Prolonged exposure of cholestatic rats to complete dark inhibits biliary hyperplasia and liver fibrosis

Yuyan Han, Paolo Onori, Fanyin Meng, Sharon DeMorrow, Julie Venter, Heather Francis, Antonio Franchitto, Debolina Ray, Lindsey Kennedy, John Greene, Anastasia Renzi, Romina Mancinelli, Eugenio Gaudio, Shannon Glaser, and Gianfranco Alpini


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Han Y, Onori P, Meng F, DeMorrow S, Venter J, Francis H, Franchitto A, Ray D, Kennedy L, Greene J, Renzi A, Mancinelli R, Gaudio E, Glaser S, Alpini G. Prolonged exposure of cholestatic rats to complete dark inhibits biliary hyperplasia and liver fibrosis. Am J Physiol Gastrointest Liver Physiol 307: G894–G904, 2014. First published September 11, 2014; doi:10.1152/ajpgi.00288.2014.—Biliary hyperplasia and liver fibrosis are common features in cholestatic liver disease. Melatonin is synthesized in the pineal gland as well as the liver. Melatonin inhibits biliary hyperplasia of bile duct-ligated (BDL) rats. Since melatonin synthesis (by the enzyme serotonin N-acetyltransferase, AANAT) from the pineal gland increases after dark exposure, we hypothesized that biliary hyperplasia and liver fibrosis are diminished by continuous darkness via increased melatonin synthesis from the pineal gland. Normal or BDL rats (immediately after surgery) were housed with light-dark cycles or complete dark for 1 wk before evaluation of 1) the expression of AANAT in the pineal gland and melatonin levels in pineal gland tissue supernatants and serum; 2) biliary proliferation and intrahepatic bile duct mass, liver histology, and serum chemistry; 3) secretin-stimulated ductal secretion (functional index of biliary growth); 4) collagen deposition, liver fibrosis markers in liver sections, total liver, and cholangiocytes; and 5) expression of clock genes in cholangiocytes. In BDL rats exposed to dark there was enhanced AANAT expression/melatonin secretion in pineal gland and melatonin serum levels; 2) improved liver morphology, serum chemistry and decreased biliary proliferation and secretin-stimulated choleresis; and 4) decreased fibrosis and expression of fibrosis markers in liver sections, total liver and cholangiocytes and reduced biliary expression of the clock genes PER1, BMAL1, CLOCK, and Cry1. Thus prolonged dark exposure may be a beneficial noninvasive therapeutic approach for the management of biliary disorders.

biliary epithelium; cholestasis; clock genes; melatonin; secretin


Melatonin is synthesized from tryptophan by activation of the enzymes serotonin N-acetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (ASMT) (62). Melatonin is released into the blood stream primarily from the pineal gland after dark exposure, and it is suppressed by light, indicating the important functions of this hormone in the regulation of circadian rhythm and several body functions (43). The hypothalamus and pituitary organs mediate many of melatonin functions including circadian rhythms, stress, and reproduction. Recently, stomach, duodenum, and the hepatobiliary system have also been identified as major sources of melatonin during the daytime (12, 44).

Abnormal melatonin synthesis and circadian rhythms are altered in patients with hepatic cirrhosis and are associated with severe liver insufficiency (58). Several studies have shown that melatonin administration has protective effects against cholestatic liver damage through its antioxidant, anti-inflammatory, and proapoptotic proprieties (19, 28, 29, 46). Recently, studies have demonstrated the antiprolifera-

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tive effects of melatonin on cholangiocytes in animal models of extrahepatic cholestasis as well as cholangiocarcinoma (26, 44, 45). Several studies have also shown that melatonin circulation during darkness is a potent oncostatic signal and light exposure at night suppresses the night peak release of melatonin and seems associated with disease processes including cancer (9–11).

Interestingly, our group has shown that administration of exogenous melatonin inhibits biliary hyperplasia and secretin-stimulated ductal secretion of BDL rats (45). The proposed mechanism involves resynchronization of circadian genes such as Per1/2, Cry1/2, BMAL1, and CLOCK, which are mediators of cell mitosis. After BDL, increased/alterned expression of clock genes in cholangiocytes has been described (45). During cholestasis, cholangiocytes also synthesize melatonin through activation of the rate-limiting enzyme AANAT (44). In vivo local inhibition of AANAT synthesis (by administration of AANAT Vivo-Morpholino) increases cholangiocyte proliferation, whereas in vitro overexpression has antiproliferative effects (44). These studies describe the local synthesis of melatonin by cholangiocytes and the pivotal role of this hormone in homeostasis of the biliary epithelium via an autocrine pathway. In this study, we tested the hypothesis that prolonged dark exposure after BDL increases melanin levels released from pineal gland inhibit cholangiocyte hyperplasia and liver fibrosis by a paracrine mechanism by inducing AANAT expression and melatonin synthesis in the pineal gland.

METHODS AND MATERIALS

Materials

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. The mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA, Sc-56) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-cytokeratin-19 (CK-19) antibody was purchased from Caltag Laboratories (Burlingame, CA). Commercially available ELISA kits for measuring melatonin levels were purchased from Cayman Chemical (Ann Arbor, MI). The RNeasy Mini Kit for RNA purification and all primers were purchased from Qiagen (Valencia, CA).

Animal Models

Male Fischer 344 rats (175–200 g) were purchased from Charles River Laboratories (Wilmington, MA) and housed in a temperature-controlled environment (22°C). Animals were fed standard rat chow and had access to drinking water ad libitum. We used normal rats, BDL or bile duct-uncannulated (BDI, for collection of bile) rats that immediately after surgery (3) were housed in a temperature-controlled environment (22°C) with 12:12-h light-dark cycles or complete dark for 1 wk. All experimental groups were used at 8:00 AM for the collection of serum, cholangiocytes, and liver samples. Before each procedure, the animals were treated with Euthasol (200–250 mg/kg body wt). Liver weight, body weight, and liver weight-to-body weight ratio (an index of the growth of liver cells including cholangiocytes) (3) were measured in all animals. All animal experiments were performed in accordance with protocols approved by Baylor Scott and White IACUC.

Freshly Isolated Cholangiocytes

Virtually pure (~98% by histochemistry for γ-glutamyltransferase, γ-GT) (49) cholangiocytes from each animal group were obtained by immunoaffinity separation (4, 5, 31) by using a monoclonal antibody, rat IgG₂a (a gift from Dr. R. Faris, Brown University, Providence, RI), against an unidentified antigen expressed by all mouse cholangiocytes (31).

Measurement of AANAT Expression in Pineal Gland and Hypothalamus Tissue and Melatonin Levels in Pineal Gland Tissue Supernatants and Serum

To validate our model, we evaluated the mRNA expression of AANAT by real-time PCR (22, 45) in total RNA (1 μg) from pineal gland and hypothalamus. Data were expressed as relative mRNA levels ± SE of AANAT-to-GAPDH ratio. To detect the mRNA expression of the selected genes, we used the RT² Real-Time assay (SA Biosciences, Frederick, MD). A ΔΔCₜ (delta delta of the threshold cycle) analysis was performed with normal cholangiocytes as control sample (22). We evaluated melatonin levels by ELISA kits (44) in the supernatant collected from short-term (6 h) cultures of pineal gland, data expressed as pg/μg protein; and 2 serum from the four groups of animals.

Evaluation of Cholangiocyte Proliferation, IBDM, Liver Histology, and Serum Chemistry

The percentage of PCNA-positive cholangiocytes was calculated by semiquantitative immunohistochemistry (45) in paraffin-
Table 1. Measurement of liver, body weight and liver-to-body weight ratio; serum chemistry; % PCNA-positive cholangiocytes and IBDM; and melatonin serum levels

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal + Light-Dark Cycles 1 wk</th>
<th>Normal + Complete Dark 1 wk</th>
<th>BDL + Light-Dark Cycles 1 wk</th>
<th>BDL + Complete Dark 1 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight, g</td>
<td>8.7 ± 0.2 (n = 9)</td>
<td>9.0 ± 0.6 (n = 7)</td>
<td>9.8 ± 0.1* (n = 8)</td>
<td>8.7 ± 0.5† (n = 12)</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>217.7 ± 5.8 (n = 12)</td>
<td>226.0 ± 5.1 (n = 7)</td>
<td>170.2 ± 4.3* (n = 12)</td>
<td>175.0 ± 2.7 (n = 12)</td>
</tr>
<tr>
<td>Liver-to-body weight ratio, %</td>
<td>4.30 ± 0.2 (n = 12)</td>
<td>3.9 ± 0.2 (n = 7)</td>
<td>5.6 ± 0.1* (n = 12)</td>
<td>4.9 ± 0.3‡ (n = 12)</td>
</tr>
<tr>
<td>Serum melatonin levels, pg/ml</td>
<td>61.52 ± 8.71 (n = 12)</td>
<td>207.0 ± 84.1 (n = 15)</td>
<td>110.1 ± 18.4* (n = 12)</td>
<td>145.3 ± 47.5 (n = 12)</td>
</tr>
<tr>
<td>SGPT, U/l</td>
<td>72.7 ± 12.1 (n = 9)</td>
<td>156.1 ± 32.5* (n = 8)</td>
<td>396.3 ± 95.6* (n = 10)</td>
<td>115.7 ± 13.9† (n = 4)</td>
</tr>
<tr>
<td>SGOT, U/l</td>
<td>185.9 ± 24.5 (n = 12)</td>
<td>270.0 ± 58.4z (n = 9)</td>
<td>1,105.8 ± 245.4* (n = 10)</td>
<td>249.2 ± 25.1† (n = 4)</td>
</tr>
<tr>
<td>Total bilirubin, mg/dl</td>
<td>&lt;0.1 (n = 7)</td>
<td>&lt;0.1 (n = 6)</td>
<td>11.5 ± 1.2* (n = 12)</td>
<td>4.7 ± 1.3† (n = 4)</td>
</tr>
<tr>
<td>ALP, U/l</td>
<td>276.4 ± 13.7 (n = 8)</td>
<td>244.2 ± 9.1‡ (n = 5)</td>
<td>400.63 ± 33.0* (n = 10)</td>
<td>357.5 ± 23.5‡ (n = 4)</td>
</tr>
<tr>
<td>Volume fraction occupied by collagen (Sirius red-stained fibers)</td>
<td>1.8 ± 0.2 (n = 5)</td>
<td>1.9 ± 0.2 (n = 5)</td>
<td>6.3 ± 1.5* (n = 5)</td>
<td>3.5 ± 0.6† (n = 4)</td>
</tr>
<tr>
<td>α-SMA+ portal MF/HSC (cells/HPF)</td>
<td>1.5 ± 0.3 (n = 5)</td>
<td>1.0 ± 0.26 (n = 5)</td>
<td>9.7 ± 1.4 (n = 5)</td>
<td>6.6 ± 0.7 (n = 4)</td>
</tr>
</tbody>
</table>

*P < 0.05 BDL vs. normal rats. †P < 0.05 BDL + dark vs. BDLCycles 1 wk. ‡not significant vs. the corresponding rats exposed to dark-light cycles. BDL, bile duct ligation; IBDM, intrahepatic bile duct mass; SGPT, serum glutamic oxaloacetic transaminases; SGOT, serum glutamate pyruvate transaminases; ALP, alkaline phosphatase; α-SMA, α-smooth muscle actin; MF, myofibroblasts; HSC, hepatic stellate cells; HPF, high-powered field.

We also evaluated 1) PCNA and CK-19 mRNA (by real-time PCR using 1 µg total RNA) (45) and PCNA protein expression (by immunoblots and/or FACS analysis (45) in purified cholangiocytes from the selected groups of animals. The selected rat primers were purchased from SABiosciences (Qiagen, Valencia, CA) and designed by using sequences with the following NCBI GenBank accession numbers: PCNA, NM_022381; CK-19, NM_199498; and GAPDH, NM_017008. Immunoblots were performed in 10 µg of protein from whole cell lysate from spleen (positive control) or purified cholangiocytes. Immunoblots were normalized to β-actin, the housekeeping gene. Band intensity of immunoblots was determined by scanning video densitometry by using the phospho-imager, Storm 860 (GE Healthcare, Piscataway, NJ) and the ImageQuant TL software version 2003.02 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). FACS analysis was also performed to measure PCNA protein expression by use of a C6 flow cytometer and analyzed by CFlow Software (Accuri Cytometers, Ann Arbor, MI) (45). At least 10,000 events in the light scatter (SSC/FSC) were acquired. The expression of PCNA was identified and gated on FL1-A/Count plots. The relative quantity of the selected protein (mean selected protein fluorescence intensity) was expressed as mean FL1-A (samples)/mean FL1-A (secondary antibodies only).

All paraffin-embedded liver sections (4–5 µm thick) were stained with hematoxylin and eosin and analyzed in a coded fashion by a board certified pathologist. Sections were evaluated by a BX-51 light microscope (Olympus, Tokyo, Japan). The serum levels of glutamate pyruvate transaminases (SGPT), glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), and total bilirubin were measured by a Dimension RXL Max Integrated Chemistry system (Dade Behring, Deerfield, IL) by the Chemistry Department, Baylor Scott & White Healthcare.

Measurement of Secretin-Stimulated Camp Levels and Bile Secrecion

Next, we evaluated 1) basal and secretin-stimulated cAMP levels (by EIA kits) in purified cholangiocytes and bile and 2) bicarbonate secretion (functional indexes of biliary growth) (3, 34) in bile fistula rats (45). Before evaluation of cAMP levels by EIA kits, purified cholangiocytes were incubated for 1 h at 37°C (to regenerate membrane proteins damaged by proteolytic enzymes during cell purification) (31) and subsequently incubated with 0.2% BSA or secretin (100 nM) for 5 min at room temperature (4, 5).

For the evaluation of bile and bicarbonate secretion, after anesthesia rats were surgically prepared for bile collection as described (3). Briefly, when steady-state bile flow was reached (60–70 min from the intravenous infusion of Krebs-Ringer-Henseleit solution,
REGULATION OF BILIARY GROWTH BY THE PINEAL GLAND

A

Normal
Normal + dark
BDL
BDL + dark

B

Normal
Normal + dark
BDL + dark
 BD L + dark

C

Normal
Normal + dark
BDL
BDL + dark

PCNA-positive cholangiocytes (%)

Intrahepatic Bile Duct Masses (%)
KRH), the animals were infused with secretin (100 nM for 30 min) (34) via a jugular vein followed by intravenous infusion of KRH for 30 min. Bicarbonate levels in bile were determined by a COBAS Mira Plus automated clinical chemistry analyzer (Bohemia, NY).

Evaluation of Liver Fibrosis in Liver Sections and Expression of Fibrotic Genes in Total Liver and Purified Cholangiocytes

Liver fibrosis was evaluated by Sirius red staining for identifying interstitial collagen with red color in paraffin-embedded liver sections (4–5 μm thick, 10 different fields analyzed from each sample obtained from 3 different animals). Slides were scanned by a digital scanner (AperioScanscope CS System, Aperio Technologies, Oxford, UK) and processed by ImageScope. An image analysis algorithm has been used to quantify the volume fraction occupied by collagen (Sirius red-stained) fibers. The algorithm was applied on the entire sections. Typical markers of fibrosis such as α-smooth muscle actin (α-SMA) were also evaluated by immunohistochemistry in paraffin-embedded liver sections (4 μm thick). We also evaluated by real-time PCR (22) the expression of COLA1, fibronectin-1, and α-SMA, matrix metalloproteinase 2 and 9 (MMP-2, MMP-9), metalloproteinase inhibitor 1 and 2 (TIMP1 and TIMP2, tissue inhibitors of metalloproteinases), and transforming growth factor-β (TGF-β) in total liver and isolated cholangiocytes. The selected rat primers were purchased from SABiosciences (Qiagen, Valencia, CA) and designed using sequences with the following NCBI GenBank accession numbers: NM_053304 (COLA1); NM_019143 (α-SMA); NM_031004 (fibronectin-1); NM_031054 (MMP-2); NM_031055 (MMP-9); NM_053819 (TIMP1); NM_021989 (TIMP2); NM_021578 (TGF-β); CK-19, NM_199498; and NM_017008 (GAPDH).

Expression of Core Clock Genes in Isolated Cholangiocytes

The expression of the core clock genes, CLOCK, PER1, Cry1, and BMAL1, was studied in isolated cholangiocytes from the selected groups of animals by real-time PCR (45). Primers used were designed against rat BMAL1, Cry1, PER1, and CLOCK according to the NCBI GenBank accession numbers: NM_024362 (BMAL1), NM_198750 (Cry1), NM_001034125 (PER1); NM_021856 (CLOCK); and NM_017008 (GAPDH). Data were expressed as relative mRNA levels ± SE of the selected gene to GAPDH ratio.

Fig. 3. Chronic exposure of BDL rats to dark for 1 wk decreases PCNA and CK-19 expression in purified cholangiocytes. PCNA (A) and CK-19 (B) expression was evaluated by real-time PCR. Data are expressed as means ± SE of 4 experiments from cumulative preparations of cholangiocytes. *P < 0.05 BDL compared with normal rats. #P < 0.05 BDL + dark compared with BDL rats. C: PCNA protein expression was evaluated by immunobLOTS. Data are expressed as means ± SE of 4 immunoblots reactions from cumulative preparations of cholangiocytes. *P < 0.05 BDL compared with normal rats. #P < 0.05 BDL + dark compared with BDL rats. D: PCNA protein expression evaluated by FACS analysis (n = 3). P1 represents the pool cholangiocytes population gated from the range of forward-scattered light (FSC) and side-scattered light (SSC). *P < 0.05 BDL + dark compared with BDL rats.
there was decreased liver-to-body weight ratio in BDL rats compared with the corresponding groups (Table 1). Consistent between normal and BDL rats exposed to complete dark over, there was no significant difference in the body weight of BDL compared with normal rats (Table 1). More-

Histology, and Serum Chemistry

Evaluation of Cholangiocyte Proliferation and IBDM, Liver

As previously shown (3), there was a decrease in the body weight of BDL compared with normal rats (Table 1). Moreover, there was no significant difference in the body weight between normal and BDL rats exposed to complete dark compared with the corresponding groups (Table 1). Consistent with reduced liver cell growth after prolonged dark exposure, there was decreased liver-to-body weight ratio in BDL rats exposed to dark compared with BDL rats exposed to light-dark cycles (controls) (Table 1). We have previously shown that in vivo administration of melatonin decreases BDL-induced biliary hyperplasia (45). Herein, we extended our previous study to determine that enhanced secretion of melatonin (from the pineal gland after prolonged exposure to dark) inhibits cholangiocyte proliferation induced by BDL (3). In agreement with previous studies (2, 3), in BDL rats there was increased percentage of PCNA-positive cholangiocytes (Fig. 2A) and IBDM (Fig. 2B) compared with normal rats (Fig. 2, A and B). Exposure of BDL rats to complete dark decreased the percentage of PCNA-positive cholangiocytes and IBDM (Fig. 2, A and B) compared with BDL controls. Prolonged dark exposure had no effect on the proliferation in normal rats. Liver architecture of normal rats exposed to 12:12-h light-dark cycles or prolonged dark was normal (score 0, no biliary proliferation or inflammation was observed) (Fig. 2C). BDL rats, which showed prominent biliary proliferation, received score 4. BDL + dark-exposed rats (that showed lower cholangiocyte proliferation compared with BDL rats) received score 3 (Fig. 2C). Serum levels of transaminases, bilirubin, and alkaline phosphatase increased in BDL compared with normal rats and significantly decreased in BDL + dark rats compared with BDL controls (Table 1); no significant difference was observed between normal rats exposed to light-dark cycles or complete dark (Table 1).

By real-time PCR, immunoblots, and FACS analysis, we demonstrated increased PCNA expression in cholangiocytes from BDL rats compared with normal rats (Fig. 3, A, C, and D). In purified cholangiocytes from BDL + dark rats, there was reduced PCNA mRNA and protein expression compared with BDL controls (Fig. 3, A, C, and D). We found a similar trend for CK-19 gene expression with a decrease in expression levels in BDL rats exposed to dark compared with BDL control rats (Fig. 3B).

Measurement of cAMP Levels in Purified Cholangiocytes and Bile and Bicarbonate Secretion In Vivo

As expected (3), secretin increased cAMP and bile and bicarbonate secretion in BDL rats but did not increase cAMP levels and bile and bicarbonate secretion in BDL that were exposed to dark (Fig. 4 and Table 2).

Evaluation of Liver Fibrosis in Liver Sections and Expression of Fibrotic Genes in Total Liver and Purified Cholangiocytes

In vitro effect of melatonin on the expression of fibrotic genes. In agreement with previous studies (18, 47), in liver sections from BDL rats there was enhanced fibrosis evaluated

<table>
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<td>BDL + Dark</td>
<td>102.02 ± 9.39</td>
<td>122.46 ± 8.38†</td>
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*P < 0.05 vs. the corresponding basal values; †nonsignificant vs. the corresponding basal values. BDL, bile duct cannulated.

Statistical Analysis

All data are expressed as means ± SE. Differences between groups were analyzed by Student’s unpaired t-test when two groups were analyzed and ANOVA when more than two groups were analyzed, followed by an appropriate post hoc test.

RESULTS

Measurement of AANAT Gene Expression in Pineal Gland and Hypothalamus Tissue and Melatonin Levels in Pineal Gland Tissue Supernatants and Serum

The transcriptional activation of the AANAT gene is the primary mechanism for the induction of melatonin biosynthesis in rodents (51). Since melatonin levels are altered by changes in light and dark cycles (35), we measured AANAT expression in the pineal gland and hypothalamus (which expresses very low levels of AANAT) and melatonin levels in pineal gland tissue supernatant and serum from the four experimental groups. There were no significant changes in AANAT expression and melatonin secretion in the pineal gland of BDL compared with normal rats (Fig. 1, A and B). As expected (27), AANAT mRNA expression and melatonin secretion increased in the pineal gland from normal and BDL + dark rats compared with their corresponding rats (Fig. 1, A and B); AANAT mRNA expression was barely detectable in the hypothalamus of normal rats and did not change significantly compared with the other groups (not shown). Similar to our previous study (45), melatonin levels were higher in the serum of BDL compared with normal rats (Table 1). Melatonin serum levels were higher in normal and BDL rats exposed to dark for 1 wk compared with the corresponding rats under light-dark cycles (Table 1).

Evaluation of Cholangiocyte Proliferation and IBDM, Liver Histology, and Serum Chemistry

As previously shown (3), there was a decrease in the body weight of BDL compared with normal rats (Table 1). Moreover, there was no significant difference in the body weight between normal and BDL rats exposed to complete dark compared with the corresponding groups (Table 1). Consistent with reduced liver cell growth after prolonged dark exposure, there was decreased liver-to-body weight ratio in BDL rats exposed to dark compared with BDL rats exposed to light-dark cycles (controls) (Table 1). We have previously shown that in vivo administration of melatonin decreases BDL-induced biliary hyperplasia (45). Herein, we extended our previous study to determine that enhanced secretion of melatonin (from the pineal gland after prolonged exposure to dark) inhibits cholangiocyte proliferation induced by BDL (3). In agreement with previous studies (2, 3), in BDL rats there was increased percentage of PCNA-positive cholangiocytes (Fig. 2A) and IBDM (Fig. 2B) compared with normal rats (Fig. 2, A and B). Exposure of BDL rats to complete dark decreased the percentage of PCNA-positive cholangiocytes and IBDM (Fig. 2, A and B) compared with BDL controls. Prolonged dark exposure had no effect on the proliferation in normal rats. Liver architecture of normal rats exposed to 12:12-h light-dark cycles or prolonged dark was normal (score 0, no biliary proliferation or inflammation was observed) (Fig. 2C). BDL rats, which showed prominent biliary proliferation, received score 4. BDL + dark-exposed rats (that showed lower cholangiocyte proliferation compared with BDL rats) received score 3 (Fig. 2C). Serum levels of transaminases, bilirubin, and alkaline phosphatase increased in BDL compared with normal rats and significantly decreased in BDL + dark rats compared with BDL controls (Table 1); no significant difference was observed between normal rats exposed to light-dark cycles or complete dark (Table 1).

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In vitro effect of melatonin on the expression of fibrotic genes. In agreement with previous studies (18, 47), in liver sections from BDL rats there was enhanced fibrosis evaluated

Table 2. Measurement of bile and bicarbonate secretion in BDL rats exposed to 12:12-h light-dark cycles and complete dark for 1 wk

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Bile Flow, μl·min⁻¹·kg body wt⁻¹</th>
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*P < 0.05 vs. the corresponding basal values; †nonsignificant vs. the corresponding basal values. BDL, bile duct cannulated.
by Sirius red staining for collagen fibers and increased expression of α-SMA compared with normal rats (Fig. 5). When BDL rats were exposed to complete dark, there was reduced fibrosis and expression of α-SMA compared with BDL rats exposed to light-dark cycles (Fig. 5). Following BDL, there was increased expression of α-SMA, COLA1, fibronectin-1, MMP-2, MMP-9, TIMP1, TIMP2, and TGF-β in total liver and cholangiocytes compared with normal rats (Fig. 6, A–D). BDL-induced increase in the expression of α-SMA, COLA1, fibronectin-1, MMP-2, MMP-9, TIMP1, TIMP2, and TGF-β was reduced by exposure of BDL rats to dark (Fig. 6, A–D).

**Expression of clock genes in isolated cholangiocytes.** There was increased expression of the clock genes, Per1, BMAL1, CLOCK, and Cry1 in total liver samples and cholangiocytes from BDL rats compared with normal rats (Fig. 7). Exposure of BDL rats to complete dark (that increases melatonin secretion and reduces biliary hyperplasia) decreases the mRNA expression of Per1, BMAL1, CLOCK, and Cry1 compared with BDL rats exposed to light-dark cycles (Fig. 7).

**DISCUSSION**

In the present study, we evaluated the effect of prolonged dark exposure (which stimulates the release of melatonin from the pineal gland) (43, 62) on BDL-induced biliary hyperplasia and liver fibrosis. In BDL rats exposed to prolonged dark there...
was 1) increased AANAT mRNA expression and melatonin levels in the pineal gland and circulating melatonin levels; 2) improved liver morphology and decreased biliary proliferation and IBDM; 3) decreased secretin-stimulated ductal secretory activity; and 4) decreased fibrosis and expression of the clock genes PER1, BMAL1, CLOCK, and Cry1 in isolated cholangiocytes compared with BDL control rats. Our findings suggest that the prolonged dark exposure may be a beneficial noninvasive therapeutic approach for the management of biliary disorders through the modulation of melatonin synthesis from the pineal gland.

Melatonin synthesis in the pineal gland shows a rhythmic fashion with high levels at night and is controlled by the rate-limiting enzyme AANAT (62). We found that there was a significant elevation in serum melatonin levels when both normal and BDL rats were exposed to prolonged complete darkness. This observation is consistent with a recent study that showed a significant increase in serum melatonin in rats exposed to constant darkness for 17 days, which was similar to the nighttime elevations in animals exposed to 12:12-h light-dark cycles (60). Several other studies have demonstrated that melatonin levels increase in serum during prolonged dark exposure in rat, pig, and chicken models (14, 35, 42). Also, consistent with our previous study and those in humans with liver cirrhosis, we found that there was significant increase in serum melatonin levels in BDL compared with normal control rats (13, 45). Since there was no significant difference in AANAT expression/melatonin secretion in the hypothalamus of normal and BDL rats, the increase in melatonin serum levels observed in BDL rats is likely due to the higher secretion of melatonin by BDL cholangiocytes as part of a compensatory mechanism as we observed during biliary proliferation (44, 45). We found that, parallel with increase melatonin serum levels, there was a significant increase in AANAT gene expression in the pineal glands of the normal and BDL animals exposed to constant darkness. Similar elevations in AANAT gene expression have been observed in rats kept in constant darkness compared with those kept in standard light-dark conditions (59). Interestingly, although there was an increase in AANAT gene expression level in the pineal gland of BDL rats kept in constant darkness, there was a smaller (compared to higher levels observed in normal rats) increase in melatonin levels in the supernatants collected from the pineal glands of the BDL rats kept in constant darkness. We postulate that this reduction may represent a compensatory mechanism in the pineal gland responding to chronically elevated melatonin levels observed in BDL compared with normal rats. Several studies have shown that daytime serum levels of melatonin are elevated in patients with liver cirrhosis, which is postulated to be due to decreased liver blood flow, lowered activity of 6β-hydroxylase, and competition with bilirubin in the intrahepatic transport system (13, 30, 52). A study has also shown that not only do patients with cirrhosis have decreased melatonin clearance, they also have decreased daily production of melatonin (33), which is consistent with our finding of decreased melatonin levels in the pineal glands of BDL rats kept in constant darkness. Further experiments are necessary to determine the contribution of the gastrointestinal tract includ-
ing the biliary epithelium (12, 44) in melatonin synthesis in response to dark exposure and the possible presence of melatonin in bile.

Since melatonin has been shown to ameliorate cholestatic-induced liver damage and systemic oxidative stress (21, 55), we next demonstrated the inhibitory effect of prolonged dark exposure (a noninvasive approach) on BDL-induced increase in biliary hyperplasia, serum chemistry, and secretin-stimulated ductal secretory activity (a functional marker of biliary proliferation) (2, 3, 5, 6). In addition to the activation of biliary hyperplasia, the BDL model is also characterized by increased biliary fibrosis, which is associated with increased collagen deposition, α-SMA, and fibronectin expression in portal areas (56). It has been shown that besides hepatic stellate cells and fibroblasts, cholangiocytes are activated, express α-SMA, and synthesize matrix (61). These findings are consistent with other studies that demonstrate that melatonin ameliorates hepatic fibrosis in a number of rodent models of fibrosis (28, 54, 55). For example, melatonin has been shown to inhibit the expression and activity of MMP-2 and MMP-9 in both in vitro and in vivo models (32, 39, 41). A recent study has shown that melatonin inhibits MMP-9 by binding to its active site, which represents another possible mechanism for the alteration in fibrosis during constant darkness (48). Melatonin has been shown to diminish the secretion of TGF-β from hepatic stellate cells (25). The beneficial effects of prolonged dark exposure on biliary hyperplasia, serum chemistry, and fibrosis are likely mediated by melatonin (as confirmed by the in vitro studies) and enhanced melatonin synthesis (45). However, our study does not rule out the possibility that other factors such as follicle-stimulating hormone (37) may be released by dark exposure that could modulate biliary function (20, 36).

Since we have previously shown (45) that melatonin inhibits biliary hyperplasia in cholestatic BDL rats through downregulation of selected core clock genes, we aimed to demonstrate that reduction of biliary injury/fibrosis by complete dark exposure is associated with reduced expression of specific clock genes in the biliary epithelium. Our present study shows decreased expression of Per1, BMAL1, CLOCK, and Cry1 and supports the concept that clock genes play a key role in the modulation of liver injury (15–17). For example, the expression of fibrosis-related genes such as TGF-β, COLA1, and TIMP1 is elevated in cholestatic Per2 knockout mice, suggesting that the clock gene Per2 plays a protective role in during liver injury (16). Also, the loss of the clock gene, mPer2, has been shown to promote liver fibrosis induced by carbon tetra-chloride (15). Furthermore, altered circadian rhythm of the clock gene, Cry2, has been demonstrated in fibrotic livers (17). Further studies are necessary to evaluate the circadian rhythm of Per1, BMAL1, and Cry1 expression (the clock genes decreased by dark exposure) during the autocrine/paracrine modulation of biliary disorders by melatonin.

We performed all the studies at 1 wk of BDL because we aimed to evaluate the proliferation and fibrosis in early stages of cholestasis. Thus further studies are needed to better understand the effect of continuous dark exposure on biliary injury and liver fibrosis during chronic cholestasis. The fact that epidemiological studies indicate that night shifts or longer light exposure is a risk factor for breast cancer (53) supports the clinical significance of dark therapy in human liver diseases. Supporting the clinical relevance of our studies, sleep-wake abnormalities have been observed in patients with cirrhosis (40). However, rodents are nocturnal animals, which is different from humans. Thus further studies (e.g., correlation between melatonin serum levels and liver functions in blind patients and patients under different dark-light cycles) are necessary to verify the effect of dark therapy in patients with chronic cholestatic liver diseases. Also, epidemiological studies are warranted to determine a correlation between circulating melatonin levels and liver functions in patients living in countries exposed to different cycles of dark/sunlight.

In summary, compared with our previous findings showing that increased biliary melatonin synthesis (by hepatic modulation of AANAT) inhibits biliary hyperplasia in an autocrine pathway (45), we now provide novel evidence that continuous dark exposure increases melatonin serum levels (through enhanced melatonin synthesis from the pineal gland) that, in turn, decrease biliary hyperplasia and liver fibrosis via a paracrine-regulated pathway. Modulation of melatonin levels through exposure to constant darkness may represent a novel and noninvasive approach for the environmental management of cholestatic liver diseases.

REFERENCES


