Material & Methods

Chemicals and Reagents

Chemicals for cell culture like Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), trypsin phosphate versene glucose (TPVG), bovine serum albumin (BSA) and antibiotic-antimycotic solution, ORO stain were purchased from Hi-media laboratories (Mumbai, India). TRIzol, SYBR select master mix, JC-1, anti-CLOCK (PA1-520) and anti-BMAL1(PA1-46118) antibodies were procured from Invitrogen (Thermo Fisher Scientific, USA). BioRad Protein Assay Dye reagent concentrate, Polyvinylidene fluoride (PVDF) membrane, Precision Plus protein ladder, iScript cDNA synthesis kit and Clarity Western ECL substrate were procured from Bio-Rad Laboratories (CA, USA). Antibodies against Nrf2 (12721S), HO-1 (70081S), β-actin (4970S) and anti-rabbit secondary antibody (7074P2) were purchased from Cell Signalling Technology (MA, USA). Antibody against Keap1 (ab139729) was purchased from Abcam (MA, USA). RNA Later stabilizing solution was purchased from Ambion Inc. (Thermo Fisher Scientific, USA). Melatonin, Haematoxylin, eosin, Oleic acid (OA) and Protease inhibitor cocktail (PIC) were purchased from Sigma Aldrich (MO, USA). Methanol, dimethyl sulphoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sisco research laboratory Pvt. Ltd. (Mumbai, India). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany).

Invitro Studies

Maintenance of HepG2 Cell Line

Human Hepatoma (HepG2) cells were procured from National Centre for Cell Science (NCCS, Pune, India). Cells were cultured in T25 flasks in DMEM growth media, supplemented with 10% FBS and 1% antibiotic antimycotic solution. The flasks were maintained in CO_2 incubator (Thermo scientific, forma series II 3110, USA) at 37°C temperature and 5% CO_2. Cells were passaged was at 80% confluency using 1X TPVG. Once the cells detached from the surface, they were collected in a 2mL centrifuge tube and spin down at 5000 rpm for 5 min. Cell pellet was resuspended in media and seeded into fresh T25 flask. For maintenance, media was changed regularly on every third day.

Treatment with Oleic acid conjugated with BSA and Melatonin

OA stock solution was prepared as described previously. 100 mM of OA was conjugated with 10% BSA to obtain 10 mM OA-conjugated BSA stock solution(Cousin et al., 2001). Further dilution was done in culture media to obtain the working concentration. Melatonin was dissolved in media to obtain a stock solution of 1 mM. Later, HepG2 cells were synchronized by serum shock (50% Fetal bovine serum) for 2 h and then treated with OA alone and in combination with melatonin for 24h(Balsalobre et al., 1998). Following treatment, cells were collected for further analysis.

Cytotoxicity Assessment

HepG2 cells were seeded in 96-well plate (10^4 cells/well) in DMEM for 24h. Later, cells were exposed to various doses of OA (0.5-2 mM) and/or melatonin (5-1000 µM). After 24h, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromideMTT (0.5 mg/ml) was added and cells were incubated for 4h. Resultant formazan crystals were solubilized in 100 µl DMSO solution and the absorbance was measured at 540 nm using multimode reader synergy HTX (Bio-Tek instruments, Inc., Winooski, VT).

Lipid Accumulation by Oil Red O staining HepG2 cells were treated with OA (0.5 mM) and/or Melatonin (100 µM) for 24 h, fixed with 4% paraformaldehyde and washed with PBS. Cells were stained with Oil red O and photographed using Floid cell imaging station (Life technologies, USA). To quantify Oil red O levels, 100% isopropanol was added in each well and measured at 510 nm using Synergy HTX Multi-Mode Microplate Reader (Bio-Tek instruments, Inc., Winooski, VT).

Intracellular Oxidative Stress by DCFDA Staining

HepG2 cells treated with OA (0.5 mM) and/or Melatonin (100 µM) for 24 h were stained with 10 µM 2, 7-dichlorodihydrofluoroscein diacetate (CM-H2-DCFDA) at 37 °C for 30 min. Cells were photographed (Floid cell imaging station; Life technologies, USA) and the intracellular fluorescence quantified using Image J software (NIH, Bethesda).

Mitochondrial membrane Potential by JC-1 staining

Cells were seeded in 6 wells plate and treated as mentioned earlier. Later, cells were washed with 1X PBS and incubated with JC-1 (5 µg/ml) in pre-warmed 1X PBS for 30 min at 37 °C. Cells were photographed using Floid cell imaging station and fluorescent intensity was quantified using ImageJ software**.**

RNA isolation and cDNA synthesis

Total RNA was isolated from HepG2 cells and liver tissue samples using Trizol reagent (Invitrogen, Thermo Scientific, USA) following manufacturer's protocol. Tissue was homogenized in Trizol reagent whereas, the cells were directly lysed using Trizol reagent in the culture dish. Lysis/homogenization was followed by phase separation

using chloroform. RNA was precipitated from aqueous phase using pre-chilled isopropanol, centrifuged to pellet that was subsequently washed once with pre-chilled 75% ethanol. The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water and the purity of same was assessed by measuring A260/A280 ratio in UV-Vis spectrometer (PerkinElmer, USA). Samples with A260/A280 within the range of 1.8-2.0 was used for gene expression studies. Samples were quantified using absorbance at 260 nm as follows:

Concentration (µg/ml) = A260 x 40 x dilution factor

where, 40 = extinction coefficient (40 µg/ml of RNA= 1 absorbance)

1 µg of RNA was used to synthesize cDNA by reverse transcription using iScript cDNA synthesis kit (Bio-Rad Laboratories, USA) according to manufacturer's protocol and cDNA was used as template for quantitative RT-PCR.

Quantitative RT-PCR analysis

Total RNA was isolated from HepG2 cells and liver tissue samples using TRIzol reagent and was reverse transcribed into cDNA using iScript cDNA Synthesis kit. The mRNA expression of various genes was quantified by qPCR analysis (QuantStudio 3, Life Technologies, CA, USA) using SYBR Select Master Mix. Gene-specific human and mice primers used for this study are listed in table 1. The data were normalized to GAPDH and analysed using $2^{-\Delta\Delta CT}$ method.

Target	**Primer**	**Sequence (5'→3')**
hClock	FP	CGAGCGCTCCCGAATTTTTA
	RP	AGGTATCTAGTGAGACTTGCC
hBmal1	FP	GGCTCATAGATGCAAAAACTGG

	RP	CTCCAGAACATAATCGAGATG
hPer2	FP	GACTCCTCGGCTTGAAACGG
	RP	GTGTCACCGCAGTTCAAACG
hCry2	FP	GTGCCTCAAATCCTGACCCA
	RP	GCCTCCCACAAGATTGACGA
hPPARα	FP	GCTTCGCAAACTTGGACCTG
	RP	GCTACCAGCATCCCGTCTTT
hSREBP1c	FP	GCGCTCAACGGCTTCAAAAAT
	RP	AAAGTGCAATCCATGGCTCC
hCPT-1	FP	ATCAATCGGACTCTGGAAACGG
	RP	TCAGGGAGTAGCGCATGGT
hNrf-2	FP	CTGCCAACTACTCCCAGGTT
	RP	TGACTGAAACGTAGCCGAAGA
hHO-1	FP	TCTTGGCTGGCTTCCTTACC
	RP	GGATGTGCTTTTCGTTGGGG
hSIRT1	FP	ACTCCAAGGCCACGGATAGG
	RP	TTCGAGGATCTGTGCCAATCA
hPGC1α	FP	AGTTCACTCTCAGTAAGGGGC
	RP	CCAGCTCCTGAATGACGCC
hGCLC	FP	AGATTAGGCTGTCCTGGGTT
	RP	AGATTAGGCTGTCCTGGGTT
hGCLM	FP	GCGAGGAGGAGTTTCCAGATG
	RP	CTGTGCAACTCCAAGGACTGA

hNQO1	FP	GTTTGGAGTCCCTGCCATTCT
	RP	GCAGAGAGTACATGGAGCCAC
hGAPDH	FP	GAGTCAACGGATTTGGTC
	RP	GACAAGCTTCCCGTTCTC

Table 2: List of primers for real time PCR of humans.

Target	**Primer**	**Sequence (5'→3')**
mClock	FP	CACTCTCACAGCCCCACTGTA
	RP	CCCCACAAGCTACAGGAGCAG
mBmal1	FP	ACATAGGACACCTCGCAGAA
	RP	AACCATCGACTTCGTAGCGT
mPer1	FP	CATGACTGCACTTCGGGAGC
	RP	CTTGACACAGGCCAGAGCGTA
mPer2	FP	GGCTTCACCATGCCTGTTGT
	RP	GGAGTTATTTCGGAGGCAAGTGT
mCry2	FP	TCGGCTCAACATTGAACGAA
	RP	GGGCCACTGGATAGTGCTCT
mPPARα	FP	TGCAAACTTGGACTTGAACG
	RP	TGATGTCACAGAACGGCTTC
mSREBP1c	FP	GCAGCCACCATCTAGCCTG
	RP	CAGCAGTGAGTCTGCCTTGAT
mCPT-1	FP	CGATCATCATGACTATGCGCTACT
	RP	GCCGTGCTCTGCAAACATC

mFAS	FP	GGAGGTGGTGATAGCCGGTAT
	RP	TGGGTAATCCATAGAGCCCAG
mCD36	FP	TGAATGGTTGAGACCCCGTG
	RP	TAGAACAGCTTGCTTGCCCA
mNrf-2	FP	CTGACGAAGTGACGCCATCTGTGAG
	RP	GCTCGACAATGTTCTCCAGCTT
mHO-1	FP	ACATCGACAGCCCCACCAAGTTCAA
	RP	CTGACGAAGTGACGCCATCTGTGAG
mSIRT1	FP	GATACCTTGGAGCAGGTTGC
	RP	CTCCACGAACAGCTTCACAA
mPGC1α	FP	AGTCTTCGGCTGTTTGGTGA
	RP	TGGAAGAACAGATGTGCCCC
mGCLC	FP	AACACAGACCCAACCCAGAG
	RP	CCGCATCTTCTGGAAATGTT
mGCLM	FP	GCCCGCTCGCCATCTCTC
	RP	GTTGAGCAGGTTCCCGGTCT
mNQO1	FP	CAGATCCTGGAAGGATGGAA
	RP	TCTGGTTGTCAGCTGGAATG
mSOD	FP	TTGGCCTGTGGAGTGATTGG
	RP	AGCCCAGTCAAAGGAGTCAC
mCYP2E1	FP	TTTCCCTAAGTATCCTC CGTGACT
	RP	GCTGGCCTTTGGTCTTTTTG
mGSS	FP	AACGAGCGAGTTGGGATG
	RP	TATGTCACCACGTCGGAGGA

mIL-6	FP	TGGATGCTACCAAACTGGAT
	RP	TGGATGCTACCAAACTGGAT
mTNFα	FP	GTGGAACTGGCAGAAGAG
	RP	AATGAGAAGAGGCTGAGAC
mNFκB	FP	GAGGTCTCTGGGGGTACCAT
	RP	AAGGCTGCCTGGATCACTTC
hGAPDH	FP	TGTGAACGGATTTGGCCGTA
	RP	ACTGTGCCGTTGAATTTGCC

Table 3: List of primers for real time PCR of mice.

Immunoblots

Autopsy of liver from control and experimental groups of mice were collected and stored in liquid nitrogen. For extracting total protein lysate, tissue was homogenized in RIPA buffer (50 mM tris (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% triton-X-100) containing protease inhibitor cocktail (Sigma Aldrich, USA) and 1mM PMSF, followed by incubation at 4°C for 2 h. The lysate was centrifuged at 10,000 rpm at 4°C for 20 min and the resultant supernatant was subjected to protein estimation using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, USA). Later, equal amount of protein (30 µg) was separated using 10% SDS gel electrophoresis and transferred onto PVDF membranes (Bio-Rad, USA) and primary antibodies against Clock (1:500), Bmal1 (1:1000), Nrf2 (1:500), HO-1 (1:1000) and Keap1 (1:1000) were added followed by HRP-linked anti-rabbit secondary antibody (1:5000). Blots were developed using Clarity western ECL reagent (Bio-Rad, CA,

USA) and X-ray films. Anti-β-actin antibody (1:5000) was used to determine equivalent loading.

Invivo Studies

Animal Ethical statements

C57BL/6J male mice (total=140, 6–8 weeks of age) were purchased from ACTREC Mumbai and maintained as per CPCSEA (The Committee for the Purpose of Control and Supervision of Experiments on Animals) standard guidelines (23 ± 2 °C, LD 12:12, laboratory chow and water ad libitum). Protocol was prior approved by the Institutional Animal Ethical Committee (IAEC; Approval no. MSU-Z/IAEC/04-2017) and experiments were conducted in CPCSEA approved animal house facility of Department of Zoology, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (827/GO/Re/S/04/ CPCSEA).

Experimental groups for animal studies Followed by a week-long acclimatization, animals were randomly divided into 7 groups (n=20 per group) viz. (i) control, (ii) HFHF (high fat + 20% Fructose diet), (iii) Jetlag (chow diet and JL - jetlag photoperiodic regimen) and (iv) HFHF+JL and maintained for 16 weeks. From 8 to 16 weeks, Groups ii, iii and iv were dosed intraperitoneally with melatonin (10 mg/kg at ZT=10) daily. JL was induced according to a published method wherein, mice were transferred from Room 1 (7:00h 19:00h light/19:00h to 7:00 h dark period) to Room 2 (11:00h to 23:00h dark/23:00h to 11:00h light period) resulting in phase advance of 8h (lights off at ZT4) and transferring back to Room 1 resulting in a phase delay of 8h (lights off at ZT20) (Fig. 1) on Mondays and Thursdays, respectively. Food intake and body weight were recorded every alternate day(Kettner et al., 2016). At the end of 16 weeks, mice were euthanized with mild isoflurane at different time points (ZT=0, 6, 12,

18, 24). Whole blood was centrifuged at 3000 rpm for 10 min at 4°C and serum was isolated and stored. Later, liver tissue samples were stored in 10% formalin (for histopathology), RNA Later (for mRNA studies) and -80°C (for immunoblot studies).

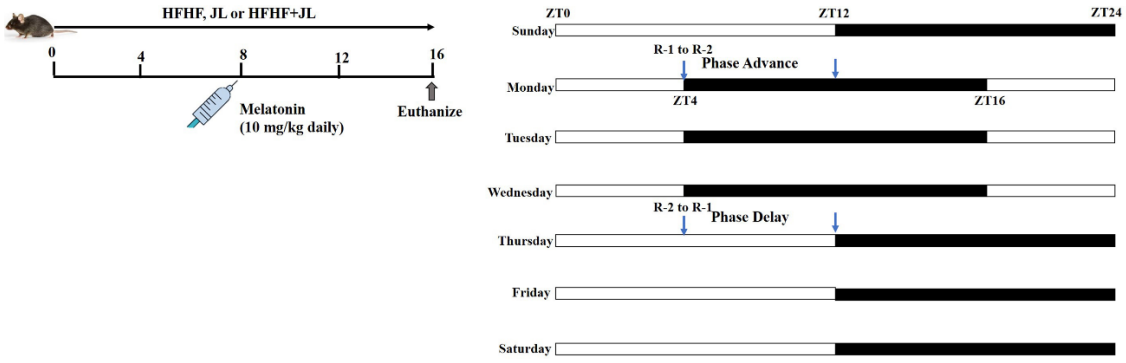

Figure 9: Melatonin treatment schedule and Jetlag protocol.

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
Soybean oil	32.3
Mineral Mix (a)	12.9
Vitamin Mix (b)	12.9
Choline Bitartrate	2.6

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

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

Serum biochemical parameters

Collected serum samples were used for detecting circulating titers of AST, ALT and ALP indicating liver function. Lipid profile, Total Lipid (TL), Total Cholesterol (TC), triglycerides (TG) quantification was done in serum samples using kits (Reckon Diagnostic kits, Vadodara, Gujarat, India). Low density lipoproteins (LDL), very low-density lipoprotein (VLDL) was determined applying Friedwald's formula. Further,

cholesterol to HDL ratio (CHL/HDL), low density lipoprotein to high density lipoprotein ratios (LDL/HDL) were determined from theoretical calculations.

Tissue samples, liver and adipose tissues were fixed in formalin (n=6). Tissue was further dehydrated and embedded in paraffin wax blocks and cut into 5µ thick sections cryotome. These sections were stained with haematoxylin and eosin (H&E). For H&E sections, the sections were deparaffinized and hydrated by passing decreasing concentration of alcohol on the slide and incubated with hematoxylin stain on the slide for about 5 minutes and washed under running water. Further differentiation was done in 1% acid and 70% alcohol for about 5 min which was followed by washing it under running water. Next, the tissue section was incubated with 1% eosin stain for 5 mins and subsequently washed under running water.

water. Further dehydration was carried out by increasing percentage of alcohol solution. Finally, the slide was mounted in mounting media. The slides were then observed and photographed under Leica DM 2500 microscope. Investigators blinded to this study conducted scoring of liver sections of control and treated mice. Adipose tissue section was observed, photographed and morphometric scoring was done for the same.

Scoring of liver

H&E staining was used to perform section evaluation. Semi-quantitative scoring system was used to access 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 (Gujral *et al.*, 2002). Evaluation of NASH was done using steatotis scoring of liver (0-5%, 1-5 to 33%, 2-34 to 66%, 3>66%) and ballooning hepatocytes

(0-none, 1- few ballooned, 2- many ballooned). The evaluation was done by two investigators blinded to the study (Liang et al., 2014).

Intra-peritoneal glucose tolerance test (IPGTT)

After 12 h of overnight fasting, A drop of whole blood was applied on test strip by amputation of tail tip. Blood glucose was measured using accu-chek active glucometer (Roche Diabetes care GmbH). Later, glucose solution was administered intraperitoneally (2 g/kg) body weight. Further glucose tolerance test was performed at 0, 30, 60, 90, and 120 min after administration of glucose load.

Statistical analysis

Data was expressed as mean ± SD. All the groups were compared with control group by one-way analysis of variance (ANOVA) and melatonin treated group was compared with its respective disease group using two-way ANOVA followed by Turkey's multiple comparison test using Graph Pad Prism 5.0 (CA, USA). Rhythmic variations in clock gene expression were analysed using Circwave software v1.4 (www.hutlab.nl). Amplitudes of curves were calculated as percentage of data mean [difference between the zenith (highest point) and nadir (lowest point) and divided by the data mean (max − min/ mean * 100%)].*P < 0.05, **P < 0.01 and ***P < 0.001 were considered to be significant.