

# Melatonin ameliorates ANIT-induced cholestasis by activating Nrf2 through a PI3K/Akt-dependent pathway in rats

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**Abstract.** Cholestasis is a devastating liver condition which is increasing in prevalence worldwide; however, its underlying pathogenic mechanisms remain to be fully elucidated. It was hypothesised that melatonin may alleviate the hepatic injury associated with cholestasis due to its established antioxidant effects. Therefore, the effect and potential anticholestatic properties of melatonin were investigated in rats with  $\alpha$ -naphthylisothiocyanate (ANIT)-induced liver injury, a common animal model that mimics the cholestasis-associated liver injury in humans. The rats received intraperitoneal injection of ANIT with or without subsequent treatment with melatonin, and were sacrificed 24 h later. The serum biochemistry parameters of the liver were measured using conventional laboratory assays, and the liver tissue was subjected to conventional histological examination, reverse transcription-quantitative

polymerase chain reaction analysis and western blotting. The levels of alanine transaminase, aspartate transaminase, total bilirubin, direct bilirubin, total bile acids, alkaline phosphatase,  $\gamma$ -glutamyl transferase and glutathione were restored in rats treated with melatonin. Histological examination provided further evidence supporting the protective effect of melatonin against ANIT-induced cholestasis. In addition, the mRNA and protein expression levels of glutamate cysteine ligase, phosphorylated Akt and nuclear factor-erythroid 2-related factor-2 were restored in rats treated with melatonin. These findings indicate that melatonin is a natural agent that appears to be promising for the treatment of cholestasis, and that the anticholestatic effects of melatonin involve the alleviation of oxidative stress.

## Introduction

Cholestasis is characterised by a reduction in bile flow and bile acid accumulation (1), and has a higher incidence in hepatopathy. The prevalence of cholestasis has been increasing globally in recent years, becoming a major public health concern. A previous study in Shanghai revealed that the total incidence of cholestasis was 10.26% among patients with chronic liver disease (2). A cross-sectional study of cholestasis in 1,000 patients with chronic viral hepatitis in China demonstrated that, following discharge of 56% of patients with chronic viral hepatitis from hospital, the main indicators of intrahepatic cholestasis, alkaline phosphatase (ALP) or  $\gamma$ -glutamyl transferase (GGT), were higher than the upper limit of normal, and the risk and severity of liver fibrosis and cirrhosis in these patients were markedly increased (3). There are several causes of cholestasis, which may be broadly classified into hepatocellular and obstructive. Obstructive cholestasis typically involves bile plugging of the interlobular bile ducts, portal expansion and bile duct proliferation in association with centrilobular cholate injury (3). In hepatocellular cholestasis, there is impaired hepatocellular bile secretion, resulting in intrahepatic accumulation of toxic bile components, including bile acids and bilirubin, leading to progressive liver injury (4,5). During cholestasis, the role of oxidative stress in hepatocellular injury has become a focus of research interest (6); however, the precise pathogenic mechanisms remain to be fully elucidated.

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**Abbreviations:** ALP, alkaline phosphatase; ALT, alanine transaminase; ANIT,  $\alpha$ -naphthylisothiocyanate; AST, aspartate transaminase; b.w., body weight; CMC, carboxymethyl cellulose; DBIL, direct bilirubin; GCL, glutamate cysteine ligase; GGT,  $\gamma$ -glutamyl transferase; GS, glutathione synthase; GSH, glutathione; i.p., intraperitoneal; Nrf2, nuclear factor-erythroid 2-related factor-2; OS, oxidative stress; ROS, reactive oxygen species; TBA, total bile acid; TBIL, total bilirubin

**Key words:** melatonin, cholestasis, nuclear factor-erythroid 2-related factor-2, phosphoinositide-3 kinase/Akt-dependent pathway

$\alpha$ -naphthylisothiocyanate (ANIT) is a hepatotoxin known to cause intrahepatic cholestasis due to selective damage of the bile duct epithelial cells. These cells, in turn, release factors that attract neutrophils, which then injure hepatocytes (7,8), leading to intrahepatic cholestasis that is pathologically similar to drug-induced cholangiolitic hepatitis in humans (9). Therefore, the administration of ANIT to experimental animals, including rats, mice and guinea pigs, may be used to generate a model accurately mimicking intrahepatic cholestasis and hepatic damage in humans (10,11).

Melatonin is a methoxyindole that is principally synthesised and secreted by the pineal gland at night under normal light/dark cycles (12,13). Melatonin is also key in a number of physiological and cellular processes, including immune response, antioxidant defence, haemostasis and glucose regulation, depending on the melatonin signalling pathway involved (14,15). In addition to its functions as a hormone, melatonin exerts antioxidant effects by scavenging reactive oxygen species (ROS) and by inhibiting lipid peroxidation (16–18), and has also been reported to possess anti-inflammatory properties (19). Melatonin has also emerged as a valuable biomarker for estimating the serotonin status in the brain, particularly for treatment monitoring purposes (20). Evidence indicates that the prolonged administration of melatonin attenuates the increase in total and low-density lipoprotein cholesterol concentration and the decrease in high-density lipoprotein cholesterol concentration in the serum of rats fed a hypercholesterolemic diet (21). In addition, orally administered melatonin was found to reduce the increase in serum total cholesterol concentration and attenuate the disruption of serum cholesterol status in rats with ANIT-induced acute liver injury with cholestasis; this protective effect may be due to its antioxidant action and its inhibitory action against neutrophil infiltration (22–24). However, the effects and potential mechanism of action of melatonin in the context of cholestasis remain to be fully elucidated.

The antioxidant defence system includes non-enzymatic and enzymatic components (25), with the latter dominated by superoxide dismutase, catalase and glutathione peroxidase (26). These antioxidant defence-related enzymes are modulated by nuclear factor-erythroid 2-related factor-2 (Nrf2) (27), which is central to the protection of cells against oxidative and/or xenobiotic damage by binding to genomic antioxidant response elements and stimulating the expression of phase II antioxidant genes (28,29). In another study, *Paeonia lactiflora* pall and paeoniflorin alleviated ANIT-induced cholestasis by activating Nrf2 through the phosphoinositide-3 kinase (PI3K)/Akt-dependent pathway (30,31).

The role of melatonin in cholestasis may be associated with resistance to oxidative stress. Although the mechanism underlying the action of melatonin in the treatment of liver disease has been widely investigated, the mechanisms underlying the alleviation of oxidative stress through the PI3K/Akt signalling pathway and Nrf2 remain to be fully elucidated. The aim of the present study was to investigate whether melatonin can alleviate ANIT-induced cholestasis. Furthermore, through metabonomics investigation of the function of melatonin against ANIT-induced cholestasis, glutathione (GSH) synthetic enzymes may be the one of the potential biomarkers. The changes in the expression of GSH synthetic enzymes

were investigated in rats with ANIT-induced cholestasis under treatment with melatonin, as were the possible contributions of the PI3K/Akt signalling pathway and Nrf2. These findings may provide novel insight into the mechanisms underlying the development of cholestasis and indicate a novel therapeutic approach to this condition.

## Materials and methods

**Animals and treatments.** A total of 18 male Sprague-Dawley rats (7–8 weeks old; weighing  $260 \pm 20$  g) were obtained from SPF (Beijing) Biotechnology Co., Ltd. (Beijing, China; certification no. SCXK-JING 2016-0002). All animals were allowed to acclimate for 1 week prior to the experiments and were maintained at a constant temperature ( $25 \pm 2^\circ\text{C}$ ) and 50% humidity with a 12:12-h light/dark cycle; all the rats had access to water and food *ad libitum*. The study protocol was performed 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) with certification no. bucm-4-2017122735-4035.

An overview of the experimental design is presented in Table I. In brief, the rats were randomly divided into three groups ( $n=6$  per group) as follows: Control group, in which the rats were treated with vehicle [75 mg/kg body weight (b.w.) olive oil] alone; ANIT group, in which the rats received intraperitoneal (i.p.) injection of ANIT (Sigma; Merck KGaA, Darmstadt, Germany) at a dose of 75 mg/kg b.w.; and the melatonin + ANIT group, in which the rats received melatonin (100 mg/kg b.w.; Sigma; Merck KGaA) orally 12 h following the initial ANIT injection. Instead of melatonin, rats in the control and ANIT groups were orally administered with the same volume of 0.25% carboxymethyl cellulose (CMC) sodium (YuanYe Biological Technology Co., Ltd., Shanghai, China) 12 h after the initial injection. All rats were fasted for 12 h prior to the injections, and each rat was weighed prior to the i.p. injection of ANIT and oral administration of melatonin or CMC sodium. ANIT was dissolved in olive oil at a dose of 75 mg/kg b.w., i.e., 1 ml of ANIT solution in olive oil (75 mg/ml) per 100 g b.w., to induce liver injury associated with cholestasis, as previously described (23). Melatonin (100 mg/kg b.w.) was suspended in 1 ml of 0.25% CMC sodium.

**Sample collection and liver function assays.** The rats were provided with standard chow and water following treatment completion. According to pilot experiments, the rats were then fasted for 12 h, and were sacrificed 36 h after the initial ANIT or vehicle injection. Blood samples were collected from the inferior vena cava and the livers were immediately removed. All efforts were made to minimise animal suffering. The serum ALP (cat. no. A059-1), aspartate aminotransferase (AST; cat. no. C010-2), alanine aminotransferase (ALT; cat. no. C0009-2), GGT (cat. no. C017-1), total bilirubin (TBIL; cat. no. C019-1), direct bilirubin (DBIL; cat. no. C019-2) and total bile acid (TBA; cat. no. E003-1) levels were detected using chemical oxidation assays. All assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Table I. Stages of the animal experimental design.

Group	Procedure/treatment				
	0 h	12 h	24 h	36 h	48 h
Control	Fast	Olive oil	0.25% CMC	Fast	Sacrifice
Model	Fast	75 mg/kg ANIT	0.25% CMC	Fast	Sacrifice
Melatonin	Fast	75 mg/kg ANIT	100 mg/kg melatonin	Fast	Sacrifice

ANIT,  $\alpha$ -naphthylisothiocyanate.

**Histological assessment of liver damage.** The liver tissues were excised and fixed in 10% phosphate-buffered formalin. The fixed tissues were cut into 1x1x0.3-cm sections, dehydrated in a graded series of alcohol and embedded in paraffin blocks. The blocks were then cut into 4-5- $\mu$ m sections, dewaxed in xylene, dipped in haematoxylin and agitated for 30 sec, rinsed in H<sub>2</sub>O for 1 min, followed by staining with 1% eosin Y solution for 30 sec with agitation, all at room temperature (20-25°C). The slides were then examined under a BX53 microscope (Olympus Corporation, Tokyo, Japan).

**GSH assay in the liver.** The active GSH was determined with a commercial kit (cat. no. A006-1; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. In brief, a portion of the liver tissue was homogenised by adding nine volumes of saline. The homogenates were centrifuged at 3,000 x g and 4°C for 10 min to collect the supernatant. The reagents were added to 0.5 ml supernatant according to the instructions, centrifuged 3,500 x g (4°C) for 10 min, and 1 ml supernatant was collected for the chromogenic reaction.

**Western blot analysis.** Nuclear and cytoplasmic extractions were accomplished using the Nuclear and Cytoplasmic Extraction kit (Biosynthesis Biotechnology Company, Beijing, China) according to the manufacturer's protocol, and then assayed for protein levels of glutamate cysteine ligase (GCL), Akt, and Nrf2 with western blotting using an automated capillary-based size sorting system (Automated Capillary Western Blot, ProteinSimple, San Jose, CA, USA). All procedures were performed with the reagents included in the kit and according to the manufacturer's protocol. In brief, 8  $\mu$ l of diluted protein lysate was mixed with 2  $\mu$ l of 5X fluorescent Master mix and heated at 95°C for 5 min. The samples, blocking reagent, wash buffer, primary antibodies, secondary antibodies, and chemiluminescent substrate were dispensed into designated wells in a microplate provided by the manufacturer. The plate was loaded into the instrument and protein was drawn into individual capillaries on a 25-capillary cassette provided by the manufacturer. Protein separation with the resulting chemiluminescent signal was performed automatically on the individual capillaries using default settings. The data were analysed using Compass software (version 3.1.7; ProteinSimple, San Jose, CA, USA). The GCL catalytic subunit (GCLC), GCL modifier subunit (GCLM) and Nrf2 antibodies used were obtained from Abcam (1:50; cat. nos. ab80841, ab124827 and ab31163, respectively; Abcam, Cambridge, UK); Akt and  $\beta$ -actin were obtained from Cell

signalling Technology (1:50; cat. nos. 4691 and 4970, respectively; CST, Danvers, MA, USA) and used as a loading control. Secondary antibodies used were obtained from ProteinSimple (1:1; cat. no. 042-206). Primary and secondary antibodies were incubated at room temperature for 30 min.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted from the liver tissues using the mirVana miRNA Isolation kit (cat. no. AM1561, Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The yield of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) and the integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide.

Quantification was performed with a two-step reaction process: Reverse transcription and PCR. Each reverse transcription reaction involved two steps. In the first step, 0.5  $\mu$ g RNA and 2  $\mu$ l 4X gDNA wiper mix were combined, and nuclease-free H<sub>2</sub>O was added up to 8  $\mu$ l. The reactions were performed in a GeneAmp® PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.) for 2 min at 42°C. In the second step, 2  $\mu$ l of 5X HiScript II Q RT SuperMix IIa was added to the mixture, and the reactions were run for 10 min at 25°C, 30 min at 50°C, and 5 min at 85°C. The 10- $\mu$ l reverse transcription reaction mix was then diluted 10 times in nuclease-free H<sub>2</sub>O and maintained at -20°C. qPCR was performed using a LightCycler® 480 II Real-time PCR instrument (Roche Diagnostics, Basel, Switzerland) with a 10- $\mu$ l PCR mixture that included 1  $\mu$ l cDNA, 5  $\mu$ l 2X QuantiFast® SYBR® Green PCR Master mix (Qiagen GmbH, Hilden, Germany), 0.2  $\mu$ l forward primer, 0.2  $\mu$ l reverse primer and 3.6  $\mu$ l nuclease-free water. The reactions were incubated in a 384-well optical plate (Roche Diagnostics) at 95°C for 5 min, followed by 40 cycles at 95°C for 10 sec and at 60°C for 30 sec. Each sample was run in triplicate. At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR product. The primer sequences (Table II) were designed in the laboratory and synthesised by Generay Biotech (Shanghai, China) based on mRNA sequences obtained from the National Center for Biotechnology Information database.

The expression levels of the target mRNAs were normalised to those of  $\beta$ -actin and calculated using the  $2^{-\Delta\Delta C_q}$  method (32).

**Statistical analysis.** All statistical analyses were conducted using SPSS 20.0 software (IBM Corp., Armonk, NY, USA).



Table II. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GCLC	CCAGGGTGATCCTCTCATAC	TGCCACTTTTCATGTTCTCG
GCLM	CCACCAGATTTGACTGCAT	TTGCCTCAGAGAGCAGTTC
pAkt	AGAACCTCATGCTGGACA	CCTTGATACCCCTCCTTGC
Nrf2	TGGGTTCACTGACTCGGA	TGTTGGCTGTGCTTTAGG
β-actin	CCACCATGTACCCAGGCATT	CGGACTCATCGTACTCCTGC

GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modifier subunit; pAkt, phosphorylated Akt; Nrf2, nuclear factor-erythroid 2-related factor-2.

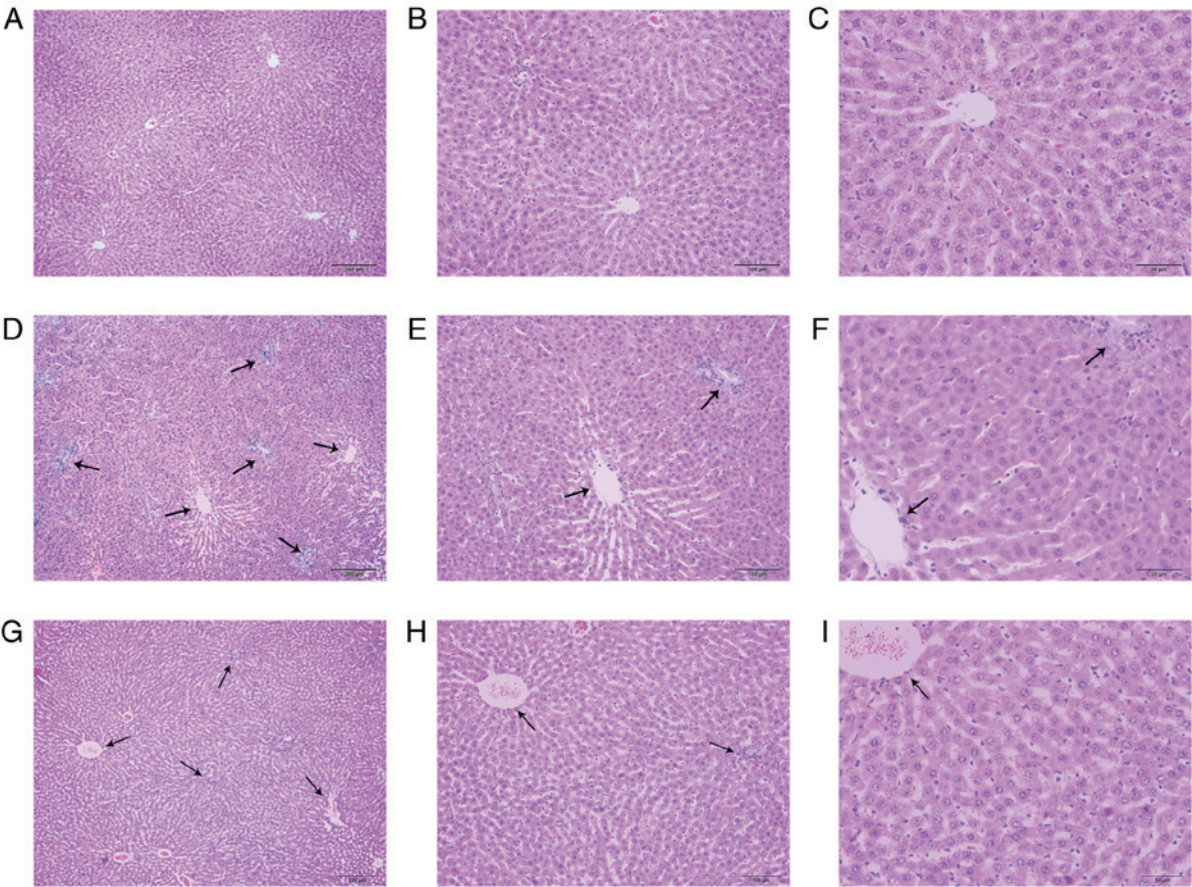


Figure 1. Melatonin reverses histological damage in the liver tissues of rats with  $\alpha$ -naphthyl isothiocyanate-induced cholestasis. Control group images at magnifications of (A) x50, (B) x100 and (C) x200; cholestasis model group images at magnifications of (D) x50, (E) x100 and (F) x200; and melatonin treatment group images at magnifications of (G) x50, (H) x100 and (I) x200. Liver tissue histological damage is indicated by black arrows.

All experiments were repeated at least three times and the obtained data are presented as the mean  $\pm$  standard deviation. Student's t-test was used for the 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 groups or more.  $P < 0.05$  was considered to indicate a statistically significant difference.

Results

*Histological examination.* Representative photomicrographs of the haematoxylin and eosin-stained liver tissues from the control,

ANIT and ANIT + melatonin groups are shown in Fig. 1. The control group exhibited a normal lobular architecture with central veins and radiating hepatic cords (Fig. 1A-C), whereas the ANIT group exhibited severe changes in liver morphology, including acute infiltration by neutrophils, metamorphosis, sinusoid congestion and hepatic necrosis and inflammation (Fig. 1D-F). However, the model rats treated with melatonin exhibited only mild bile duct epithelial damage and hepatocyte hydropic degeneration, and relatively milder neutrophil infiltration (Fig. 1G-I).

*Effects of melatonin on serum biochemistry.* As shown in Fig. 2, the ANIT-treated rats exhibited a marked increase

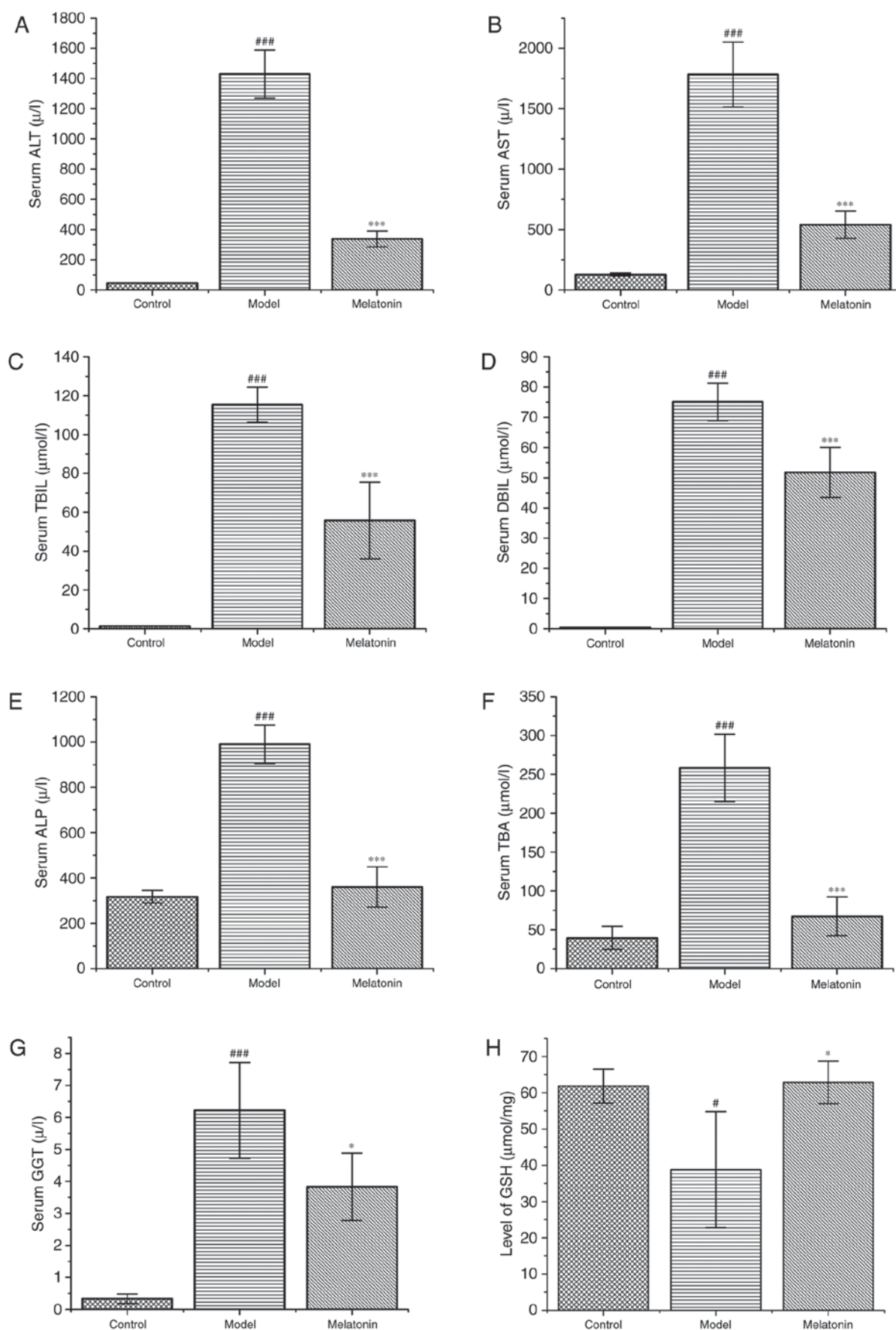


Figure 2. Effects of melatonin on serum biochemistry. The rats were treated with ANIT (75 mg/kg) with and without melatonin. The following liver function markers in the serum were assayed: (A) ALT, (B) AST, (C) TBIL, (D) DBIL, (E) ALP, (F) TBA, and (G) GGT. (H) Levels of GSH in liver tissues. Data are expressed as the mean  $\pm$  standard error of the mean (n=6 per group). ###P<0.001 and #P<0.05 compared with the control group; \*\*\*P<0.001 and \*P<0.05 compared with the ANIT group. ANIT,  $\alpha$ -naphthyl isothiocyanate; ALT, alanine transaminase; AST, aspartate transaminase; TBIL, total bilirubin; DBIL, direct bilirubin; ALP, alkaline phosphatase; TBA, total bile acids; GGT,  $\gamma$ -glutamyl transferase; GSH, glutathione.

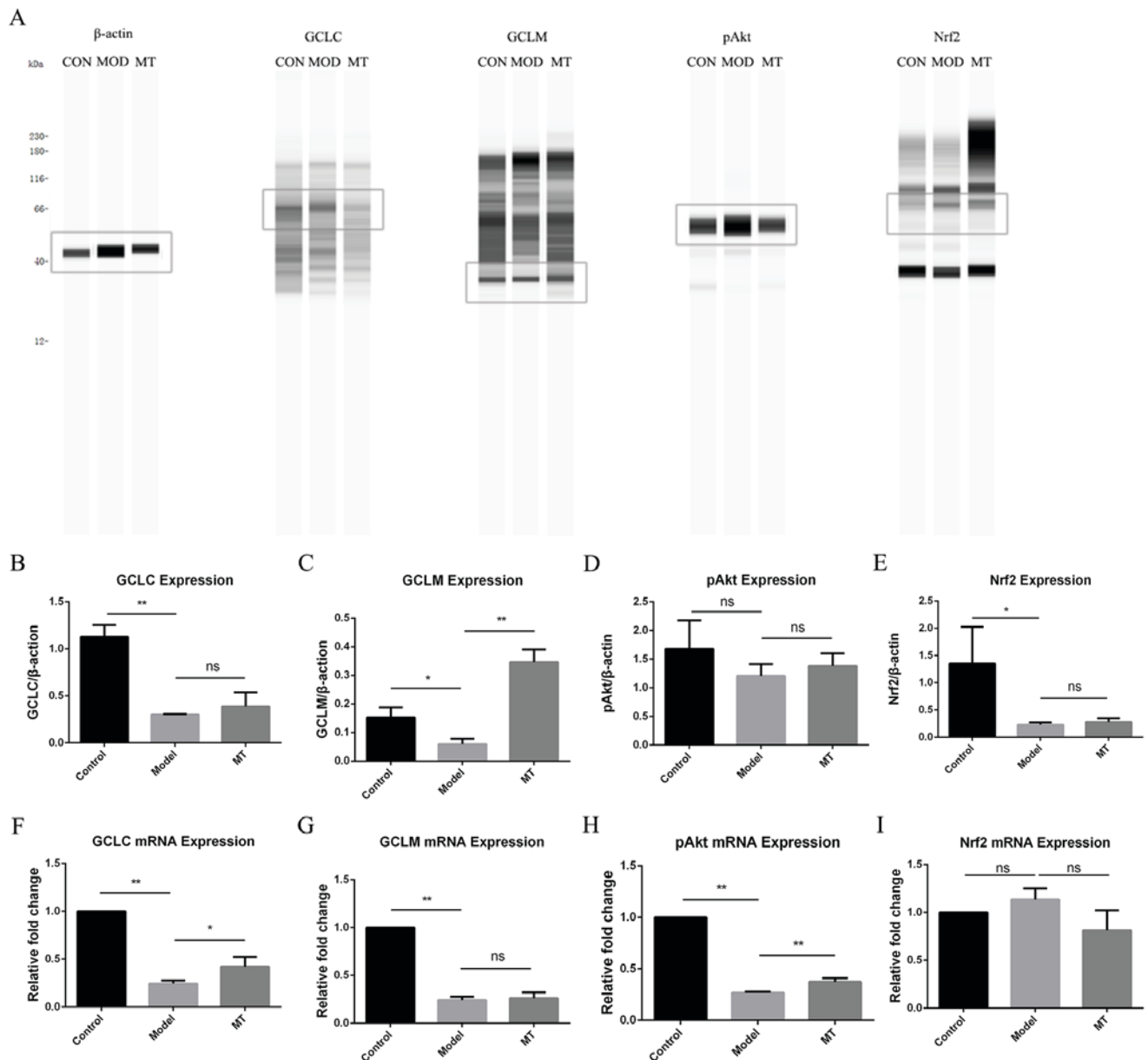


Figure 3. Effects of melatonin on hepatic protein and mRNA expression. (A) Effect of melatonin on hepatic protein expression. Effect of melatonin on hepatic protein expression of (B) GCLC, (C) GCLM, (D) pAkt and (E) Nrf2. Effect of melatonin on hepatic mRNA expression of (F) GCLC, (G) GCLM, (H) pAkt and (I) Nrf2. \*\* $P < 0.01$  and \* $P < 0.05$ . CON, control; MOD, model; MT, melatonin; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modifier subunit; Nrf2, nuclear factor-erythroid 2-related factor-2; ns, not significant.

in ALT and AST levels, which were significantly reduced following treatment with melatonin (Fig. 2A and B). Similarly, the levels of TBIL, DBIL, ALP, TBA and GGT were markedly increased in ANIT-treated rats compared with the control group, and were effectively reduced following melatonin administration (Fig. 2C-G).

**Effects of melatonin on hepatic GSH.** Compared with the control group, the model groups exhibited markedly reduced concentrations of GSH in the liver tissue; the GSH levels were increased in the melatonin-treated group compared with those in the ANIT group (Fig. 2H).

**Effects of melatonin on the activation of GCLC, Nrf2, Akt and GCLM.** The results of protein analysis (Fig. 3A-E) showed that melatonin upregulated the protein expression of GCLM. Melatonin increased the mRNA levels of GCLC and pAkt, the expression levels of which were reduced by administration of ANIT (Fig. 3F-I).

## Discussion

Serum liver enzymes (including ALT, AST, ALP and GGT), TBA and serum bilirubin (TBIL and DBIL) are important indices of the clinical manifestations of cholestatic hepatitis.



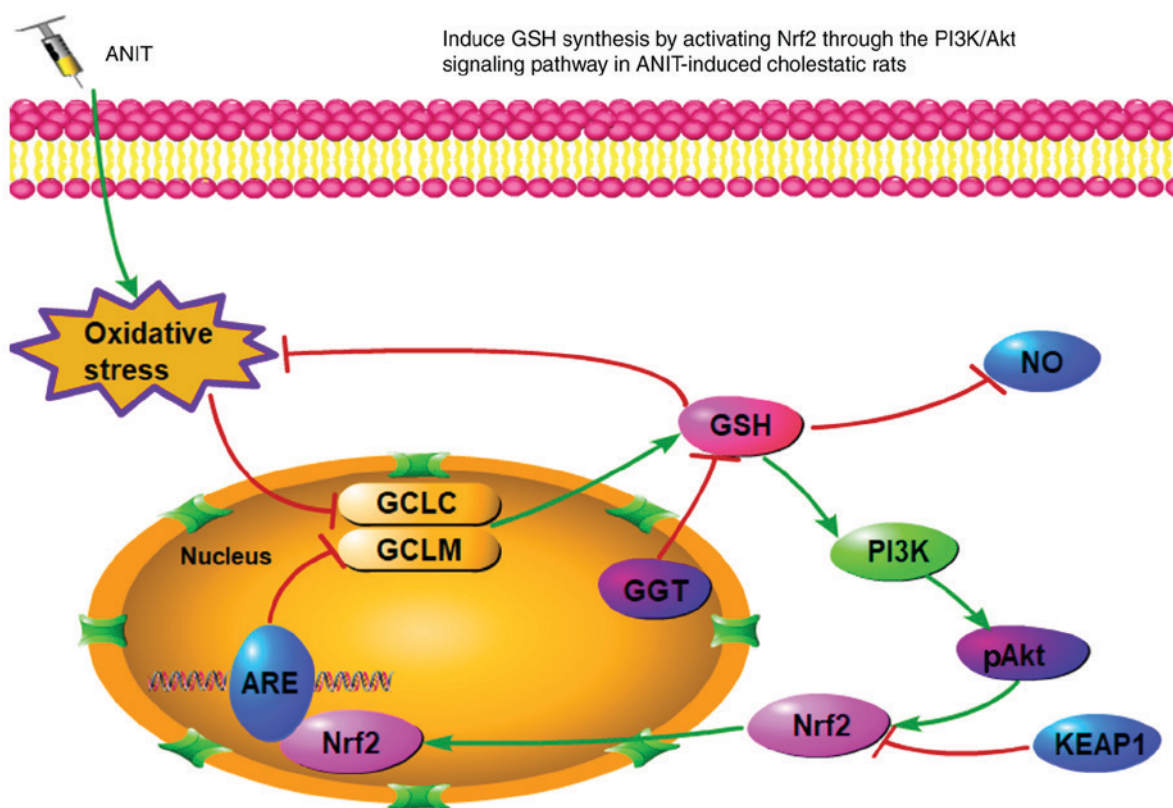


Figure 4. Schematic presentation of the proposed effects of melatonin against ANIT-induced intrahepatic cholestasis. ANIT,  $\alpha$ -naphthyl isothiocyanate; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modifier subunit; Nrf2, nuclear factor-erythroid 2-related factor-2; ARE, antioxidant response element; GSH, glutathione; GGT,  $\gamma$ -glutamyl transferase; PI3K, phosphoinositide-3 kinase; KEAP1, Kelch-like ECH-associated protein 1.

These markers tend to increase following ANIT administration and commonly peak at 48 h (33). The results of the present study indicate that treatment with melatonin notably decreased the ANIT-induced serum levels of serum ALT, AST, TBA, TBIL, DBIL and ALP. Furthermore, the histological manifestations of liver damage were reduced following melatonin treatment. Overall, these results indicate that melatonin may be a candidate drug exerting protective effects against ANIT-induced cholestasis and ensuing liver injury.

The activation of Nrf2 protects the liver from xenobiotic toxicity by regulating the expression of several detoxifying and antioxidant enzymes, in addition to transporters (34-37). The PI3K/Akt pathway has been suggested as the key signaling pathway in this system by regulating the expression of Nrf2, with the activity of the GCL subunit in hepatocytes also regulated by PI3K/Akt signalling (38). GSH is involved in the detoxification of chemical substances conjugated by the catalytic action of GSH and the extracellular transport of conjugated compounds, acting as one of the major cellular antioxidant defence molecules against ROS production (39). GSH synthesis occurs in the cytosol of all mammalian cells via two enzymatic steps: The formation of  $\gamma$ -glutamylcysteine from glutamate and cysteine catalysed by GCL, and the formation of GSH from  $\gamma$ -glutamylcysteine and glycine catalysed by GSH synthase (40). Akt, as a major regulator of PI3K signalling, exerts an anti-apoptotic effect, and may be phosphorylated and activated during several different types of cell death (41). Nrf2 acts as a key transcription factor and a regulator of the expression of GCL in response to oxidative

stress, along with other anti-oxidative stress genes. Therefore, the present study also examined the effects of melatonin on the expression of GSH and its synthetic enzymes. It was demonstrated that melatonin markedly increased the levels of GSH, and upregulated the mRNA and protein expression levels of GCLC and GCLM. These findings indicated that the effect of melatonin on the increase in GSH may be associated with upregulation of the expression of GCLM and GCLC, thereby contributing to the protection of cells against oxidative damage and against ANIT-induced cholestasis. A schematic summary of the proposed effects of melatonin against ANIT-induced intrahepatic cholestasis is presented in Fig. 4. Overall, the results of the present study indicate that melatonin markedly attenuated cholestasis through regulating oxidative stress via targeting the Nrf2-PIK/Akt axis, highlighting this natural product as an antioxidant candidate for the treatment of cholestasis.

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## Availability of data and materials

The datasets generated and analysed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

XZ and XD conceived and designed the study. YL, HY and ZX acquired, analysed and interpreted the data. DW and SS were responsible for handling the animals and obtaining tissue specimens. XS and YW performed statistical analyses. BZ and HD performed histopathological analyses. 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 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). All efforts were made to minimise animal suffering.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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