Chemicals and reagents:

Cell culture reagents such as Dulbecco's Modified Eagle's Medium (DMEM), trypsin phosphate versene glucose (TPVG), bovine serum albumin (BSA) and antibiotic-antimycotic solution were purchased from HiMedia Laboratories, Mumbai, India. Fetal bovine serum (FBS) was procured from Gibco. TRIzol and RNA-Later stabilizing solution were purchased from Invitrogen, USA and Ambion, Inc. USA respectively. Protease inhibitor cocktail (PIC), hematoxylin, eosin, Oil Red O (ORO), cell culture grade melatonin, oleic acid and miR-122-specific inhibitor (NSC-5476) were sourced from Sigma Aldrich, USA. Precision plus protein ladder, polyvinylidene fluoride (PVDF) membrane, Protein Assay Dye reagent concentrate, iScript cDNA synthesis kit, SYBR green master mix, Clarity Western ECL blotting substrate were procured from Bio-Rad Laboratories, USA. Primary antibodies against NOCT (SC-376584) and β-actin (E-AB-40338) were purchased from Santa Cruz Biotechnology, USA and E-Labscience, USA respectively. Secondary antibodies (SC-516102 and 32469) were acquired from Santa Cruz Biotechnology, USA and Invitrogen, USA respectively. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl tetrazolium bromide), dimethyl sulfoxide (DMSO), chloroform, methanol, ethanol and isopropanol were obtained from Sisco Research Laboratory, Pvt. Ltd. Mumbai, India. miRNeasy kit (217004) and miRScript II RT kit (218161) were purchased from Qiagen. Regular chow diet and high fat diet were procured from VRK Solutions. Other chemicals used were of analytical grade and purchased from Merck, Germany.

Molecular docking studies:

NOCT protein structure predictions

Since the full-length protein structure of mmu-NOCT and hsa-NOCT are not available in protein databases, i-TASSER (Iterative Threading Assembly Refinement) was used. Herein, the amino acid sequences of mmu-NOCT and hsa-NOCT were obtained from NCBI. The sequences were formatted in

FASTA format and uploaded to the i-TASSER server (https://zhanggroup.org/I-TASSER/). No additional templates or constraints were provided, and the default settings of the server were used for all predictions. The query protein sequences were first analysed by i-TASSER using Position-specific iterated BLAST (PSI-BLAST) to identify homologous proteins from a non-redundant sequence database. Based on these results, a sequence profile was generated by multiple sequence alignment. Further, secondary structure prediction was carried out using PSIPRED, that analyses the sequence profile generated in the previous step. The sequences were then threaded through a representative library of Protein Data Bank (PDB) structures using LOMETS (local meta-threading server) pipeline, that integrates multiple threading algorithms. LOMETS aligned the query sequences with structurally similar proteins in the PDB to identify potential structural templates. After threading, i-TASSER assembled full-length 3D models by iterative fragment assembly simulations. The server generated top 5 models, ranked according to their C-scores (represent confidence levels of the prediction; range from -5 to 2 with higher values indicating higher confidence). The accuracy of the predicted models was evaluated using the C-score provided by i-TASSER. Additionally, the predicted models were visually analysed using molecular visualization tools such as PyMol to confirm their structural features.

Validation of NOCT protein structures by Ramachandran Plot analysis

The predicted protein structures of mmu-NOCT and hsa-NOCT in PDB format were obtained from i-TASSER and prepared for analysis. All the water molecules and heteroatoms were removed from the PDB files to focus on the backbone torsion angles. Subsequently, the PDB files for each model were uploaded to the PROCHECK web server wherein; the φ (phi) and ψ (psi) backbone torsion angles for all non-glycine and non-proline residues were calculated. Ramachandran Plot was generated for each model that displayed the allowed, generously allowed and disallowed regions of the torsional angle

space. Further, the Ramachandran plots were analysed to evaluate the structural quality of the models, and the following metrics were assessed:

- Percentage of residues in favoured regions comprises of residues falling in the most sterically favourable regions of the plot.
- Percentage of residues in allowed regions includes residues in regions that are generally permissible but less favourable.
- Percentage of residues in disallowed regions: this parameter indicates residues that adopt sterically unfavourable conformations, that could point to structural errors in the model.

Molecular docking between melatonin and mmu-NOCT

Docking between melatonin and mmu-NOCT (generated through i-TASSER) was performed using AutoDock Vina, PyRx and PyMol softwares. Mmu-NOCT protein structure was imported into PyMol and non-protein molecules such as water molecules and ions were removed. The protein was further checked for missing residues and corrections were made as necessary. Hydrogen atoms were added using PyMol and the structure was saved in PDB format for further use. The 3D structure of melatonin was downloaded from PubChem (CID:896) in SDF format and PyRx was used to convert the SDF format of melatonin to PDBQT format. Using Open Babel feature in PyRx, the lignad's geometry was optimized to ensure correct bond lengths and angles. The prepared protein structure (in PDB format) and the prepared melatonin structure were loaded into PyRx. A grid box was defined around the protein's binding site wherein; the grid parameters were chosen to enclose the active site or potential ligand binding regions. The docking parameters for AutoDock Vina were left at their default settings and the docking simulation was performed using AutoDock Vina integrated within PyRx. The 9 protein-ligand complexes were generated and were analysed. The binding affinity values for each docking pose were compared and the best pose was selected based on the lowest energy conformation.

Computational studies for miRNAs

miRNAs with a potential seed sequence in *Noct* mRNA in humans, mice and rats were investigated using computational prediction algorithms (miRDB V 6.0 and TargetScan V 7.0). Potential miRNAs implicated in NAFLD/NASH were identified in miRNet and Human MicroRNA Disease Database HMDD) and were subjected to enrichment analysis in miRNet. The two datasets (miRNAs with potential complementarity to *Noct* gene and miRNAs implicated in NAFLD/NASH) were merged in CytoScape 3.9.1 software using the default parameters. From the resultant miRNAs, miR-122 was selected as the most relevant epigenetic regulator of Noct gene in NAFLD/NASH and was further scrutinized in TargetScan.

Functional analysis of Nocturnin

RNA-seq dataset of wildtype (WT) and Noct knockout (*Noct*$^{-/-}$) A549 was retrieved from the Gene Expression Omnibus (GEO) repository of NCBI (accession ID: GSE123477). Raw gene expression data were pre-processed using TPM/FPKM and was followed by probe-to-gene mapping using platform annotation files. Samples were categorized into two groups (Control vs Noct KO) based on the provided metadata. Differential gene expression analysis was performed using DESeq2 in R and statistical significance was determined using a Benjamini-Hochberg corrected p-value <0.05 and a log2 fold change threshold of ± 1.5. Results were visualized via volcano plot. Protein-protein interaction (PPI) networks of these differentially expressed genes were generated using STRING and Cytoscape 3.9.1. Additionally, pathway enrichment analysis was conducted using the Webgestalt online tool.

In vivo studies:

Animal maintenance:

6-8 weeks old male C57BL/6J mice were procured from ACTREC, Mumbai, India and housed according to CCSEA guidelines (23± 2° C, 12:12 light-dark cycle, with ad libitum access to laboratory chow and water). The mice were allowed to acclimatize for 7 days before initiation of the experiment. The experimental design was approved from the Institutional Animal Ethical Committee (IAEC; approval no. MSU-Z/IAEC06/01-2022) and the experimental regime was conducted in the CCSEA-approved animal house facility of the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, India. Mice were randomly assigned to seven experimental groups after completion of the acclimatization period.

Photoperiodic shifts induced chronodisruption (CD) and experimental design:

Chronodisruption (CD) in mice was induced by phase-advance-phase-delay photoperiodic shifts for 16 weeks. In brief, mice were housed in two separate rooms, designated as Room 1 and Room 2. Room 1 comprised of light period from ZT0 to ZT12 and dark period from ZT12 to ZT24 and Room 2 consisted of dark period from ZT4 to ZT16 and light period from ZT16 to ZT4. Transferring mice from Room 1 to Room 2 on Monday resulted in an 8-hours phase advance (lights off at ZT4) and moving them back to Room 1 on Thursday caused an 8-hours phase delay (see Fig. M1). All transfers occurred at 10.55 am.

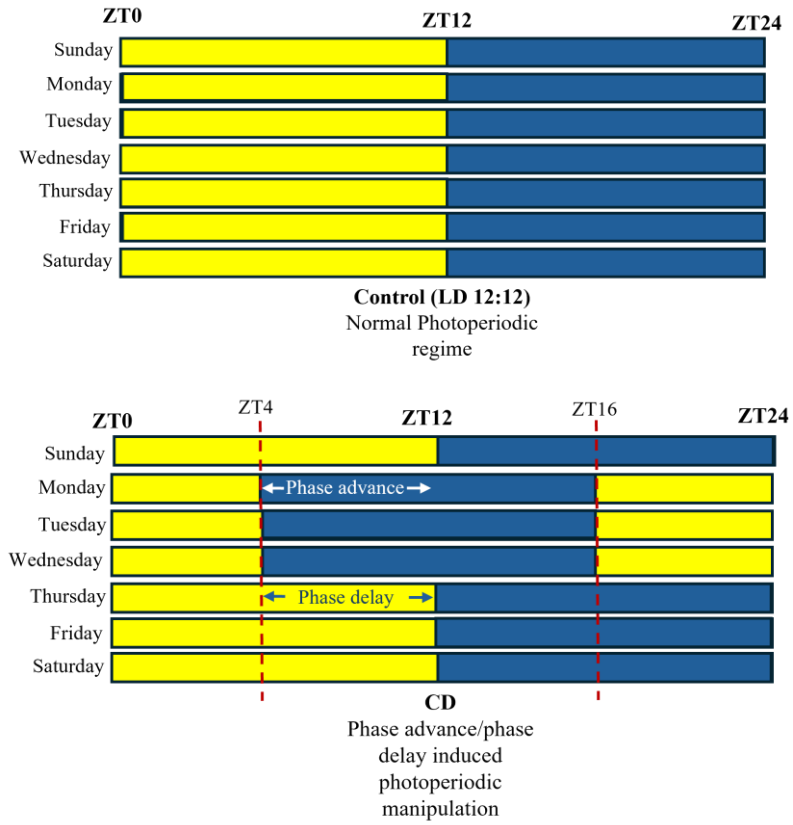


Figure M1. Schematic representation of normal and phase-advance-phase-delay induced photoperiodic regime in mice that was followed for 16 weeks.

Table M1. Composition of high fat diet (Joshi et al., 2021)

Macronutrient composition	
Protein % of energy	20
Carbohydrate % of energy	20
Fat % of energy	60
Energy (MJ/kg)	21.8
Ingredient (g/kg)	
Casein	258.4
L-Cystine	3.9

Corn Starch	0
Maltodextrin	161.5
Sucrose	88.9
Cellulose	64.6
Lard	316.6
Soyabean oil	32.3
Vitamin mix (a)	12.9
Mineral mix (b)	12.9
Choline Bitartrate	2.6

(a) Vitamin mix: comprises of the following components (per g vitamin mix): retinyl acetate (0.8 mg), DL-a-tocopheryl acetate (10.0 mg), menadione sodium bisulfite (0.05 mg), cholecalciferol (1.0mg), biotin (0.02 mg), cyanocobalamin (1 µg), folic acid (0.2 mg), nicotinic acid (3mg), calcium pantothenate (1.6 mg), pyridoxine- HCl (0.7mg), riboflavin (0.6 mg), thiamin HCl (0.6 mg).

(b) Mineral mix: comprises of the following components (per g mineral mix): sodium chloride (259 mg), magnesium sulphate (257.6 mg), magnesium oxide (41.9 mg), cupric carbonate (1.05 mg), chromium K sulphate (1.925 mg), sodium fluoride (0.2 mg), ferric citrate (21 mg), potassium iodate (0.035 mg), manganous carbonate (12.25 mg), sodium selenite (0.035 mg), ammonium molybdate (0.3 mg) and zinc carbonate (5.6 mg).

Melatonin preparation and administration:

Melatonin was freshly dissolved in less than 1% volume of ethanol and subsequently 0.9% saline (NaCl) was added to achieve a concentration of 10 mg/kg body weight. Sterile tubes containing melatonin solution were enclosed in aluminium foil to avoid light-induced degradation. Melatonin was intraperitoneally injected daily (in evenings) from 9th week till the end of 16 weeks.

Validation of experimentally induced NASH

Mice were randomly assigned to seven experimental groups and each group comprised of 15 animals.

Table M2: Experimental design

Sr. No.	Experimental Groups	Experimental manipulations
1.	Group I (C)	Standard chow diet; LD 12:12
2.	Group II (H)	High fat diet + 20% fructose water; LD 12:12
3.	Group III (CD)	Standard chow diet; photoperiodic shifts induced CD.
4.	Group IV (HCD)	High fat diet + 20% fructose water (H diet); photoperiodic shifts induced CD.
5.	Group V (HM)	High fat diet + 20% fructose water (H diet); LD 12:12; melatonin injection (10mg/kg B.W.) from 9th week till 16 weeks.
6.	Group VI (CDM)	Standard chow diet; photoperiodic shifts induced CD; melatonin injection (10mg/kg B.W.) from 9th week till 16 weeks.
7.	Group VII (HCDM)	High fat diet + 20% fructose water (H diet); photoperiodic shifts induced CD; melatonin injection (10mg/kg B.W.) from 9th week till 16 weeks.

High-fat-high-fructose (H) diet and/or chronodisruption (CD) induced NAFLD in C57BL/6J mice is a established model in our laboratory. Further, melatonin mediated re-entrainment of clock genes and improvement in liver function via corrections in the Nrf2-HO1 pathway intermediates has been reported.

Blood and tissue collection:

At the end of 16 weeks, mice were fasted overnight and euthanized with mild isoflurane at five time points (ZT = 0,6,12,18, and 24). Whole blood was collected via retro-orbital sinus under mild isoflurane anaesthesia and was allowed to clot at room temperature for 30 minutes. Subsequently, serum was isolated by centrifugation at 3000 rpm at 4^{o}C for 10 minutes was carefully

separated and stored at -80ºC for further analysis. Liver tissues samples were harvested in 4% PFA (for histochemical analysis) and in RNA-Later solution and snap frozen for qPCR analysis.

Serum biochemical analysis:

Serum samples were analysed to measure circulating titres of liver function markers (ALT and AST) and lipid profile (total lipids- TL, total cholesterol-TC, triglycerides-TG, HDL-cholesterol) were quantified using commercially available kits (Reckon Diagnostic kits, Vadodara, Gujarat, India). Low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) were calculated using Friedwald's formula. Additionally, cholesterol to HDL ratio and LDL to HDL ratio were determined through theoretical calculations.

Disease control groups (H, CD and HCD) recorded significant increment in the circulating titres of liver function markers (ALT and AST) [Fig. M2] and serum lipid profile whereas HDL-cholesterol (HDL-c) recorded a significant decrement [Fig. M3]. However, melatonin treatment (HM, CDM and HCDM groups) accounted for a significant decrement in liver function markers and lipid profile [Fig. M2 and M3].

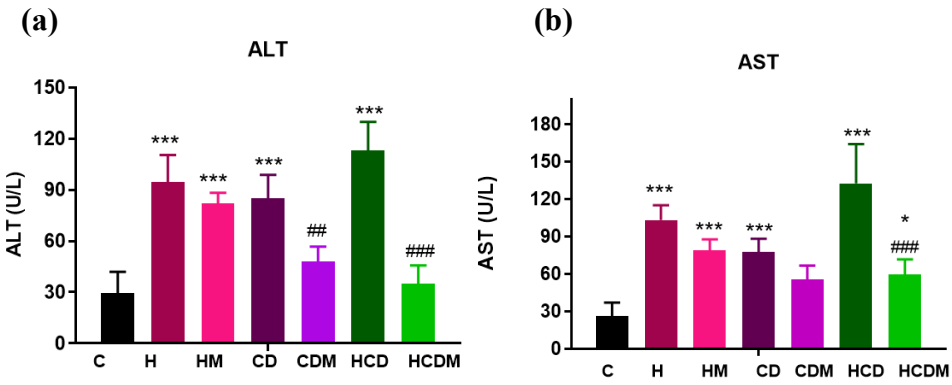


Figure M2. Alterations in serum liver function markers: (a) ALT and (b) AST in C57BL/6J mice subjected to H and/or CD regime for 16 weeks and melatonin-mediated improvements. ***$p<0.001$ when H, CD and HCD are compared with control (C) and ##$p<0.01$, ###$p<0.001$ when melatonin-treated groups (HM, CDM and HCDM) are compared to the respective disease-control group.

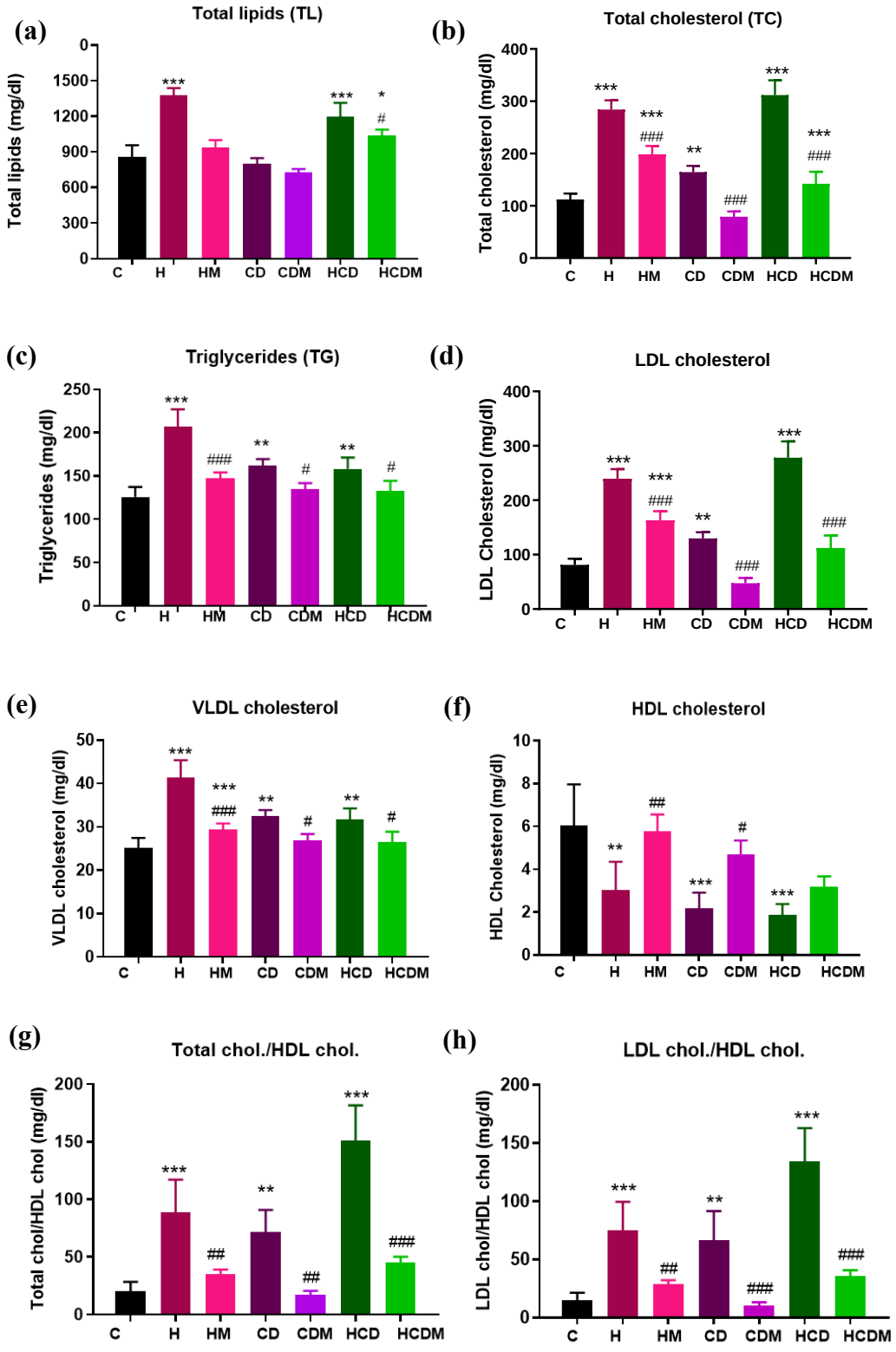


Figure M3. High-fat-high-fructose (H) diet alone or in combination with chronodisruption (CD) for 16 weeks significantly alter lipid profile parameters: (a) Total lipids-TL, (b) Total cholesterol-TC, (c) Triglycerides-TG), (d) LDL-cholesterol, (e) VLDL-cholesterol, (f) HDL-cholesterol, (g) Total cholesterol/

HDL-cholesterol and (h) LDL cholesterol/HDL cholesterol. *p<0.05, **p<0.01 and ***p<0.001 when H, CD and HCD are compared with control (C) and #p<0.05, ##p<0.01 and ###p<0.001 when melatonin-treated groups (HM, CDM and HCDM) are compared to respective disease-control groups.

Microscopic evaluation of hepatic tissues and H&E staining

Liver tissue samples were fixed in 4% paraformaldehyde (PFA) and was subsequently dehydrated, embedded in paraffin wax blocks and cut into 5µm thick sections in a cryotome. Sections were then stained with haematoxylin and eosin (H&E) wherein; the sections were deparaffinized and rehydrated by immersing them in decreasing concentrations of alcohol. The sections were then incubated with hematoxylin stain for approximately 5 minutes, followed by rinsing under running water. Differentiation was performed using 1% acid alcohol (70% alcohol and acid) for 5 minutes, after which the sections were again rinsed under running water. Finally, the tissue sections were stained with 1% eosin for 5 minutes and washed under running water. Further, sections were dehydrated by passing them through increasing concentrations of alcohol. The slides were then mounted using a mounting medium. Observations and photographs were taken using Leica DM 2500 microscope. Liver sections from control and treated mice were scored by investigators who were blinded to the study.

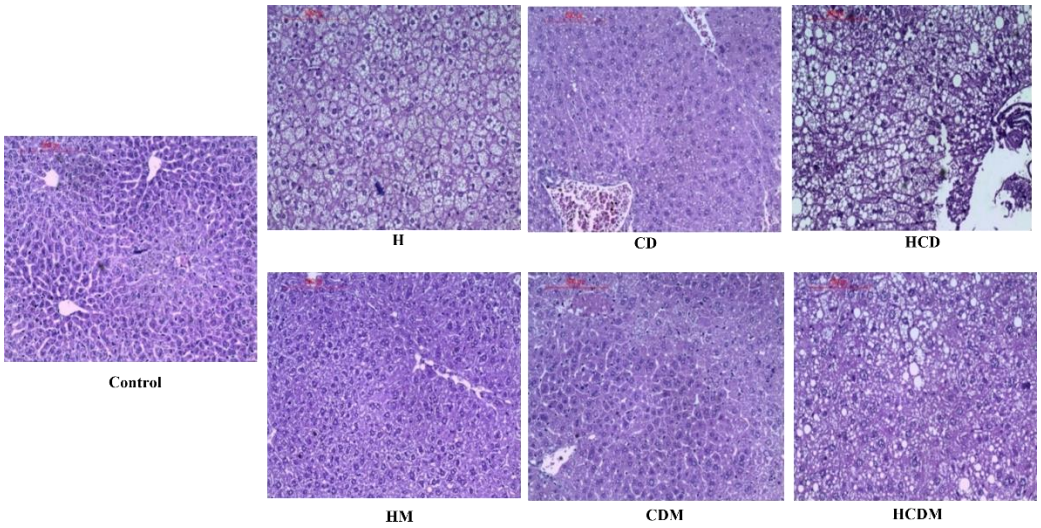
Scoring of liver sections

Sections of liver tissues were evaluated by H&E staining. A semi-quantitative scoring system was utilized to assess hepatocyte necrosis and intrahepatic haemorrhage (0- none, <10% of total area-1, <30% of total area-2, less than 40% of total area-3, more than 50% of total area-4. Ballooning of hepatocytes was graded as follows: 0- none, 1- few ballooned, 2- many ballooned. The assessment was conducted by two investigators blinded to the study.

In the present study, microscopic analysis of liver tissue sections of H, CD and HCD mice revealed varying levels of hepatocyte ballooning, cellular derangement and hepatic cords [Fig. M4(a)]. Random scoring of liver sections

demonstrated higher hepatocyte ballooning and steatosis scores in the H, CD, and HCD groups [Fig. M4(b) & (c)]. Melatonin treatment resulted in a marked reduction in hepatic abnormalities and intracellular fat accumulation in the HM, CDM, and HCDM groups [Fig. M4(b) & (c)].

(a)



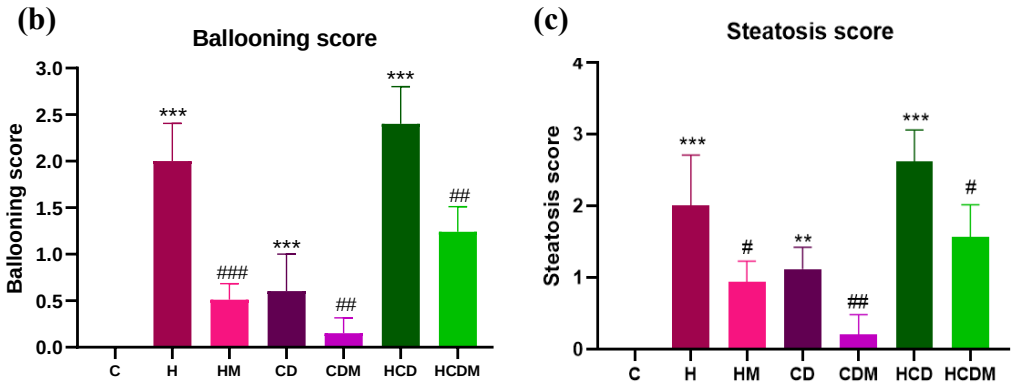


Figure M4. (a) Microscopic evaluation of liver tissues of H and/or CD mice for 16 weeks and melatonin-mediated improvements in: (b) Ballooning scores and (c) steatosis scores. **p<0.01, ***p<0.001 when H, CD and HCD is compared to control. #p<0.05, ##p<0.01 and ###p<0.001 when melatonin-treated groups (HM, CDM and HCDM) are compared to their respective disease-control groups.

In vitro studies

Maintenance of HepG2 cells

Human Hepatoma (HepG2) cells were obtained from NCCS, Pune, India and were maintained in DMEM growth medium containing 10% FBS and 1% antibiotic-antimycotic solution. Cells were maintained at 37^{o}C and 5% CO^2. Cells were sub-cultured at 70% confluency using 1X TPVG wherein, once the cells detached from the flask surface, they were collected in a sterile 2ml tube and centrifuged at 2000 rpm for 5 minutes. The resultant cell pellet was re-suspended in fresh media and transferred to new cell culture flask. Further, the media was changed every third day. All the experiments were conducted at ~70% confluency and respective treatments were given in incomplete cell culture medium.

Preparation of oleic acid (OA) conjugated with BSA and melatonin solutions

Stock solution of OA was prepared by conjugating 100 mM of OA with 10% BSA solution to obtain a stock solution of 10 mM OA-conjugated BSA (ref…). Melatonin was dissolved in minimal volume of 100% ethanol and the volume was made up to prepare a stock solution of 1mM. Subsequent dilutions of OA and melatonin were prepared in incomplete cell culture media. Subsequent dilutions of both OA and melatonin were prepared in incomplete cell culture media to achieve the desired concentrations for working solutions.

For synchronization of individual cellular clocks, HepG2 cells were subjected to serum shock (50% FBS) for 2 h and then treated with respective compounds for 24 h. After 24 h, cells were collected for further analysis.

Cytotoxicity assessment

HepG2 cells were seeded in a 96-well plate (cell seeding density: 10^4 cells/well) in DMEM and incubated for 24 h. Subsequently, the cells were subjected to increasing concentrations of OA (0.5-2mM) and/or melatonin (5-1000µM). After 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml) was added and the cells were incubated

at 37°C for 4 h. The resultant formazan crystals were dissolved in 100µl of DMSO and absorbance was measured at 540 nm using a Synergy HTX multimode reader (Bio-Tek Instruments, Inc., Winooski, VT).

Intracellular lipid accumulation by Oil Red O (ORO) staining

HepG2 cells were treated with OA (0.5 mM) alone or in combination with melatonin (100 µM) for 24 h, then fixed with 4% PFA, and then rinsed with PBS. Cells were further stained with Oil Red O (ORO) stain and the ORO levels were quantified by adding 100% isopropanol to each well. Absorbance was measured at 510 nm in Synergy HTX multimode microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Intracellular miR-122 inhibition

Intracellular miR-122 was inhibited in HepG2 cells by subjecting them to a combination of OA and synthetic miR-122-specific inhibitor procured from Sigma (Cat. No. NSC-5476) for 24 h. Concentration of the inhibitor was chosen by performing MTT assay as described above with increasing concentrations of the miR-122 inhibitor (0.05 µM to10 µM). Efficacy of the inhibitor was evaluated by checking intracellular miR-122 levels by qPCR.

Total RNA isolation and cDNA synthesis

Total RNA from HepG2 cells and liver tissue samples were isolated using TRIzol reagent (Invitrogen, Thermo Scientific, USA). Tissue samples were homogenized in TRIzol whereas, cells were lysed directly in the culture dish using the same reagent. Following lysis and homogenization, phase separation was carried out with chloroform. RNA was precipitated from aqueous phase using pre-chilled isopropanol and centrifuged to form a pellet which was subsequently washed once with pre-chilled 75% ethanol. The resultant RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water and its purity was assessed by measuring absorbance at 260nm and 280 nm (A_{260} and A_{280}) and calculating their ratios (A_{260}/ A_{280}) nanodrop. Samples with A_{260}/ A_{280}

ratio between 1.8 and 2.0 were used for gene expression analysis. RNA concentration was determined by absorbance at 260 nm using the following formula:

$$\text{Concentration } (\mu g/ml) = A_{260} * 40 * \text{dilution factor}$$

Where 40 is the extinction co-efficient (40 µg/ml of RNA = 1 absorbance unit).

To synthesize cDNA, 1 µg of RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories, USA), following the manufacturer's instructions. The resulting cDNA was used as a template for quantitative RT-PCR.

Isolation of miRNA and cDNA synthesis

Total miRNA from liver tissue samples and HepG2 cells was isolated using miRNeasy Kit (Qiagen, Germany). Tissue samples were homogenized, and cells were directly lysed in QIAzol lysis reagent. The lysates were processed according to the manufacturer's instructions and miRNA was quantified by measuring ratios of absorbance at 260 nm, 280 nm and 230 nm (A_{260}, A_{280} and A_{230} respectively) using nanodrop. The miRNAs were subsequently transcribed into cDNA using miScript II RT kit following the manufacturer's instructions for template quantity. The resultant cDNA was subsequently used as a template for quantitative RT-PCR reactions.

Quantitative RT-PCR

Quantitative RT-PCR was carried out using SYBR Select Master Mix (Bio-Rad, USA) according to the manufacturer's instructions, with the reactions performed on QuantStudio- 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, USA). mRNA expression of target genes was analysed using $2^{-\Delta\Delta CT}$ method, with 18S and 5S serving as endogenous control

for RNA and miRNA, respectively. Primer sequences used herein are listed in Table M3.

Table M3. List of primer sequences used in the study.

Target	Primer	Sequence (5' to 3')
mmu-Noct	FP	ACCAGCCAGACATACTGTGC
	RP	CTTGGGGAAAAACGTGCCT
mmu-18S rRNA	FP	GCAATTATTCCCCATGAACG
	RP	GGCCTCACTAAACCATCCAA
mmu-miR-122	FP	AGCTGTGGAGTGTGACAATGGT
	RP	AATGGCGTTTGATGGTTTGGAC
mmu-5S	FP	TCTCGTCTGATCTCGGAAGC
	RP	AGCCTACAGCACCCGGTATT
hsa-NOCT	FP	TGTTCCCCGACTTGCCTGGG
	RP	TGTAACCAACATAGTAACTCGCGG
hsa-18S rRNA	FP	CGTTCAGCCACCCGAGATT
	RP	GACCCGCACTTACTGGGAATT
hsa-miR-122	FP	CTTAGCAGAGCTGTGGAGTGTG
	RP	TTGATAGTTTAGACACAAACAC
hsa-5S	FP	GGCCATACCACCCTGAACGC
	RP	CAGCACCCGGTATTCCCAGG

Western Blotting

For extracting protein lysate from HepG2 cells, cell pellets were washed twice with ice-cold PBS, lysed in 1X RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100) containing protease inhibitor cocktail (PIC) and 1 mM PMSF, and incubated on ice for 30 minutes. Cell lysates were then centrifuged at 10,000 rpm for 20 minutes at 4^{o}C, and the resulting supernatants were used for protein quantification using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, USA). Equal amounts of protein from each sample were denatured in 6X loading dye (12% SDS, 30% β-mercaptoethanol, 60% glycerol, 0.012% bromophenol blue and 0.375 M Tris-HCl pH 6.8) at 95-100^{o}C for 5 minutes. A total of 30µg of each protein sample was separated by SDS-PAGE and transferred onto PVDF membrane using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories,

USA). Complete transfer of proteins was confirmed using 0.05% Ponceau S staining. The membrane was destained in distilled water, followed by blocking with 5% milk in Tris-buffered saline (TBS) for 1 hour at room temperature (RT) and was subsequently incubated overnight with primary antibody prepared in 3% BSA. After 3 washes with TBS-T (TBS + 0.1% Tween 20), the membrane was incubated with HRP-linked anti-mouse secondary antibody (1:2000) for 1 h at RT. Protein expression was detected using Clarity™ Western ECL Substrate (Bio-Rad Laboratories, USA) following manufacturer's instructions and the chemiluminescence was captured on X-ray films. Membrane was subsequently stripped using stripping buffer, re-blocked, and re-probed overnight with primary β-actin antibody.

CircWave analysis

Rhythmicity analysis was performed using CircWave Batch v1.4 (designed by Dr. Roelof Hut, University of Groningen, Netherlands), that detects rhythmic patterns by fitting a sine wave model to time-course data. Normalized gene/protein expression values were arranged in a spreadsheet format, with rows representing individual genes/proteins and columns representing the time points. The data was prepared as a .csv file and each time point were entered as an independent variable and the gene/protein expression levels as dependent variables. The period was set at 24 hours. For each gene/protein, the significance of rhythmicity was evaluated by comparing models with and without additional harmonics using an F-test. A p-value threshold of 0.05 was used to determine statistical significance of the rhythm. CircWave calculated parameters such as CONST, SIN and COS that were used to plot the fitted sine/cosine function at 0.1 h interval till 24 h). The peak and trough values were extracted by half the difference (delta) by CONST. The value thus obtained represented percentage (%) relative amplitude. Further, peak time (i.e. time at which the gene/protein reached its peak expression; represented as "Centre of Gravity- CoG), waveform plots (rhythmic data presented as a combinatory

sine-cosine waveform) and cumulative expression (gene/protein expression over a period of 24 h) was extracted from the output file of CircWave.

Statistical analysis

Data were presented as mean ± SD. Comparisons between all groups and the control group were performed using one-way analysis of variance (ANOVA). The treatment groups were compared with their corresponding disease control groups using two-way ANOVA, followed by Tukey's multiple comparison test. All the statistical analyses were performed in GraphPad Prism 5.0 (CA, USA).