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RESEARCH ARTICLE

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# Melatonin induces Nrf2-HO-1 reprogramming and corrections in hepatic core clock oscillations in Non-alcoholic fatty liver disease

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**Abstract**

Melatonin pleiotropically regulates physiological events and has a putative regulatory role in the circadian clock desynchrony-mediated Non-alcoholic fatty liver disease (NAFLD). In this study, we investigated perturbations in the hepatic circadian clock gene, and Nrf2-HO-1 oscillations in conditions of high-fat high fructose (HFHF) diet and/or jet lag (JL)-mediated NAFLD. Melatonin treatment (100 μM) to HepG2 cells led to an improvement in oscillatory pattern of clock genes (*Clock, Bmal1*, and *Per*) in oleic acid (OA)-induced circadian desynchrony, while *Cry, Nrf2*, and *HO-1* remain oblivious of melatonin treatment that was also validated by circwave analysis. C57BL/6J mice subjected to HFHF and/or JL, and treated with melatonin showed an improvement in the profile of lipid regulatory genes (*CPT-1, PPARa, and SREBP-1c*), liver function (AST and ALT) and histomorphology of fatty liver. A detailed scrutiny revealed that hepatic mRNA and protein profiles of Bmal1 (at ZT6) and Clock (at ZT12) underwent corrective changes in oscillations, but moderate corrections were recorded in other components of clock genes (Per1, Per2, and Cry2). Melatonin induced changes in oscillations of anti-oxidant genes (Nrf2, HO-1, and Keap1) subtly contributed in the overall improvement in NAFLD recorded herein. Taken together, melatonin induced reprograming of hepatic core clock and Nrf2-HO-1 genes leads to an improvement in HFHF/JL-induced NAFLD.

**KEYWORDS**
clock genes, melatonin, NAFLD, Nrf2-HO-1

## 1 | INTRODUCTION

Circadian rhythms are the internal biological clock that orchestrates various physiological events of metabolic processes in mammals.[1] The suprachiasmatic nucleus (SCN) is the central pacemaker of the biological clock, while peripheral clocks in various organs are operated by autoregulatory expression of clock genes.[2] The molecular network comprises of circadian locomotor output cycles kaput (Clock) and brain and muscle ARNT-like 1 (Bmal1) as activators,

**Abbreviations:** ARE, antioxidant response elements; Bmal1, brain and muscle ARNT-like 1; Clock, circadian locomotor output cycles kaput; Cry1-2, cryptochrome circadian regulator 1; HFHF, high fat high fructose; HO-1, heme oxygenase 1; JL, jet lag; Keap1, Kelch-like ECH-associated protein 1; NAFLD, non-alcoholic fatty Liver disease; Nrf2, nuclear factor erythroid 2-related factor 2; OA, oleic acid; Per1-2, period circadian protein homolog 1 and 2; SCN, suprachiasmatic nucleus; ZT, zeitgebers time.

and period circadian protein homolog 1 (Per1), Per2, cryptochrome circadian regulator 1 (Cry1), and Cry2 as repressors, which work in a transcriptional–translational feedback loop.[3] Like various other organs, the liver has its own circadian rhythm and clock gene expression patterns. The hepatic clock can be modulated by external clues, wherein light serves as an important synchroniser. However, food also acts as a crucial clue which reprograms the hepatic clock.[4,5] Clock genes expression is known to differ before and after feeding in tissues such as the hypothalamus, liver, and skeletal muscles.[6] High-fat diet leads to alterations in the expression and cycling of canonical circadian clock genes, nuclear receptors, and clock-controlled genes.[6-8] Palmitate, a saturated fatty acid at low doses inhibits the molecular clock activity and destabilizes protein–protein interaction between Bmal1 and Clock.[9-11]

Epidemiological studies have shown that shift workers with frequent changes in timings over a considerable span and transcontinental travellers exposed to photoperiodic changes amounting to jetlag have a high risk of metabolic disorders.[12,13] Clock gene mutant animals display impaired glucose and lipid metabolism and are susceptible to diet-induced obesity and metabolic dysfunction.[14] Also, there are reports on marked activation in the expression of Per1 and Per2 and suppression of clock expression in chronic jetlagged CBA/N mice.[15] Chronic jetlag has also been reported to aggravate steatohepatitis and even induce hepatocellular carcinoma in $Fxr^{-/-}$ mice.[16] These compelling evidences point toward a strong connection between the circadian clock and metabolic homeostasis.

NAFLD is associated with hepatic dysregulation of energy metabolism, lipid accumulation, oxidative stress and inflammation.[17] The pathophysiology of NAFLD is best explained by the multiple hit model, wherein oxidative stress plays a primary role in initiating hepatic damage.[18] The cellular response against oxidative stress is mainly regulated by the Kelch-like ECH-associated protein 1 (Keap1)—nuclear factor erythroid 2-related factor 2 (Nrf2) - antioxidant response elements (ARE) genes.[19] In conditions of NAFLD, Nrf2 has been shown to be downregulated which is accompanied by an increased oxidative stress.[20] Xu and co-workers had investigated the expression patterns for antioxidant genes in mice livers and found that Nrf2 expression exhibited circadian variations that altered cellular oxidative stress response.[21] Circadian clock-dependent regulation of redox status, ROS homeostasis and antioxidant defence has been investigated by various research groups wherein, evidence suggesting Bmal1 as a transcriptional regulator of Nrf2 has been showcased.[22-25]

Melatonin is secreted by the pineal gland, responsible for regulating the circadian rhythm. However, there is an increasing evidence showing its involvement in many other key physiological functions.[26-28] Melatonin is known to protect against obesity and hepatic steatosis by improving lipid dysmetabolism and attenuating inflammation in high-fat diet-fed mice.[29,30] Melatonin attenuates dysregulation of the circadian clock pathway in mice with $CCl_4$ induced liver fibrosis. One of the recent findings has shown the regulatory role of melatonin in lipid homeostasis and clock gene regulation in mice exposed to constant light.[31]

Although, a major body of evidence shows the correlation of circadian clock with NAFLD in clock gene ablation models, the role of clock genes and melatonin in metabolic rewiring under conditions of HFHF and/or JL models is a lacuna in the available scientific information. The role of melatonin in modulating circadian rhythm is well established, but its role in re-entrainment of the altered circadian cycle by HFHF and/or JL in NAFLD is not known. This study is the first to investigate in detail the shift in clock gene oscillations and Nrf2-HO-1 in HFHF and/or JL-induced NAFLD, wherein merits of exogenous melatonin in making corrective changes have been contemplated.

## 2 | MATERIAL AND METHODS

### 2.1 | 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 were purchased from Hi-media laboratories (Mumbai, India). TRIzol, SYBR select master mix, anti-CLOCK (PA1-520), and anti-BMAL1(PA1-46118) antibodies were procured from Invitrogen (Thermo Fisher Scientific, USA). 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, and oleic acid (OA) were purchased from Sigma Aldrich (MO, USA). Methanol, dimethyl sulphoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphen yl tetrazolium bromide (MTT) were purchased from Sisco research laboratory Pvt. Ltd. (Mumbai, India).

### 2.2 | Maintenance of HepG2 cells and their treatment

Human Hepatoma (HepG2) cells were procured from National Centre for Cell Science (NCCS, Pune, India). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% antibacterial-antimycotic solution at 37°C in a humidified atmosphere with 5% $CO_2$. Passaging was done using 1X TPVG at about 80% confluency.

### 2.3 | Treatment with oleic acid conjugated with BSA and/or melatonin

OA stock solution was prepared as described previously.[32] About 100 mM of OA was conjugated with 10% BSA to obtain 10 mM OA-conjugated BSA stock solution. Further dilution was done in culture media to obtain a working solution. 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 hours and then treated with OA alone and in combination with Melatonin for 24 hours. Following treatment, cells were collected for further analysis.

### 2.4 | Cytotoxicity analysis

HepG2 cells were seeded in a 96-well plate ($10^4$ cells/well) in DMEM and exposed to various doses of OA (0.5-2 mM) and/or melatonin (5-1000 µM). After 24 hours, MTT (0.5 mg/mL) was added, and cells were incubated for 4 hours. Resultant formazan crystals were solubilized in 100 µL DMSO solution and absorbance was measured at 540 nm using multimode reader synergy HTX (Bio-Tek instruments, Inc, Winooski, VT).

### 2.5 | Intracellular lipid accumulation

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

### 2.6 | Intracellular oxidative stress

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

### 2.7 | Mitochondrial membrane potential

Cells were seeded in a six-well 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 minutes at 37°C. Cells were photographed using Floid cell imaging station, and fluorescent intensity was quantified using ImageJ software.

### 2.8 | Animal studies

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

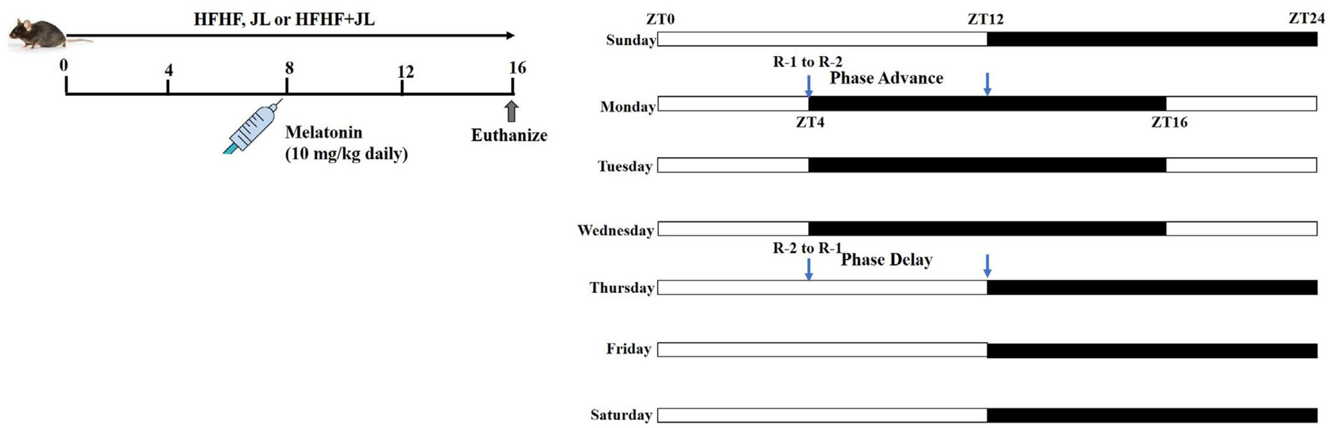


**FIGURE 1** Schematic representation of photoperiodic regime. C57BL/6J mice of Control and HFHF groups were maintained with L:D 12:12 regime; JL were subjected to 8 hours phase advance on Monday and 8 hours phase delay on Thursday for 16 weeks; HFHF + JL were fed with high fat high fructose (HFHF) diet and maintained on JL photoperiodic regimen

libitum). The protocol was prior approved by 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).

## 2.9 | Experimental groups for animal studies

Followed by a week-long acclimatization, animals were randomly divided into seven 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. A dose of 10mg/kg body weight was preferred as it is widely used in rodent models.[33] JL was induced according to a published method[16] wherein, mice were transferred from Room 1 (7:00 to 19:00 hours light/19:00 to 7:00 hours dark period) to Room 2 (11:00 to 23:00 hours dark/23:00 to 11:00 hours light period) resulting in a phase advance of 8 hours (lights off at ZT4) and transferring back to Room 1 resulting in a phase delay of 8 hours (lights off at ZT20) (Figure 1) on Mondays and Thursdays, respectively. Food intake and body weight were recorded every alternate day (Figure S6). 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 minutes at 4°C, and serum was isolated and stored. Later, liver tissue samples were snap-frozen (ORO staining), stored in 10% formalin (for histopathology), RNA Later (for mRNA studies) and −80°C (for immunoblot studies).

## 2.10 | Serum biochemical analysis

Levels of circulating enzymes indicative of liver function (AST and ALT) and serum lipid profile (TL, TC, TG, LDL, VLDL, CHL/HDL, and LDL/HDL ratio) were estimated using commercially available kits (Reckon Diagnostic kits, Vadodara, Gujarat, India).

## 2.11 | Histopathological analysis

Formalin-fixed liver samples were dehydrated and embedded in paraffin wax blocks, cut into 5 μm thick sections, stained with hematoxylin and eosin (H&E). Observations were recorded and photographed (Leica DM 750 microscope). Investigators blinded to this study conducted scoring of ballooning hepatocytes sections of control and treated mice.[34]

**TABLE 1** Primer sequences for quantitative PCR

| Gene Name | Forward Primer (5′→3′) | Reverse Primer (5′→3′) |
|---|---|---|
| *hClock* | CGAGCGCTCCCGAATTTTA | AGGTATCTAGTGAGACTTGCCA |
| *hBmal1* | GGCTCATAGATGCAAAAACTGG | CTCCAGAACATAATCGAGATGG |
| *hPer2* | GACTCCTCGGCTTGAAACGG | GTGTCACCGCAGTTCAAACG |
| *hCry2* | GTGCCTCAAATCCTGACCCA | GCCTCCCACAAGATTGACGA |
| *hPPARα* | GCTTCGCAAACTTGGACCTG | GCTACCAGCATCCCGTCTTT |
| *hSREBP1c* | GCGCTCAACGGCTTCAAAAAT | AAAGTGCAATCCATGGCTCC |
| *hCPT-1* | ATCAATCGGACTCTGGAAACGG | TCAGGGAGTAGCGCATGGT |
| *hNrf-2* | CTGCCAACTACTCCCAGGTT | TGACTGAAACGTAGCCGAAGA |
| *hHO-1* | TCTTGGCTGGCTTCCTTACC | GGATGTGCTTTTCGTTGGGG |
| *hGAPDH* | GAGTCAACGGATTTGGTC | GACAAGCTTCCCGTTCTC |
| *mClock* | CACTCTCACAGCCCCACTGTA | CCCCACAAGCTACAGGAGCAG |
| *mBmal1* | ACATAGGACACCTCGCAGAA | AACCATCGACTTCGTAGCGT |
| *mPer1* | CATGACTGCACTTCGGGAGC | CTTGACACAGGCCAGAGCGTA |
| *mPer2* | GGCTTCACCATGCCTGTTGT | GGAGTTATTTCGGAGGCAAGTGT |
| *mCry2* | TCGGCTCAACATTGAACGAA | GGGCCACTGGATAGTGCTCT |
| *mPPARα* | TGCAAACTTGGACTTGAACG | TGATGTCACAGAACGGCTTC |
| *mSREBP1cmc* | GCAGCCACCATCTAGCCTG | CAGCAGTGAGTCTGCCTTGAT |
| *mCPT-1* | CGATCATCATGACTATGCGCTACT | GCCGTGCTCTGCAAACATC |
| *mGAPDH* | TGTGAACGGATTTGGCCGTA | ACTGTGCCGTTGAATTTGCC |

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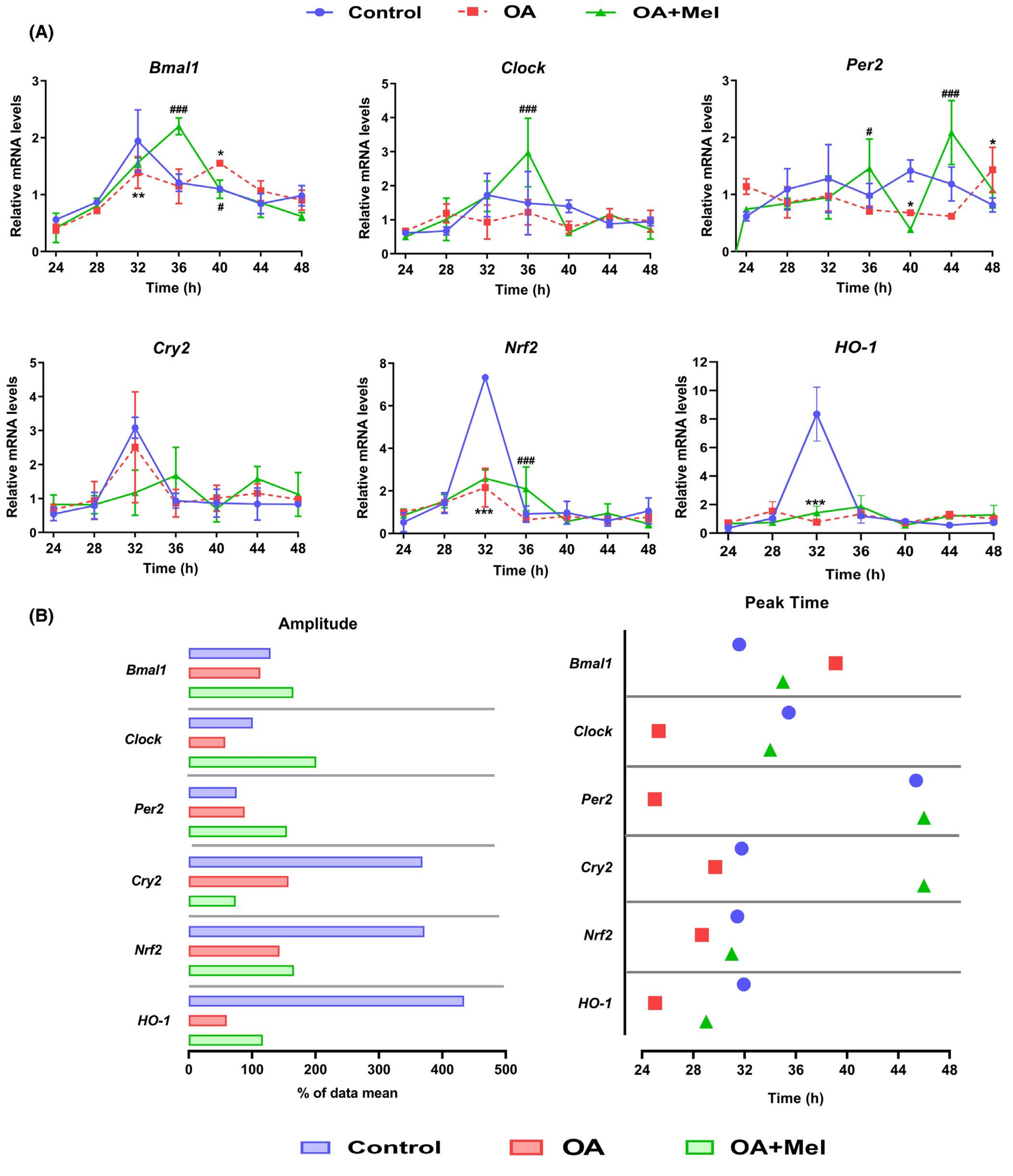


**FIGURE 2** Melatonin modulates oscillation of clock genes in OA-induced circadian desynchrony. A, After 2 hours of serum shock, HepG2 cells were treated with OA and/or melatonin for 24 hours. Cells were collected for mRNA analysis at an interval for 4 hours between 24 to 48 hours. Transcription levels were measured by RT-PCR and normalized to GAPDH. Data represented as mean ± SD. $^{*}P < .05$, $^{***}P < .001$ vs control, $^{\#}P < .05$, $^{\#\#\#}P < .001$ vs OA group. n = 3. B, Circwave analysis shows correction in amplitude and peak time in HepG2 cells treated with OA + Mel

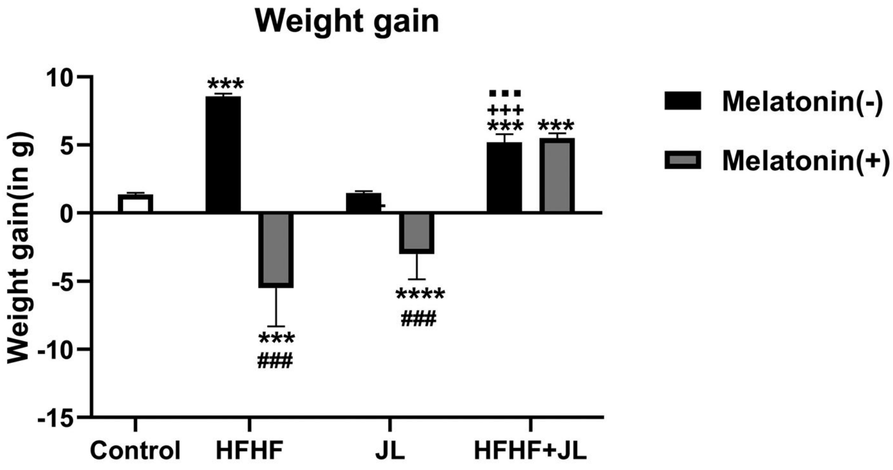


**FIGURE 3** Melatonin treatment favorably reduces body weight of HFHF and/or JL treated mice at the end of 16 weeks. Data represented as mean ± SD ***$P < .001$ vs control, ###$P < .001$ vs HFHF, JL and HFHF + JL, +++$P < .001$ vs HFHF and …$P < .001$

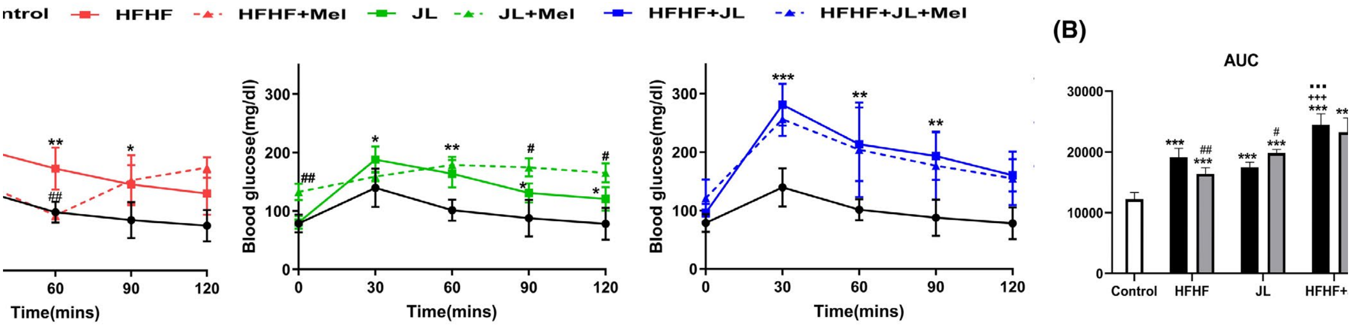


**FIGURE 4** Exogenous melatonin treatment does not improve blood glucose levels in HFHF and/or JL exposed C57BL/6J mice. A, Blood glucose levels at various time intervals B, area under curve (AUC). Data represented as mean ± SD. *$P < .05$, **$P < .01$, ***$P < .001$ vs control, #$P < .05$, ##$P < .01$, ###$P < .001$ vs HFHF, JL and HFHF + JL, +++$P < .001$ vs HFHF and …$P < .001$. n = 6

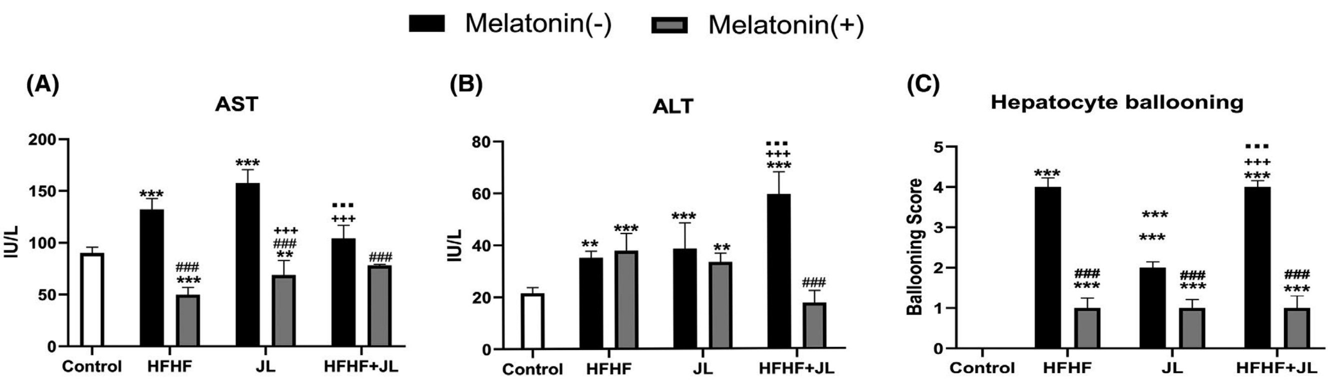


**FIGURE 5** Melatonin treatment improves Liver function and histopathology in HFHF and/or JL exposed mice. Graph represent (A) AST and (B) ALT levels in serum and (C) Scoring of fatty manifestation in liver Data represented as mean ± SD. *$P < .05$, **$P < .01$, ***$P < .001$ vs control, #$P < .05$, ##$P < .01$, ###$P < .001$ vs HFHF, JL and HFHF + JL, +++$P < .001$ vs HFHF and …$P < .001$. n = 6

## 2.12 | Oil Red O staining analysis

Four-micrometer-thick frozen liver sections were washed in deionized water for three times followed by blocking with oil red O solution for 10 minutes at room temperature, then washed with deionized water and stained with hematoxylin. In the end, tissue sections were mounted in glycerin gelatin, and analyzed by light microscopy (Leica DM 750 microscope).

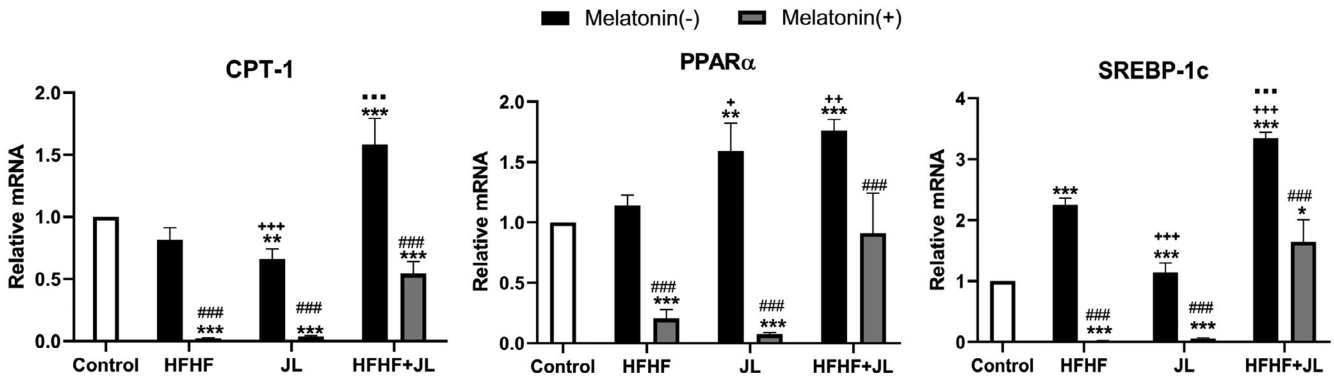


**FIGURE 6** Protective effect of melatonin on genes governing lipid metabolism in HFHF and/or JL exposed mice liver. Graphs represents mRNA expression of lipid metabolism genes as analyzed by RT-qPCR. Data represented as mean ± SD. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs control, $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ vs HFHF,JL and HFHF + JL, $^{+}P < .05$, $^{+++}P < .001$ vs HFHF and …$P < .001$

## 2.13 | Glucose metabolism analysis

At the end of the 15th week, mice were fasted for 6 hours and an intraperitoneal (IP) glucose tolerance test (IGTT) was performed. An IP glucose solution (2 g glucose/kg body weight) was administered, followed by blood glucose monitoring at 30, 60, 90, and 120 minutes using Accucheck active glucometer.

## 2.14 | mRNA studies by RT-qPCR

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 analyzed using the $2^{-\Delta\Delta CT}$ method.

## 2.15 | Immunoblot analysis

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 hours. The lysate was centrifuged at 10 000 rpm at 4°C for 20 minutes, and the resultant supernatant was subjected to protein estimation using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, USA). Later, an 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.

## 2.16 | Statistical analysis

Data were expressed as mean ± SD. All the groups were compared with the 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 analyzed using Circwave software v1.4 (www.hutlab.nl). Amplitudes of curves were calculated as the percentage of data mean [difference between the zenith (highest point) and nadir (lowest point) and divided by the data mean (max − min/mean * 100%)].$^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ were considered to be significant.

# 3 | RESULTS

## 3.1 | Melatonin improves OA-induced fat accumulation in HepG2 cells

HepG2 cells were treated with OA (0.5 mM) alone or in combination with Melatonin (100 µM). Decrement in cytotoxicity, fat accumulation, oxidative stress and an improved mitochondrial membrane potential were inferred via MTT assay, ORO, DCFDA, and JC1 stainings, respectively

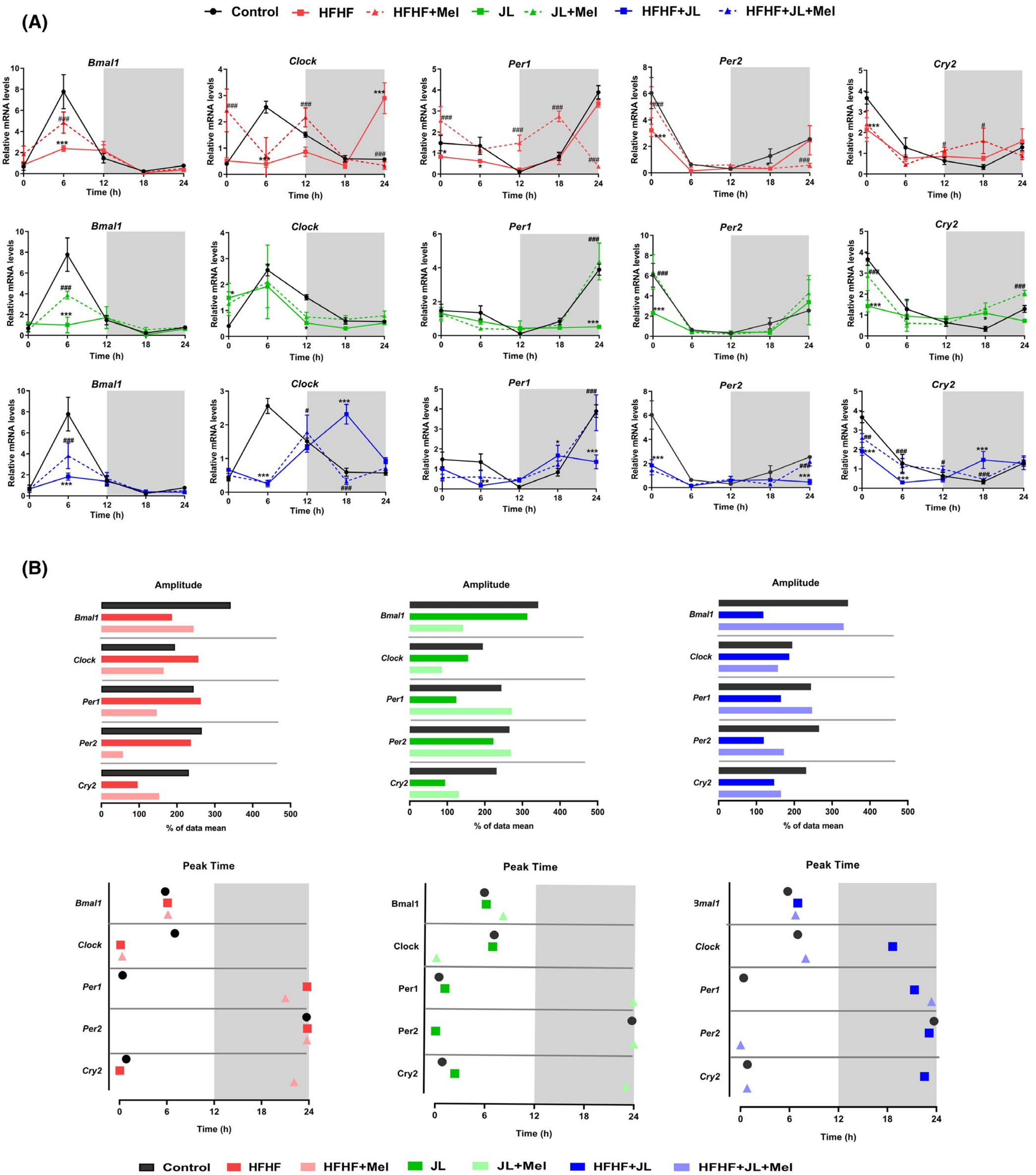


**FIGURE 7** Melatonin reprograms the circadian clock gene in HFHF and/or JL mice liver as evidenced by their mRNA profiles. A, Grey shaded area indicates dark phase (ZT12 to ZT24). Data represented as mean ± SD. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs control, $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ vs HFHF, JL and HFHF + JL. n = 4 for each time point. B, Circwave analysis of clock genes in liver of HFHF and/or JL treated mice shows an improvement following melatonin treatment

(Figures S1-S4). Also, Melatonin treatment accounted for significant ($P < .001$) increment in mRNA levels of lipolytic genes (*CPT-1 and PPARα*) and decrement in lipogenic genes (*SREBP-1c*) ($P < .001$) (Figure S5). These findings are in agreement with reports of other research groups[35] and hence forms the basis of our study.

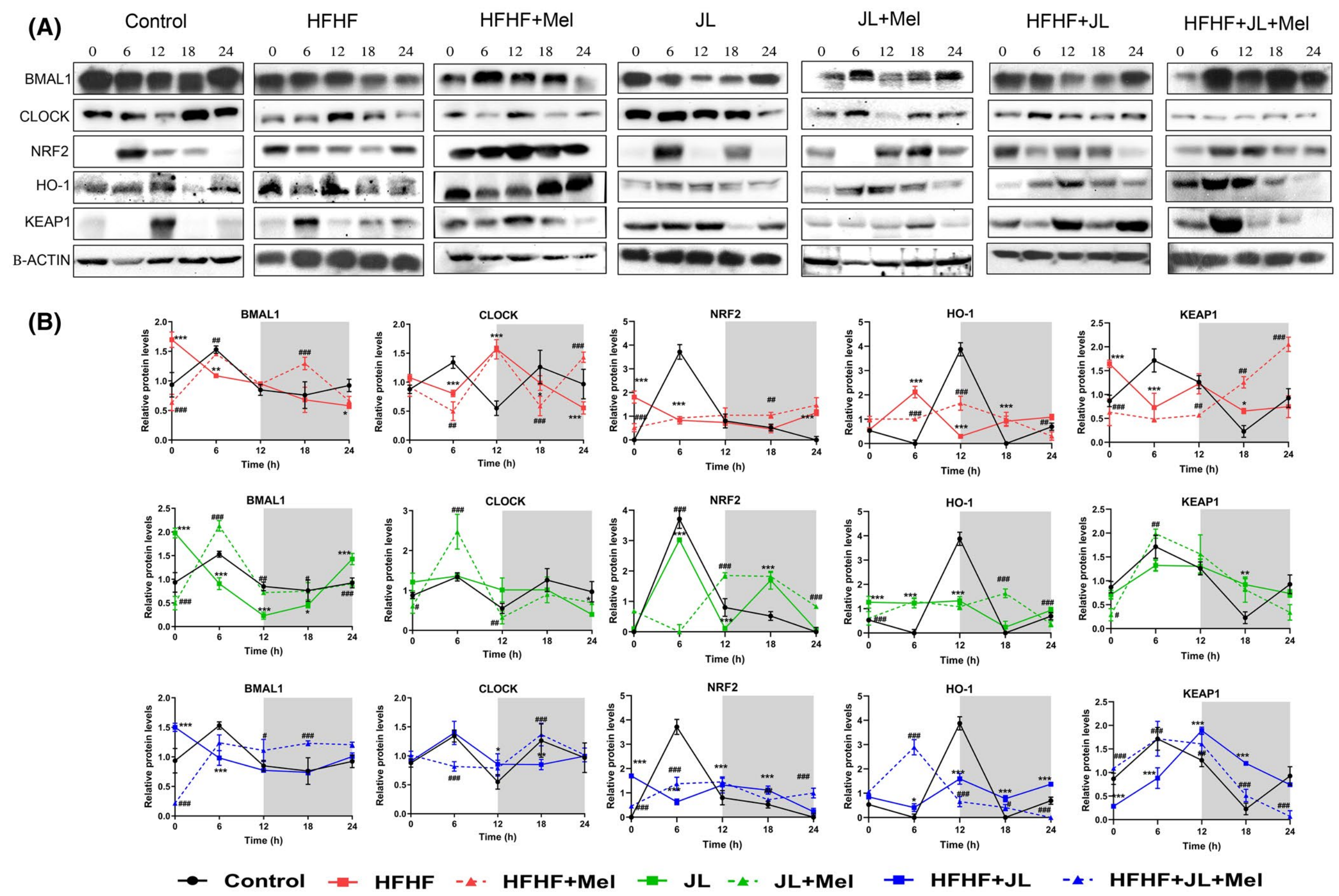


**FIGURE 8** A, Melatonin modulates protein expression pattern of Clock-Bmal1 and NRF2-ARE pathway genes in HFHF and/or JL exposed mice liver. B, Densitometric analysis of western blot. Blots were normalized by β-actin as endogenous control. Grey shaded area indicates the dark phase (ZT12 to ZT24). Data represented as mean ± SD. **P* < .05, ***P* < .01, ****P* < .001 vs control, $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ vs HFHF, JL and HFHF + JL

## 3.2 | Melatonin synchronizes core clock genes oscillations and antioxidant genes in OA-treated HepG2 cells

To study the potential of melatonin in modifying the circadian clock following OA treatment to HepG2 cells, mRNA levels were studied at 4 hours intervals (24, 28, 32, 36, 40, 44, and 48 hours). Synchronized HepG2 cells showed a robust oscillatory pattern of core clock genes (*Clock, Bmal1, Per2, Cry2*) and antioxidant pathway genes (*Nrf2 and HO-1*). After serum shock, cells were treated with OA (0.5 mM) for 24 hours. The presence of OA dampened the oscillation of *Clock, Bmal1, Per2, Nrf2, and HO-1,* while oscillation of *Cry2* was unaltered. Intriguingly, melatonin treatment significantly improved oscillation of *Bmal1, Clock* and *Per2* (*P* < .001). However, expression levels of *Cry2, Nrf2, HO-1* were not adequately corrected (Figure 2A). Circwave analysis also showed a strong positive shift in the amplitude of *Bmal1, Clock,* and *Per2* following melatonin treatment. But the amplitude of *Cry2, Nrf2, and HO-1* were not restored (Figure 2B).

## 3.3 | Melatonin improves patho-physiological changes in NAFLD

C57BL/6J mice were subjected to HFHF, JL, or a combination of both. A significant increase in body weight was noted in HFHF and HFHF + JL group (*P* < .001), while JL showed no change as compared to the control. Furthermore, melatonin treatment accounted for the decrement in body weight of the HFHF and JL group (Figure 3). HFHF/JL alone or in combination accounted for a significant increment in blood glucose and AUC (Area under curve) (*P* < .001). Melatonin treatment to these three experimental groups did not account for a decrement in AUC as the values were significantly higher than the control group (Figure 4). The markers of liver function (AST and ALT) were significantly elevated in HFHF, JL and HFHF + JL groups (*P* < .001). Melatonin treatment accounted for significant decrement (*P* < .001) in serum AST levels in these experimental groups, whereas decrement in ALT levels was recorded only in HFHF and JL groups (Figure 5A,B). Microscopic evaluations of liver tissue showed hepatocyte ballooning, cellular derangement, and Mallory hyaline in HFHF, JL and HFHF + JL groups, with more prominent changes seen

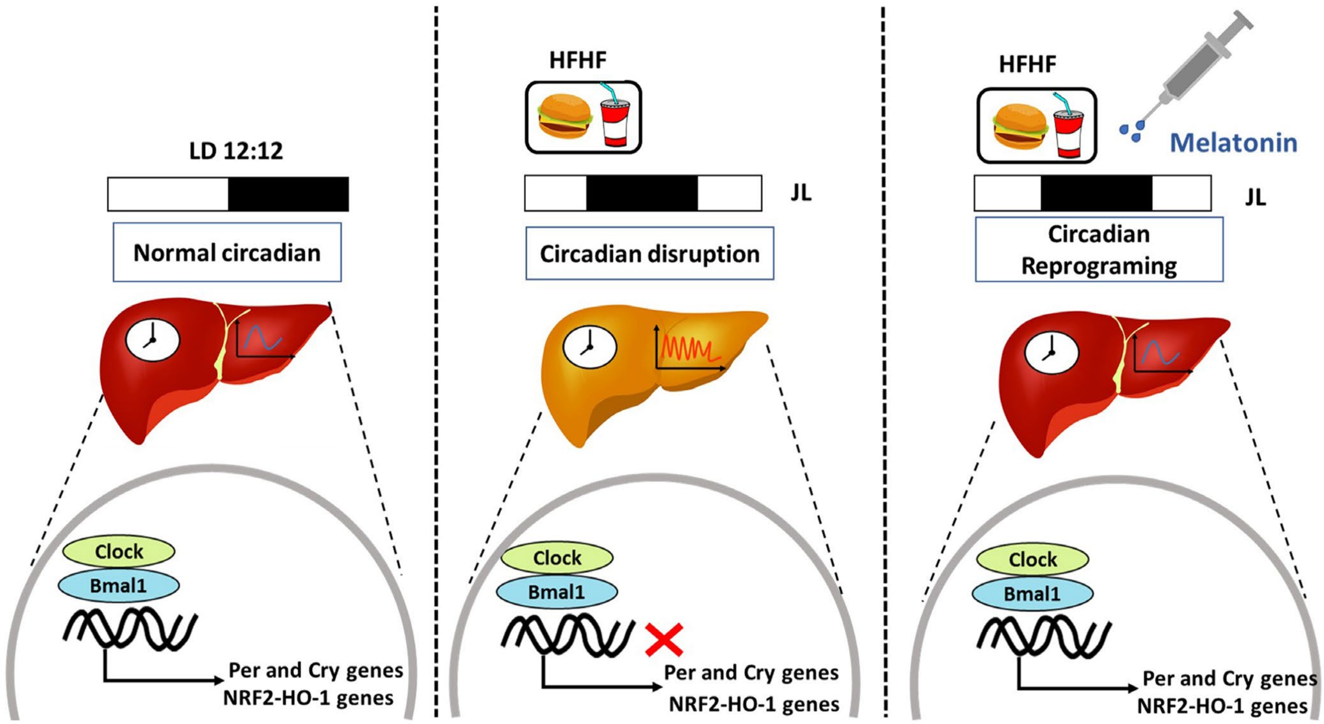


**FIGURE 9** Schematic representation illustrating the modulatory effect of melatonin in circadian desynchrony induced by HFHF and/or JL leading to improvement in pathophysiological condition of NAFLD

in the HFHF + JL group (Figure S7). Random scoring of the liver tissue sections revealed that HFHF + JL group accounted for maximum indices of fatty manifestations in the liver. Melatonin treatment accounted for a significant decrement in indices of hepatic manifestations and intracellular fat accumulation with reparative changes even in the HFHF + JL + Mel group (Figure 5C). HFHF and HFHF + JL groups recorded significant increment in fatty changes in hepatocytes as evidenced by ORO staining, but melatonin treatment showed a beneficial effect in decreasing the lipid content in the liver (Figure S7).

## 3.4 | Melatonin makes corrective changes in the mRNA profile of lipid regulatory genes

Experimental groups viz. HFHF, JL, and HFHF + JL showed alterations in lipid profile wherein TG, TC, LDL, and VLDL were found to be significantly elevated ($P < .01$) and HDL significantly lowered ($P < .001$). Melatonin treatment to these experimental groups accounted for a significant ($P < .01$) improvement in TG and TC but LDL, VLDL, and HDL did not record the said favorable changes. However, relative significant decrement ($P < .001$) in LDL and VLDL and relative improvement in HDL were observed following melatonin treatment (Figure S8). Further, mRNA levels of *CPT-1, PPARα, and SREBP-1c* were significantly elevated in HFHF, JL and HFHF + JL groups ($P < .01$). Melatonin treatment accounted for a significant decrement ($P < .001$) in mRNA of the said genes (Figure 6).

## 3.5 | Melatonin resynchronises hepatic clock gene expression pattern desynchronized by HFHF and/or JL mice

A time point study (ZT = 0, 6, 12, 18, 24 hours) was conducted to assess possible alterations in core clock genes (*Clock, Bmal1, Per1, Per2, and Cry2*) in the liver of control and experimental groups. Both *Bmal1* and *Clock* mRNA peaked at ZT6 in the liver of control mice. HFHF feeding resulted in a flattened peak of *Bmal1* and *Clock* mRNA. Melatonin treatment accounted for the improvement in *Bmal1* oscillation at ZT6, whereas the clock showed a peak shift from ZT6 to ZT12. The oscillations of *Per1, Per2,* and *Cry2* did not show significant variations in HFHF-fed mice. However, *Per1 and Cry2* oscillations were elevated at ZT18 in melatonin-supplemented HFHF-fed group (Figure 7A). A positive shift in amplitude observed in circwave analysis further justified restoration of *Bmal1* oscillation by melatonin treatment (Figure 7B). The similarity in oscillations of *Bmal1* and *Clock* genes in the liver of HFHF- and JL-treated mice is a key observation. Oscillations of *Per1, Per2,* and *Cry2* appeared to be dampened as evidenced by nearly flattened curve (Figure 7A). Exogenous melatonin appears to restore the oscillations of said genes as evidenced by the circwave analysis (Figure 7B). Additionally, HFHF + JL group noted completely arrhythmic expression in mRNA of Bmal1, *Per1, Per2,* and *Cry2*, whereas *Clock* mRNA showed phase shift from ZT6 to ZT18. Melatonin treatment preserved the diurnal variation in expression of *Bmal1, Per2* and *Cry2*, but *Per1* remained asynchronous. However, melatonin treatment resulted in a moderate restoration of the peak in *Clock* mRNA at ZT12 (Figure 7A). Thus, HFHF reported change only in the positive arm of the transcriptional–translational feedback loop, whereas JL and HFHF + JL resulted in more prominent disruption of the circadian clock. Overall, melatonin supplementation alleviated the expression of core clock gene transcripts in HFHF, JL and HFHF + JL groups with the results being most prominent in *Bmal1, Clock, Per1,* and *Cry2*.

### 3.6 | Immunoblots show melatonin-induced circadian reprogramming in Clock-Bmal1 and NRF2-HO-1 of HFHF and/or JL mice

Rhythmic oscillations of Bmal1 and Clock protein in the liver of control mice showed a peak at ZT6 and ZT18, respectively. HFHF diet feeding resulted in flattening of the peak of Bmal1 and a shift was seen at ZT12 in Clock proteins. Melatonin treatment to HFHF-fed mice resulted in the restoration of ZT6 peak of Bmal1 whereas the peak of ZT12 of Clock remained unchanged. Furthermore, Nrf2 and Keap1 proteins showed a peak at ZT6 and HO-1 peaked at ZT12 in the liver of healthy mice. Feeding of HFHF diet caused flattening of ZT6 peak of Nrf2 and Keap1. Also, the peak of HO-1 shifted from ZT12 to ZT6 in this group. Melatonin treatment did not result in corrections of the oscillations of Nrf2, HO-1, and Keap1 proteins wherein Nrf2 and HO-1 showed further flattening of the peaks whereas Keap1 underwent a shift from ZT6 to ZT24. JL induced significant distortion in the oscillation of clock genes (Bmal1 and Clock) and HO-1 whereas, Nrf2 and Keap1 oscillations did not undergo significant changes. Melatonin treatment restored the oscillations of Bmal1 and Clock proteins at ZT6, but HO-1 oscillations were not restored. Oscillations of Nrf2 and Keap1 proteins were oblivious to the treatment schedule. A combination of HFHF + JL caused a lack of oscillations in Bmal1 as evidenced by the flattened curve. Also, Clock oscillation at ZT6 were unchanged but ZT18 was lacking. Furthermore, Nrf2 and HO-1 proteins witnessed the flattening of the curve in the HFHF + JL group, whereas Keap1 recorded a shift from ZT6 to ZT12. Melatonin treatment could restore the ZT6 oscillation of Bmal1 and Keap1 protein and were comparable to control. Oscillations of the Clock at ZT18 was restored following melatonin treatment, but ZT6 was flattened. HO-1 noted a change in peak from ZT12 to ZT6, but Nrf2 was oblivious to melatonin treatment (Figure 8A,B).

## 4 | DISCUSSION

The circadian clock regulates an array of pathophysiological processes in the liver, wherein epidemiological studies have highlighted implications of circadian misalignment caused by chronic jetlag in metabolic disorders including NAFLD. In our study, OA induced circadian misalignment and its subsequent impact on antioxidant regulatory genes (Nrf2, HO-1, Keap1) has thrown light on the importance of core clock genes in NAFLD. In diseases like pulmonary fibrosis and diabetes, circadian control of Nrf2 is well established,[23,25] but these studies lack clarity on the oscillatory pattern of said genes. Findings of the present study reveal that OA treatment to HepG2 cells leads to circadian misalignment in Nrf2 and HO-1, while exogenous administration of melatonin moderately re-entrain circadian oscillations of Nrf2 and HO-1 genes. The therapeutic role of melatonin in re-entrainment of disturbed circadian rhythm following constant light exposure has been reported.[31] Our previous study had reported perturbations in NRF2-ARE pathway genes in HFHF-induced NAFLD. Herein, our hypothesis was further validated in chronic HFHF and/or JL-treated C57BL/6J mice that showed significant variations in oscillatory patterns of Nrf2, HO-1, and Keap1 (on a 24 hours scale) with HFHF + JL group showing maximal variations. Protein expression of Nrf2, HO-1, and Keap1 in control mice are in agreement with these published findings.[21] But variations recorded in HFHF and JL groups provide an insight into the dynamic state of the hepatic antioxidant defense system under conditions of NAFLD. Nuclear transfer of Nrf2 following administration of a test therapeutant[20] or melatonin[36] is crucial for Nrf2 activation. Hence, the corrective changes obtained in the oscillatory patterns of Nrf2 and associated genes observed herein, is attributable to the said mechanism. The re-entrainment of disturbed rhythms of antioxidant genes in the HFHF + JL group provides testimony to the efficacy of therapeutic potential of melatonin.

SCN in the hypothalamus of mammals synchronizes subsidiary peripheral clocks in the body[37] but the liver contains its own clock[38] that regulates fatty acid, glucose and Xenobiotic metabolisms. Both food restriction and high-calorie diet are known to entrain hepatic clocks; a physiological response that is independent of SCN.[39] In our study, the HFHF mediated poor circadian oscillation of hepatic core clock genes in the liver are in agreement with other studies.[8,11] But the same was not observed in negative regulators (Per and Cry) suggesting that the core feedback loop was markedly reduced by HFHF feeding. A variety of photoperiodic regime have been experimented to induce chronodisruption, that amounts to the phase advance-phase delay (lights off at ZT4 and lights on at ZT20, respectively). The Jetlag (JL) photoperiodic schedule used herein has been reported to cause a subdued circadian oscillation of clock genes, making $Fxr^{-/-}$ mice prone to

NAFLD and further leading to hepatocellular carcinoma.[16] In our study, melatonin mediated corrective changes in the oscillatory pattern of clock genes in mice subjected to JL has been reported. The findings of the other research groups and the data showcased herein establish the potency of HFHF or JL in manifesting desynchrony of clock genes, that is also a key cause in the epidemiology of NAFLD. Such a combination of HFHF and JL has never been studied. Based on our findings in the HFHF + JL group we hypothesize that a high-calorie diet in combination with chronodisruption has a synergistic effect on core clock regulators and antioxidant-related genes.

Melatonin is extensively reported for its multifaceted physiological role and also in improving hepatic pathophysiology in liver disease including NAFLD.[40] Lowered hepatic fat accumulation and corrections in physiological perturbations of fatty acid synthesis and transportation have been attributed to melatonin-mediated improvement of NAFLD. In our study, observations on melatonin mediated decreased OA uptake in HepG2 cells, lowered intracellular oxidative stress and improved mitochondrial membrane potential are in agreement with other research groups[35,41] and hence forms the basis of our study. Evening injections of melatonin have been associated with a high degree of physiological relevance in mice[42] and hence, the same was used in our study that resulted in improved levels of AST and ALT, key lipid metabolism genes (CPT-1, PPARα, SREBP-1c) and microscopic evaluations (H&E and ORO stainings) that comprehend the improved status of NAFLD in HFHF and/or JL mice. The perturbations in Nrf2-HO-1 genes in HFHF and/or JL has never been reported and our results throw light on the same. Although melatonin treatment in HFHF, JL, or HFHF + JL does not appear to accurately restore the said oscillations, the recorded observations appear to be adequate in improving the functional status of the fatty liver.

Primary hepatocytes are the "gold standard" for studying hepatic cellular metabolism because of their obvious relevance to an in vivo situation albeit, a short life span is a cause of concern. Hence, transformed cell lines derived from hepatocellular carcinoma are used as an alternative wherein; HepG2 cells are popular due to their easy availability, less variation resulting due to handling and a longer life span. But, investigation of the HepG2 proteome had revealed discrepancies related to their gluconeogenic pathway and a greater reliance on non-oxidative glucose metabolism compared with primary human hepatocytes. Alterations in the metabolic phenotype of HepG2 cells are its limitation that can be attributed to its origin from a tumor tissue.[43,44] A comparative study on the oscillatory pattern of circadian clock genes in HepG2 and mouse liver cells had shown similar periodicity but variations in their dynamics.[45] Hence, in our study, the HepG2 cells were used to generate a prima facie evidence on the merits of melatonin in correcting OA-induced circadian desynchrony whereas; the crux of the findings was based on results obtained in liver tissue of C57BL/6J mouse treated with HFHF and/or JL. Taken together, this study unravels the relevance of clock gene oscillations and Nrf2-HO-1 in the liver that culminate in NAFLD (Figure 9). The HFHF-JL synergy symbolizes the actual scenario of a lifestyle disorder and exogenous melatonin mediated corrective changes in the oscillatory pattern of core clock genes and associated genes strongly implies toward the use of melatonin as a therapeutant in lifestyle disorders including NAFLD.

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## CONFLICT OF INTEREST

Authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

A. Joshi carried out the experiment, analyzed the data, and wrote the manuscript. K.K. Upadhyay contributed in study design and manuscript editing. A. Vohra contributed in animal experimentation and histological analysis. K. Shirsath contributed in experimentation and editing the manuscript. R. Devkar analyzed the data, contributed, and edited manuscript. All the authors revised and approved the manuscript.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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