Abstract. The therapeutic effects of melatonin on cholestatic liver injury have received widespread attention recently. The aim of the present study was to investigate the mechanisms of the anti-cholestatic effects of melatonin against α-naphthyl isothiocyanate (ANIT)-induced liver injury in rats and to screen for potential biomarkers of cholestasis through isobaric tags for relative and absolute quantitation (iTRAQ) proteomics. Rats orally received melatonin (100 mg/kg body weight) or an equivalent volume of 0.25% carboxymethyl cellulose sodium salt 12 h after intraperitoneal injection of ANIT (75 mg/kg) and were subsequently sacrificed at 36 h after injection. Liver biochemical indices were determined and liver tissue samples were stained using hematoxylin-eosin staining, followed by iTRAQ quantitative proteomics to identify potential underlying therapeutic mechanisms and biomarkers. The results suggested that the expression levels of alanine transaminase, aspartate aminotransferase, total bilirubin and direct bilirubin were reduced in the rats treated with melatonin. Histopathological observation indicated that melatonin was effective in the treatment of ANIT-induced cholestasis. iTRAQ proteomics results suggested that melatonin-mediated reduction in ANIT-induced cholestasis may be associated with enhanced antioxidant function and relieving abnormal fatty acid metabolism. According to pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes, the major metabolic pathways for the metabolism of melatonin are fatty acid degradation, the peroxisome proliferator-activated receptor signaling pathway, fatty acid metabolism, chemical carcinogenesis, carbon metabolism, pyruvate metabolism, fatty acid biosynthesis and retinol metabolism, as well as drug metabolism via cytochrome P450. Malate dehydrogenase 1 and glutathione S-transferase Yb-3 may serve as potential targets in the treatment of ANIT-induced cholestasis with melatonin.

Introduction

Cholestasis is a disease in which bile flow is impaired. Cholestasis may be caused by either intrahepatic or extrahepatic dysfunction (1), and may lead to a range of clinical hepatobiliary diseases, such as liver failure, hepatobiliary malignancy and bile fibrosis, cirrhosis (2). Ursodeoxycholic acid (UDCA) is currently the only treatment approved by the US Food and Drug Administration for the treatment of patients with cholestasis (3). However, according to the Primary Biliary Cholangitis Treatment and Management Guidelines from the British Society of Gastroenterology from 2018, oral UDCA is not an ideal therapeutic option as it may aggravate liver injury and one-third of patients are unresponsive to it (4,5).
Melatonin is a hormone secreted primarily by the pineal gland (6,7). Melatonin is involved in the regulation of circadian rhythms, such as the sleep/wake rhythm, neuroendocrine rhythm and body temperature cycles (8,9). There are numerous studies suggesting that melatonin may be used to treat liver disease and the hepatoprotective effects of melatonin may be associated with its antioxidant properties (10). Previous studies by our group demonstrated that the therapeutic effects of melatonin against α-naphthyl isothiocyanate (ANIT)-induced acute cholestasis are associated with the attenuation of oxidative stress (11,12).

ANIT is a widely utilized chemical substance able to induce acute cholestasis by injuring bile duct epithelium and hepatic parenchymal cells (13,14). However, the mechanisms underlying ANIT-induced acute cholestasis have remained to be fully elucidated. Due to its similarities with drug-induced cholangiolic hepatitis in humans, ANIT-induced acute cholestasis has been widely used as a model of acute cholestasis in susceptible species, such as rats (15-17).

Proteomics provides essential information on the quantity, function and relationship of protein complexes (18). Isobaric tags for relative and absolute quantitation (iTRAQ) combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis has been used to determine protein quantities and their compositions (19-22). In the present study, the anti-cholestatic effect of melatonin in rats was assessed using iTRAQ combined with LC-MS/MS to identify the differentially expressed proteins in the samples. The differentially expressed proteins were subjected to Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses in order to understand their effects and mechanisms.

Materials and methods

Chemicals and drugs. Table I provides details of chemicals and drugs. AST, ALT, TBIL and DBIL were detected by chemical oxidation assays. All other reagents were of analytical grade or higher.

Experimental animals. Male Sprague-Dawley rats (weight, 240-280 g; age, 7 weeks) were procured from SPF-JD-SPF Biotech Co., Ltd. (Beijing, China; certification no. SCXK-JING 2016-0002) and were allowed to acclimatize for 1 week. All rats were maintained in cages with free access to rat chow and water in a temperature-controlled (22-24˚C) environment under a 12-h dark/light cycle with 50% humidity. The present study was performed in accordance with the guidelines of the animal care regulations of Beijing University of Chinese Medicine and was approved by the Ethics Committee for Animal Care and Use (Approval No. bucm-4-2017122735-4035).

Proteomics and reagents. Urea, DL-dithiothreitol (DTT), iodoacetamide (IA), IPG buffer and formic acid (FA) were purchased from GE Healthcare. SDS, Tris-(hydroxymethyl)-amino-methane, trichloroacetic acid, ammonium persulfate and N,N,N',N'-tetramethylmethylenediamine were obtained from Sigma-Aldrich (Merck KGaA). Trypsin was from Promega Corp. and acetonitrile (ACN; HPLC grade) and H₂O were purchased from Thermo Fisher Scientific, Inc.

Protein extraction. Samples were immediately ground in liquid nitrogen and subsequently, 300 μl SDS lysis buffer was added. Protease inhibitor (phenylmethylsulfonylfluoride) was added to a final concentration of 1 mM, followed by mixing. The samples were sonicated in an ice bath at 80 W with 1-sec on/off cycles for 3 min, which was repeated three times. The supernatant was collected after centrifugation at 12,000 × g for 20 min at 4˚C. The concentration of extracted protein in the supernatant was determined using a bicinchoninic acid assay. The protein extract was stored at -80˚C.

Protein digestion and iTRAQ labeling. A total of 100 μg protein extract was mixed in 120 μl reducing buffer [10 mM DTT, 8 mM urea and 100 mM TEAB, pH 8.0] and the solution was incubated at 60˚C for 1 h. IA was added to a final concentration of 50 mM and left in the dark at room temperature for 40 min. The supernatant was collected after centrifugation at 12,000 × g for 20 min at 4˚C, and the supernatant was collected. This step was repeated twice, and after a final centrifugation at 12,000 × g for 20 min at 4˚C and the supernatant was collected, the peptides were collected. A total of 50 μl 100 mM TEAB was added, the
mixture was centrifuged at 12,000 x g for 20 min at 4°C again and the supernatant was collected and lyophilized. The sample was reconstituted in 100 µl 100 mM TEAB and subsequently, 40 µl sample was transferred to a new tube for labeling. A total of 100 µl of iTRAQ reagent was transferred to the sample tube and 200 µl water was added to quench the labeling reaction. The solution was lyophilized and the sample was stored at -80°C.

**MS analysis.** All analyses were performed on a Triple time of flight 5600 MS machine (SCIEX) equipped with a Nanospray III source (SCIEX). Samples were loaded onto a capillary C18 trap column (3 cm x 100 µm) and then separated by a C18 column (15 cm x 75 µm) on an Eksigent nanoLC-1D plus system (SCIEX). The flow rate was 300 nl/min and a linear gradient was applied over 90 min (from 5-85% B over 90 min; mobile phase A was 0.1% FA in water and phase B was 95% ACN/0.1% FA in water). Data were acquired using a 2.4-kV ion spray voltage, 35 psi curtain gas, 5 psi nebulizer gas and an interface heater temperature at 150°C. The MS scanned between 400 and 1,500 m/z with an accumulation time of 250 msec. For Information-Dependent Acquisition, 30 MS/MS spectra (80 msec each; mass range, 100-1,500 m/z) of MS peaks above an intensity of 260 with a charge state between 2 and 5 were acquired. A rolling collision energy voltage was used for collision induced dissociation fragmentation and MS/MS spectra acquisitions. Mass was dynamically excluded for 22 sec.

**Bioinformatics analysis of proteomic data.** Functional analysis of the differentially expressed proteins identified was performed using GO annotation in the categories molecular function (MF), biological process (BP) or cellular component (CC) with the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (david.ncifcrf.gov/). The overall steps consisted of Sequence alignment, GO entry extraction mapping, GO annotation and data augmentation. Subsequently, KEGG (genome.jp/kegg/pathway.html) was used to predict the metabolic pathways of proteins in cells and the function of these proteins. The data were obtained using the Search Tool for the Retrieval of Interacting Genes and proteins (STRING) network analysis (string-db.org/) and Cytoscape version 3.7.1. To be considered differentially expressed proteins, the criteria were P<0.05 and a fold-change >1.5 or <0.67.

**Western blot analysis.** Western blots were performed using automated capillary western blot, an automated capillary-based size sorting system (ProteinSimple). All procedures were performed according to the manufacturer's protocol. In brief, 8 µg of diluted protein lysate was mixed with 2 µg fluorescent Master Mix (mixed 5 times) and heated at 95°C for 5 min. The samples, blocking reagents, wash buffer, primary antibodies, secondary antibodies and chemiluminescent substrate were dispensed into designated wells in a manufacturer-provided microplate. The plate was loaded into the instrument and protein was drawn into individual capillaries using a 25-capillary cassette, provided by the manufacturer. Protein separation and obtainment of the resulting chemiluminescent signal were performed automatically on the individual capillaries using the default settings. The data were analyzed using Compass software (ProteinSimple; v2.7.1).

Malate dehydrogenase 1 (MDH1) and glutathione S-transferase Yb-3 (GSTM3) antibodies were purchased from ProteinTech (1:50 dilution; cat. no. 15904-1-AP and 15214-1-AP, respectively); β-actin antibody was obtained from Cell Signaling Technology, Inc. (1:50 dilution; cat. no. 4970; Cell Signaling Technology,

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**Table I. Details of chemicals and drugs.**

| Chemicals/drugs                                      | Company                                      |
|------------------------------------------------------|----------------------------------------------|
| α-naphthyl isothiocyanate                             | Sigma-Aldrich (Merck KGaA)                   |
| Melatonin                                            | Sigma-Aldrich (Merck KGaA)                   |
| Carboxymethyl cellulose sodium salt                  | Yuanye Biological Technology Co., Ltd.       |
| Aspartate aminotransferase (cat. no. C010-2)         | Nanjing Jiancheng Bioengineering Institute  |
| Alanine aminotransferase (cat. no. C0009-2)          | Nanjing Jiancheng Bioengineering Institute  |
| Total bilirubin (cat. no. C019-1)                    | Nanjing Jiancheng Bioengineering Institute  |
| Direct bilirubin (cat. no. C019-2)                   | Nanjing Jiancheng Bioengineering Institute  |

**Table II. Details of the animal experimental design.**

| Group | Treatment at each time-point (h) |
|-------|----------------------------------|
|       | 0      | 12     | 24     | 36     | 48     |
| Control | Fasting | Olive oil | 0.25% CMC | Fasting | Sacrifice |
| Model   | Fasting | 75 mg/kg ANIT | 0.25% CMC | Fasting | Sacrifice |
| MT      | Fasting | 75 mg/kg ANIT | 100 mg/kg MT | Fasting | Sacrifice |

MT, melatonin; ANIT, α-naphthyl isothiocyanate; CMC, carboxymethyl cellulose sodium salt.
Inc.) and used as the loading control. Secondary antibody was purchased from ProteinSimple (cat. no. 042-206). Incubation with primary and secondary antibodies was performed at room temperature for 30 min.

Statistical analysis. Data analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc.). The individual statistical tests used were the Mann-Whitney U-test, Wilcoxon's matched-pairs signed-rank test and Spearman's test for correlations. Student's t-test was used for analysis of statistical significance between two groups and one-way analysis of variance followed by Dunnett's post-hoc test was applied to analyse statistical significance among three or more groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Histological observations. The histology results are provided in Fig. 1. H&E staining of the control group indicated a normal hepatic lobular structure with hepatic cell cords arranged radially outward from the terminal venules, as well as a uniformly distributed portal area (Fig. 1D). The specimens from the ANIT-induced model group displayed evidence of neutrophil infiltration in the bile duct between the hepatic lobule, sinusoid congestion and necrotic inflammation (Fig. 1E). A small amount of neutrophil infiltration was observed in the melatonin-treated group, with mild inflammation and mild cell edema (Fig. 1F). These results were consistent with those of previous studies by our group (11,12). For each group, 6 random fields of view were statistically evaluated for inflammatory lesions (examples in Fig. 1A-C) and the quantitative results are presented in Fig. 1G. The quantitative results indicated that a significant amount of inflammatory lesions appeared in the model vs. control group, which was significantly decreased in the melatonin vs. model group.

Effects of melatonin on serum biochemistry. The levels of ALT, AST, TBIL and DBIL were significantly increased in the rats intraperitoneally injected with ANIT and were significantly
reduced following treatment with melatonin (Fig. 2). These results were consistent with those of previous studies by our group (11,12).

**ITRAQ-based quantitative proteomics analysis of the hepatoprotective effect of melatonin.** The hepatic tissue proteins from the three groups with technical duplicates were pooled for the iTRAQ coupled LC-MS/MS analysis. A total of 2,059 proteins were detected and quantified with a minimum confidence level of 95% and all proteins contained 2 or more peptides. After screening the data, 327 dynamically changed proteins were obtained in each different group. There were 63 differentially expressed proteins between control group vs. model group (Model-Control) and model group vs. melatonin group (Melatonin-Model) (Figs. 3 and 4). Therefore, further research should assess the function of these proteins.

**Results of classification of differential protein analysis.** GO analysis indicated that the functional terms in the BP category were ‘small molecule metabolic process’, ‘organic acid metabolic process’, ‘oxoacid metabolic process’, ‘single-organism metabolic process’, ‘carboxylic acid metabolic process’ and ‘oxidation-reduction process’ (Fig. 5A). In the category CC, the most relevant enriched terms by the differentially expressed proteins were ‘mitochondrion’, ‘extracellular exosome’, ‘cytosol’, ‘organelle membrane’, ‘endoplasmic reticulum membrane’ and ‘cytoplasm’ (Fig. 5B). The most relevant enriched terms in the category MF were ‘oxidoreductase activity’, catalytic activity, cofactor binding and acting on CH-OH group of donors (Fig. 5C).

KEGG pathway analysis indicated that the main pathways enriched by the differentially expressed proteins were ‘metabolic pathways’, ‘fatty acid degradation’, ‘PPAR signaling pathway’, ‘fatty acid metabolism’, ‘chemical carcinogenesis’, ‘carbon metabolism’, ‘pyruvate metabolism’, ‘fatty acid biosynthesis’ and ‘retinol metabolism’ (Fig. 6). A network of the top 10 most significantly enriched pathways, the top 38 most significantly enriched proteins and their interactions is provided in Fig. 7. Table III shows the details of the 63 differentially expressed proteins of α-naphthyl isothiocyanate-induced cholestasis rats with or without melatonin treatment.

**Western blot analysis.** Western blot experiments were performed to validate the results of iTRAQ combined with the LC-MS/MS analysis. The two proteins MDH1 and GSTM3 were selected for this comparison. The results indicated that the ratios of MDH1 and GSTM3 were decreased following intraperitoneal injection of ANIT and increased with melatonin treatment. These results were consistent with the proteomics data (Fig. 8).

**Discussion**

The pathogenesis of acute cholestasis has remained to be fully elucidated. Long-term biliary obstruction may cause liver damage, even induce biliary cirrhosis or liver fibrosis and, in
severe cases, may lead to liver failure (25). In recent years, it has been indicated that oxidative stress and abnormal fatty acid metabolism may be involved in the pathogenesis of intrahepatic cholestasis (11,12,24,26). Previously, melatonin has been demonstrated to improve liver fibrosis and liver damage caused by various diseases through inhibiting oxidative damage (10). In the present study, the differential expression protein profile following melatonin treatment for ANIT-induced liver injury in rats was determined using iTRAQ-coupled LC-MS/MS analysis. A total of 63 significantly differentially expressed proteins were identified and two representative proteins were analyzed by western blot based on the iTRAQ results.

The results of the KEGG pathway analysis indicated that the PPAR signaling pathway was associated with fatty acid degradation (Fig. 9), metabolism and biosynthesis. In addition, the PPAR signaling pathway was involved in pyruvate metabolism and citrate cycle, and linked to carbon metabolism by ACAT2, MDH1 and ME1. The PPAR signaling pathway and fatty acid degradation were also associated with retinol metabolism via CYP4A2, and carbon metabolism via CYP2C22, CYP4A2 and CYP2C13. In carbon metabolism, GSTM3 was linked to drug metabolism by cytochrome P450.

It has been hypothesized that excessive accumulation of bile acids may induce oxidative stress. This may result in an abnormally oxidized state of the internal environment, inducing mitochondrial generation of reactive oxygen species (ROS) by interfering with compounds such as those involved in mitochondrial respiratory chain complexes. These ROS are highly toxic and cause damage to liver cells (24). Melatonin is a mitochondrial targeting antioxidant, which is synthesized in mitochondria, and the mitochondria are also the sites of melatonin metabolism (27). The treatment of liver diseases is constantly being explored and several novel drugs are based on anti-oxidative strategies. They interfere with the pathological mechanisms underlying mitochondrial damage, oxidative stress and ROS production (28-30). Oxidoreductase activity was among the top MF terms enriched by the differentially expressed proteins was. Thus, it was speculated that redox reactions were enhanced, and the imbalance between oxidation...
Figure 5. Gene Ontology analysis of the differentially expressed proteins of cholestasis rats with or without melatonin treatment. Functional terms enriched by the proteins were determined in the three major categories (A) biological process, (B) cellular component and (C) molecular function. The y-axis indicates the enrichment ratio of a specific category of proteins in each major category.

Figure 6. Changes in metabolome profiles in the cholestasis rats with or without melatonin treatment. Bubble chart displaying Kyoto Encyclopedia of Genes and Genomes pathways enriched. The y-axis represents the pathways and the x-axis represents the enrichment factor. The color and size of the bubble in the chart represent the enrichment significance and the amount of differentially expressed genes enriched in the pathway, respectively. PPAR, peroxisome proliferator-activated receptor.
and antioxidant systems was reduced following mitochondrial synthesis enhanced by melatonin treatment. Thus, it was hypothesized that the therapeutic mechanism underlying melatonin treatment of acute cholestasis may be associated with the alleviation of oxidative stress by enhancing antioxidant function reducing ROS levels. A previous study by our group indicated that glutathione (GSH) serves a pivotal role in the antioxidant defense in intrahepatic cholestasis. In addition, GSH participates in oxidative defense through its catalysis by glutathione S-transferase (GST) and glutathione peroxidase. GSTM3, a member of the GST family that upregulates the expression of proteins, participates in oxidative defense to repair liver damage (31). In the present study, the expression levels of GSTM3 were increased and the results were consistent with those of the western blot analysis. Thus, GSTM3 may be a potential biomarker for the treatment of intrahepatic cholestasis by melatonin.

Mitochondrial \( \beta \)-oxidation, peroxisome \( \beta \)-oxidation, \( \omega \)-oxidation and microsome pathways are oxidized forms of fatty acids in humans. Among these, \( \beta \)-oxidation of mitochondrial long-chain fatty acids is the primary pathway of cellular oxidation. \( \omega \)-3 polyunsaturated fatty acids have been reported to be effective in the prevention and treatment of cholestasis and n-3 polyunsaturated fatty acids may also exhibit therapeutic potential (32). In the present study, KEGG analysis suggested that fatty acid degradation, biosynthesis and metabolism were significantly different prior to and after treatment. Protein lysine acetylation is a type of post-translational modification and acetylated proteins regulate important metabolic processes such as fatty acid metabolism (33). In cells, acetyl-CoA either enters the tricarboxylic acid cycle or is used to synthesize fatty acids. MDH1 catalyzes the conversion of malic acid to oxaloacetate (34). Glucose is known to undergo glycolysis

Figure 7. Protein-protein interaction network analysis of the differentially expressed proteins of cholestasis rats with or without melatonin treatment.
Table III. Details of the 63 differentially expressed proteins of α-naphthyl isothiocyanate-induced cholestasis rats with or without melatonin treatment.

| Uniprot ID | Protein name (gene symbol) | F-value | P-value |
|------------|---------------------------|---------|---------|
| A0A0G2K151 | Apolipoprotein E (APOE)   | 0.5889  | 0.0090 |
| A0A0G2K1S6 | Malic enzyme 1 (ME1)      | 1.9905  | 0.0150 |
| A0A0G2K3Z9 | N/A                       | -       | -       |
| A0A0G2K6H2 | Glutathione S-transferase zeta 1 (GSTZ1) | 1.6831 | 0.0295 |
| A0A0G2QC06 | Transferrin (TF)          | 0.6179  | 0.0091 |
| A0A0H2UHP1 | Aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) | 0.6223 | 0.0039 |
| A0A140TAC7 | Gulosolactone (L-)- oxidase (GULO) | 1.8658 | 0.0162 |
| B0BNJ4     | ETHE1, persulfide dioxygenase (ETHE1) | 1.5354 | 0.0857 |
| D3ZIC2     | Urocanate hydratase 1 (UROC1) | 1.7511 | 0.1896 |
| F1LMK6     | Serine dehydratase (SDS)  | 1.7656  | 0.0766 |
| F1LQ55     | Sterol carrier protein 2 (SCP2) | 2.0842 | 0.0795 |
| G3V6H4     | Mitochondrial amidoxime reducing component 1 (MARCl) | 1.5733 | 0.1288 |
| G3V715     | Aldehyde dehydrogenase 1 family, member B1 (ALDH1A1) | 3.5411 | 0.0442 |
| G3V730     | Aldehyde dehydrogenase 6 family, member A1 (ALDH6A1) | 1.5926 | 0.0348 |
| G3V836     | Clusterin (CLU)            | 0.6256  | 0.0209 |
| M0R4N4     | Dehydrogenase/reductase (SDR family) member 7 (DHR7) | 1.6838 | 0.0487 |
| O35077     | Glycerol-3-phosphate dehydrogenase 1 (GPD1) | 2.4498 | 0.1107 |
| O88813     | Acyl-CoA synthetase long-chain family member 5 (ACSL5) | 1.6460 | 0.0447 |
| O88989     | Malate dehydrogenase 1 (MDH1) | 4.6705 | 0.0673 |
| P04182     | Ornithine aminotransferase (OAT) | 2.3116 | 0.0180 |
| P05182     | Cytochrome P450, family 2, subfamily e, polypeptide 1 (CYP2E1) | 2.1076 | 0.0205 |
| P07379     | Phosphoenolpyruvate carboxykinase 1 (PCK1) | 1.6217 | 0.0094 |
| P07687     | Epoxide hydrolase 1 (EPHX1) | 0.6197 | 0.0010 |
| P08009     | Glutathione S-transferase, mu 3 (GSTM3) | 1.6969 | 0.0045 |
| P09118     | Urate oxidase (UOX)        | 2.1557  | 0.0020 |
| P09811     | Phosphorylase, glycogen, liver (PYGI) | 2.1289 | 0.0647 |
| P10760     | Adenosylhomocysteinase (AHCY) | 2.1517 | 0.0252 |
| P12785     | Fatty acid synthase (FASN) | 1.9435 | 0.0444 |
| P14141     | Carbonic anhydrase 3 (CAR3) | 1.5513 | 0.0001 |
| P14173     | Dopa decarboxylase (DDC)   | 1.6658  | 0.0224 |
| P15083     | Polymeric immunoglobulin receptor (PIGR) | 0.6010 | 0.0010 |
| P17712     | Glucokinase (GCK)          | 1.9240  | 0.0404 |
| P17988     | Sulphotransferase family 1A member 1 (SULT1A1) | 0.4074 | 0.0080 |
| P18162     | Acyl-CoA synthetase long-chain family member 1 (ACSL1) | 1.6682 | 0.0318 |
| P19225     | Cytochrome P450, family 2, subfamily c, polypeptide 22 (CYP2C22) | 0.6401 | 0.0064 |
| P20814     | Cytochrome P450, family 2, subfamily c, polypeptide 13 (CYP2C13) | 0.6094 | 0.2323 |
| P20816     | Cytochrome P450, family 4, subfamily a, polypeptide 2 (CYP4A2) | 0.6297 | 0.0231 |
| P22789     | Sulphotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 6 (SULT2A6) | 2.1173 | 0.0255 |
| P25093     | Fumarylacetocacetate hydrolase (FAH) | 1.6438 | 0.0894 |
| P27867     | Sorbitol dehydrogenase (SORD) | 1.5635 | 0.0658 |
| P36201     | Cysteine-rich protein 2 (CRIP2) | 0.6203 | 0.0038 |
| P37397     | Calponin 3 (CNN3)          | 0.5362  | 0.0339 |
| P50123     | Glutamyl aminopeptidase (ENPEP) | 1.5400 | 0.1557 |
| P50237     | Sulphotransferase family 1C member 3 (SULTLC3) | 1.8028 | 0.2110 |
| P55006     | Retinol dehydrogenase 7 (RDH7) | 1.5913 | 0.0071 |
| P80254     | D-dopachrome tautomerase (DDT) | 1.5901 | 0.2094 |
| Q03336     | Regucalcin (RGN)           | 2.4009  | 0.1904 |
| Q10758     | Keratin 8 (KRT8)           | 0.6412  | 0.0021 |
| Q3MHS3     | Aldo-keto reductase family 1, member C1 (AKR1C1) | 1.7571 | 0.0756 |
| Q4KLZ6     | Triokinase and FMN cyclase (TKFC) | 2.2275 | 0.0849 |
(producing pyruvate), pyruvate subsequently produces acetyl-CoA in the mitochondria and MDH1 promotes the citrate shuttle to provide NADPH and acetyl-CoA for fat synthesis. In the present study, MDH1 was significantly differentially expressed and associated with carbon and pyruvate metabolism. MDH1 is downregulated by ANIT.

Table III. Continued.

| Uniprot ID | Protein name (gene symbol) | F-value | P-value |
|------------|-----------------------------|---------|---------|
| Q5BJ9      | Keratin 18 (KRT18)          | 0.5850  | 0.0080  |
| Q5FVR2     | Thymidine phosphorylase (TYMP) | 2.0643  | 0.0869  |
| Q5HZE3     | Thyroid hormone responsive (THRSP) | 1.7476  | 0.0140  |
| Q5IOM4     | Aldo-keto reductase family 1, member C13 (AKR1C13) | 1.5343  | 0.1205  |
| Q5RJP0     | Aldo-keto reductase family 1, member B7 (AKR1B7) | 4.2623  | 0.0012  |
| Q5K22      | Acetyl-CoA acetyltransferase 2 (ACAT2) | 0.6613  | 0.0034  |
| Q62730     | Hydroxysteroid (17-beta) dehydrogenase 2 (HSD17B2) | 0.6607  | 0.0004  |
| Q62120     | ATP binding cassette subfamily C member 2 (ABCC2) | 1.5017  | 0.0126  |
| Q64656     | Alanine-glyoxylate aminotransferase 2 (AGXT2) | 1.9370  | 0.0044  |
| Q66HT1     | Aldolase, fructose-bisphosphate B (ALDOB) | 1.9087  | 0.0535  |
| Q6K4C0     | Flavin containing monoxygenase 5 (FMO5) | 0.6264  | 0.0156  |
| Q69JC6     | TAP binding protein (TABP) | 0.6588  | 0.0114  |
| Q9Z2Y0     | Glycine-N-acyltransferase-like 2 (GLYATL2) | 0.6144  | 0.0026  |

N/A, not available.

Figure 8. Immunoblotting analysis of GSTM3, MDH1 and β-actin in the (A) control group, (B) model group and (C) melatonin group. β-actin was used as the loading control. (D and E) The levels of protein expression are presented as the mean ± standard deviation. **P<0.01 control vs. model group; #P<0.05 melatonin vs. model group. MDH1, malate dehydrogenase 1; GSTM3, glutathione S-transferase Yb-3.
and upregulated following melatonin treatment. However, its function in the treatment of cholestasis with melatonin requires further study (35).

The liver is a complex system and its nuclear receptors have the ability to limit bile concentration and siltation by coordinating the stabilization of bile acids and bile secretion (36). One of these nuclear receptors is PPAR. It affects bile balance and cholestatic liver damage in humans (37). The role of PPARs in the liver and fatty acid metabolism primarily include modulation of peroxisome fatty acid oxidation and mitochondrial function (38). The KEGG pathway prediction of the present study suggested that the activity of the PPAR signaling pathway changed significantly between the model vs. control group and returned to near normal in the melatonin group. Therefore, the role of the PPAR signaling pathway in the treatment of cholestasis by melatonin requires further validation.

Cytochrome P450 is a broad-spectrum biocatalyst with catalytic activity. It primarily occurs in the liver but also in other tissue types and is the primary enzyme involved in melatonin metabolism (39). Cytochrome P450 was hypothesized to be relevant to the physiological production of $O_2^-$, which may result in oxidative stress under pathological conditions (40). Cytochrome P450 is also involved in the regulation of bile acids and may participate in melatonin metabolism in rat livers (41). Studies have indicated that a lack of cytochrome P450 may affect the metabolism of endogenous substances, such as cholesterol and bile acids (42).

In the present study, there was a significant difference in metabolism of xenobiotics by cytochrome P450 based on the KEGG pathway analysis between the melatonin group and model group. Some of these proteins, such as CYP1A2 and CYP2E1, are known to serve a role in the treatment of liver disease.

In conclusion, the results of the present study suggest that the therapeutic mechanism of melatonin in the treatment of acute cholestasis may be associated with enhancing antioxidant function and relieving abnormal fatty acid metabolism. The primary metabolic pathways of melatonin in the treatment of cholestasis were fatty acid degradation, the PPAR signaling pathway, fatty acid metabolism, chemical carcinogenesis, carbon metabolism, pyruvate metabolism, fatty acid biosynthesis and retinol metabolism, as well as drug metabolism mediated by cytochrome P450. MDH1 and GSTM3 may be potential biomarkers for melatonin treatment of intrahepatic cholestasis in the ANIT-induced animal model. However, these proteomic results are preliminary data and further studies are required to determine the role of these proteins in the treatment of cholestasis with melatonin.

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Availability of data and materials

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomeexchange.org) via the iProX partner repository with the dataset identifier PXD023155. The data can be accessed online at: https://www.iprox.org/page/project.html?id=IPX0002663000.

Authors’ contributions

XZ and XD made substantial contributions to the study conception. DW, HY and YL performed most of the experiments. ZZ, SS and DD performed histopathological analyses. LS, ZZ and XS participated in data analysis and interpretation. DW and HY confirm the authenticity of all the raw data. All authors have read and approved the final version of this 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 Anim als of the Ministry of Science and Technology of China and was approved by the Medical and Experimental Animal Ethics Committee of Beijing University of Chinese Medicine (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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