributed in human tissues (13) and serves an important role in a number of physiological processes (15,16). For instance, MT exerts an anti-inflammatory effect by scavenging reactive oxygen species and inhibiting lipid peroxidation (13,17-19). In addition, oral administration of MT was reported to abolish the increase in the total cholesterol concentration in the serum of rats with α-naphthyl isothiocyanate (ANIT)-induced acute liver injury and cholestasis by restoring cholesterol metabolism and transport in the liver (20). However, few studies have investigated the effects of orally administered MT on serum metabolome profiles in rats with cholestasis.

Metabolomics analysis has been widely used to evaluate the therapeutic effects of herbal medicines (21), as it provides a global view of low molecular weight metabolites in biological networks and signaling pathways. It has been suggested that the development of cholestasis may be associated with alterations in endogenous metabolite profiles (22). Metabolomics has previously been applied to investigate the therapeutic effects of Paeonia lactiflora Pall (23), yinchenhao (24) and rhubarb (25) as treatment approaches for cholestasis.

ANIT is a well-known hepatotoxicant that causes bile duct epithelial cells to release factors that attract neutrophils, leading to hepatic injury (26-30). ANIT-induced intrahepatic cholestasis shares similarities with drug-induced cholangiolic hepatitis in humans. Transient intrahepatic cholestasis can be induced in animal models with a single dose of ANIT, which has been demonstrated in rats (30-34) as well as mice and guinea pigs (31).

The present study investigated the anti-cholestatic effect of MT in rats. Gas chromatography-mass spectrometry (GC-MS) analysis was conducted to evaluate the metabolite profiles, while multivariate data analysis was performed in order to identify biomarkers and biochemical signaling pathways associated with cholestasis.

Materials and methods

Materials and reagents. Carboxymethyl cellulose sodium salt (CMC) was obtained from Yuanye Biological Technology Co., Ltd. (Shanghai, China). ANIT and MT were purchased from Sigma-Aldrich (Merck KGaA; Darmstadt, Germany). ANIT was dissolved in olive oil and administered at a dose of 75 mg/kg body weight. The rats were injected with the same volume of vehicle or ANIT. Rats in the MT group were orally administered MT (100 mg/kg body weight) 12 h after the initial ANIT injection. The model and control groups received the same volume of 0.25% CMC at 12 h after the initial injection. Each rat was weighed prior to treatment with intraperitoneal injections and oral administration.

Sample collection and liver function assays. The rats were provided with standard chow and water following the completion of the treatments. Rats were then fasted for 12 h prior to being sacrificed at 36 h after the initial ANIT or vehicle injection. Blood samples were collected from the inferior vena cava, and the liver was removed from each rat immediately after sacrifice. The blood samples were collected and centrifuged at 3,500 x g and 4°C for 15 min to obtain the serum. Sterile, hemolysis-free serum samples were stored at -80°C prior to determination of biochemical parameters and metabolomics analysis. All serum samples were used within 1 month. Serum ALT, AST, TBIL, DBIL, GGT and ALP levels were measured with commercial test kits according to the manufacturer's protocol.

Histological analysis of liver damage. Liver tissues were excised and fixed in 10% phosphate-buffered formalin. Fixed tissues were cut into 1x1x0.3 cm sections. Sections were dehydrated in a gradient alcohol series, and embedded in paraffin wax blocks. The embedded wax blocks were fixed to the slicer and were cut into 4-5 μm thick slices. Following dewaxing slides in xylene, The slides were dipped into hematoxylin and agitated for 30 sec, rinsed in H₂O for 1 min, followed by staining with 1% eosin Y solution for 30 sec with
agitation, all at room temperature (20-25°C). Slides were examined under a BX53 microscope (Olympus Corporation, Tokyo, Japan).

Sample preparation for metabolome profiling. Serum samples stored at -80°C were thawed at room temperature, and then 50 µl of each serum sample was added to a 1.5-ml Eppendorf tube and stored at -80°C. The samples were then centrifuged at 12,000 x g for 10 min. The quality control (QC) sample was prepared by mixing aliquots of all samples to obtain a pooled sample. A 150-µl volume of supernatant was then transferred to a glass sampling vial and vacuum-dried at room temperature. Following the addition of 80 µl of 15 mg/ml methoxylamine hydrochloride in pyridine, the samples were vortexted for 2 min and derivatized at 70°C for 60 min. The samples were then allowed to stand at ambient temperature for 30 min prior to analysis.

GC-MS analysis. Derivatized samples were analyzed on a 7890B gas chromatograph paired with a 5977A mass-selective detector system (Agilent Technologies, Inc., Santa Clara, CA, USA). A DB-5MS fused-silica capillary column (30 m x 0.25 mm x 0.25 µm; Agilent Technologies, Inc.) was used to separate the derivatives. Helium (>99.999%) was used as the carrier gas at a constant flow rate of 1 ml/min through the column. The injector temperature was maintained at 260°C, the injection volume was 1 µl in the splitless mode, and the solvent delay time was set to 5 min. The oven temperature was initially set at 60°C, and was then increased to 125°C at a rate of 8°C/min, 210°C at a rate of 5°C/min, 270°C at a rate of 10°C/min and 305°C at a rate of 20°C/min, and was finally held at 305°C for 5 min. The temperature of the MS quadrupole and electron impact ion source were set to 150°C and 230°C, respectively. The collision energy was 70 eV. MS data were acquired in full-scan mode (m/z 50-500). The QC was injected at regular intervals (every 10 samples) throughout the analytical run to obtain a set of data for assessing reproducibility.

Data pre-processing and statistical analysis. Chem Station version E.02.02.1431 software (Agilent Technologies, Inc.) was used to convert the file format (D) of raw data to a common data format. ChromaTOF version 4.34 software (LECO Corporation, St. Joseph, MI, USA) was used to analyze the data. Metabolites were identified using Fiehn databases (http://fiehnlab.ucdavis.edu/projects/fiehnlib) in ChromaTOF.

Identification of differentially expressed metabolites. Differentially expressed metabolites were selected based on a combination of a statistically significant threshold of VIP values obtained from the OPLS-DA model and P-values from one-way analysis of variance (followed by Tukey's test) of normalized peak areas by SIMCA version 14.0 (Umetrics, Umeå, Sweden). Metabolites with VIP>1 and P<0.05 were considered to be significantly differentially expressed.
Identification of pathways. Pathways were identified with the Kyoto Encyclopedia of Genes and Genomes (https://www.kegg.jp/kegg/) and MBRole (http://csbg.cnb.csic.es/mbrole2/). The candidate biomarkers were mapped to KEGG by MBRole ID number shifting function. Pathways containing the candidate biomarkers were considered by KEGG pathway enrichment result.

Results

Therapeutic effect of MT on cholestasis in rats. ANIT administration resulted in significantly increased ALT and AST serum levels in rats compared with those in the control group (Fig. 1). However, treatment with 100 mg/kg MT was observed to reverse this effect (Fig. 1). Similarly, TBIL, DBIL, ALP and GGT levels were markedly increased in the ANIT group when compared with the control group. The high serum levels of these molecules indicated that ANIT successfully induced cholestasis. However, this effect was significantly reduced by MT treatment (Fig. 1).

Histological observations. Representative photomicrographs of HE-stained liver tissue from control rats and the cholestasis rats with or without MT (100 mg/kg) treatment are presented.
in Fig. 2. The liver in the control group had a normal lobular architecture with central veins and radiating hepatic cords (Fig. 2A-C). By contrast, rats with cholestasis exhibited marked changes in liver morphology, including acute infiltration of polymorphonuclear neutrophils, fatty metamorphosis, sinusoid congestion and necrotic inflammation (Fig. 2D-F). Rats treated with MT exhibited mild bile duct epithelial damage and hydropic degeneration of hepatocytes with fewer invading neutrophils (Fig. 2G-I).

Multivariate statistical analysis. PCA was conducted to assess the differences in the metabolome profiles of the three groups. A score plot allowed for visualization of observational clusters, which differed significantly between the control, ANIT, MT and QC groups (Fig. 3A). The results of PCA indicated that multivariate statistical analysis was necessary to clarify the differences among the groups.

OPLS-DA was applied to eliminate and classify uncorrelated noise and identify potential biomarkers based on differences in metabolome profiles. The ANIT, MT (100 mg/kg) and control groups were distinguished in the models (Fig. 3B and C). The R²X, R²Y and Q²(cum) of the ANIT and control groups were 0.437, 0.996 and 0.974 respectively, as compared with the values of 0.349, 0.983 and 0.909 respectively which from ANIT group and MT group. These results indicated that the model was of good quality and provided reliable confidence predictions. Permutation tests with 200 iterations and validation plots indicated that the original models were valid (Fig. 3D and E). An analysis of OPLS-DA loading and score plots revealed several critical variables that were far removed from the center of the coordinate of the loading plot (Fig. 3F and G), suggesting that they were important for clustering.

Metabolite identification in MT-treated rats with cholestasis. Metabolites whose concentration varied among groups were selected based on the combination of a statistically significant threshold of VIP values obtained from the OPLS-DA model and P-values from a two-tailed Student’s t-test of normalized peak areas. Metabolites with values of VIP>1 and P<0.05 were included. The number and trend of metabolites in different groups were presented in Fig. 4. Candidates that significantly differed among the groups with P<0.05 and |log fold change|>2 were identified as candidate biomarkers for cholestasis and MT treatment (Table II). Variations in the trends of seven biomarkers, which included 5-aminopentanoate, 5-methoxytryptamine, L-tryptophan, threonine, glutathione (GSH), L-methionine and indolelactate, were presented in...
Fig. 5. Cholestasis model rats had markedly elevated serum L-tryptophan, L-methionine and threonine levels, as well as significantly lower levels of the other four metabolites, when compared with the control group. By contrast, the MT group exhibited markedly elevated serum 5-methoxytryptamine, indolelactate, GSH and 5-aminopentanoate levels, and reduced levels of the other three metabolites when compared with the model group.

Figure 3. OPLS-DA, 200-permutation test and OPLS-DA score plot of cholestasis rats with or without MT treatment. (A) Principle component analysis score plot of the Con, Mod (ANIT), MT and QC groups. (B) OPLS-DA of Cholestasis model (ANIT) vs. Con group. (C) OPLS-DA of MT treatment vs. ANIT group. (D) 200-permutation test of Cholestasis model (ANIT) and Con group. (E) OPLS-DA score plot of Cholestasis model (ANIT) and Con group. (G) OPLS-DA score plot of MT treatment and ANIT group. OPLS-DA, orthogonal partial least-squares discriminant analysis; MT, melatonin; Con, control; Mod, model; QC, quality control; ANIT, α-naphthyl isothiocyanate.
Pathway analysis in MT-treated rats with cholestasis. Bubble diagrams of the control, ANIT and MT groups are presented in Fig. 6, where the x-axis shows the Rich factor, which is the ratio of the differential metabolite number to the total number of metabolites in model organisms in each pathway. The color and size of each circle reflect the P‑value and the variation in the metabolite number in each pathway, respectively. The results revealed that the seven metabolites with a significantly different expression among the groups appeared in the following four KEGG pathways: i) Glycine, serine and threonine metabolism; ii) cysteine and methionine metabolism; iii) tryptophan metabolism; and iv) arginine and proline metabolism. Specifically, those associated with amino acids were focused on.

Discussion

Cholestasis is characterized by intrahepatic accumulation of potentially toxic bile acids resulting from hepatocellular dysfunction or bile duct obstruction (2). However, the detailed mechanism underlying this condition has not been fully elucidated. Identifying sensitive and specific biomarkers is important for the early detection of hepatic fibrosis. In the present study, the GC-MS-based serum metabolomics analysis revealed significant changes in pathways associated with the metabolism of the amino acids arginine/proline, glycine/serine/threonine, tryptophan and cysteine/methionine. Significant differences were also observed in the levels of 5-aminopentanoate, 5-methoxytryptamine, L-tryptophan, threonine, glutathione (GSH), L-methionine and indolelactate among the control rats and the cholestasis model rats with or without MT treatment.

Based on the KEGG database results, L-tryptophan, 5-methoxytryptamine and indolelactate in the serum are by-products of tryptophan metabolism, while GSH and L-methionine are by-products of the cysteine and methionine metabolism. In addition, threonine is a by-product of the glycine, serine and threonine metabolism, and 5-aminopentanoate is
Figure 5. Changes in metabolome profiles in the cholestasis rats with or without melatonin treatment. The levels of (A) 5-aminopentanoate, (B) 5-methoxytryptamine, (C) L-tryptophan, (D) threonine, (E) glutathione, (F) L-methionine, and (G) indolelactate are displayed. The squares indicate the mean value of expression. *P<0.01 vs. control group; ^P<0.05 and ^^P<0.01 vs. cholestasis model group.
Figure 6. Bubble diagrams of the control, cholestasis model and MT treatment groups. (A) Cholestasis model (α-naphthyl isothiocyanate-treated) vs. control group; and (B) MT treatment vs. model group. The x-axis shows the Rich factor, the color of each circle indicates the P-value, and the size of each circle reflects the varying metabolite number of each pathway. MT, melatonin.
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Levels of these metabolites were also demonstrated to be altered in serum. These results suggested that the metabolism of arginine, proline, glycine, serine, threonine, tryptophan, cysteine and methionine may be disrupted in cholestasis (Fig. 7).

GSH is a thiol compound that is associated with catabolism and intracellular transport. Alterations in plasma GSH levels are associated with Parkinson's and Alzheimer's diseases, diabetes, macular degeneration and human immunodeficiency virus infection (36). GSH participates in a number of cellular functions, including protection from free radicals during oxidative stress (37). It has been reported that, for partial hepatectomy in rats, hepatic GSH levels have increased after 24 h (38,39). however, due to the short half-life of GSH, liver GSH levels return to baseline despite this elevation within 48 h (40). 5-Methoxytryptamine is the main metabolite produced in the transformation of MT by MT deacetylase, which is subsequently metabolized to 5-methoxyindoleacetaldehyde, 5-methoxyindole acetic acid or 5-methoxytryptophol (41). A previous study reported that only a small fraction (approximately 0.3-0.8%) of the concentration of the MT present in the incubation medium was converted to 5-MT (42). This indicates that deacetylation of MT is one possible pathway for the biosynthesis of 5-MT. Indolepropionic acid is a product of the microbial degradation of L-tryptophan (43). MT and indolepropionic acid function as endogenous electron donors, primarily detoxifying reactive radicals; however, they do not undergo autooxidation in the presence of transition metals (44). Furthermore, previous results have indicated that indolepropionic acid is effective in protecting rat hepatic microsomal membranes against rigidity and against lipid peroxidation caused by iron (45). Additionally, 5-aminopentanoate has been reported to serve an important role in proline metabolism and may participate in lysine degradation in Phaeobacter inhibens DSM 17395 (46). Lee et al (47) also found that lysine degradation is specifically associated with stroke occurrence, and that low expression of 5-aminopentanoate may increase the risk of thrombotic stroke. In the present study, serum GSH,

Figure 7. Schematic illustration of metabolic pathways associated with cholestasis that were altered by MT treatment. Red and light blue boxes indicate metabolites with significantly higher and lower levels, respectively, in the cholestasis model when compared with the control group. Boxes bordered in red and blue represent metabolites with significantly higher and lower levels, respectively, in the MT treatment group when compared with the control rats. MT, melatonin.
5-methoxytryptamine, indolepropionic and 5-aminopentanoate levels were decreased in cholestasis model rats when compared with the control rats; however, these levels were increased by MT treatment. Thus, these four metabolites may be useful biomarkers for diagnosing cholestasis and evaluating responses to MT treatment.

L-Tryptophan, a precursor of serotonin and MT, serves a role in depression, schizophrenia and somatization (48). Lower levels of plasma tryptophan are associated with enhanced pain, autonomic nervous system responses, gut motility, peripheral nerve function, and ventilation and cardiac dysfunction (49,50). Threonine is an essential amino acid that is incorporated into intestinal mucosal proteins and is required for the synthesis of secretory glycoproteins (51). Threonine and alanine share the same amino acid transporter, which is responsible for threonine uptake into different cell types, including hepatocytes (52).

Threonine is an essential amino acid that is incorporated into the intermediate metabolite between MT and methionine. In the present study, serum L-tryptophan levels were increased when compared with the control rats; however, these levels were increased by MT treatment. Thus, these four metabolites may be useful biomarkers for diagnosing cholestasis and evaluating responses to MT treatment.

In conclusion, the results of the present study demonstrated that MT has a significant anti-cholestatic effect. Seven metabolites, including 5-aminopentanoate, 5-methoxytryptamine, L-tryptophan, threonine, GSH, L-methionine and indolelactate, were identified as potential biomarkers of cholestasis, which may be useful for disease diagnosis and for assessing the therapeutic efficacy of MT treatment.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HY, YL and ZX contributed equally to the present study. XZ and XD conceived and designed the study. HY, YL and ZX acquired, analyzed and interpreted the data. DW and SS were responsible for handling the animals and obtaining tissue specimens. HD and BZ performed histopathological analyses. ZZ and LS performed statistical analyses. All authors approved the final manuscript.

Ethics approval and consent to participate

The study protocol was in strict accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China, and was approved by Beijing University of Chinese Medicine Medical and Experimental Animal Ethics Committee (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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