Research Article
ISSN (Print): 0971-913X
ISSN (Online): 2455-1244
J Endocrinol Reprod. *Vol 28(2), 103-115, June 2024, DOI: 10.18311/jer/2024/44777*

Exogenous Melatonin Corrects Hepatic Nocturnin Levels in Experimentally Induced MASLD

Shruti Kulshrestha[1], Rhydham Karnik[1,2], Aliasgar Vohra[1,3], Apeksha Joshi[1] and Ranjitsinh Devkar[1*]

[1]Chronobiology and Metabolic Endocrinology Lab, Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara – 390002, Gujarat, India; rv.devkar-zoo@msubaroda.ac.in

[2]Dr. Vikram Sarabhai Institute of Cell and Molecular Biology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara – 390002, Gujarat, India

[3]Department of Neurology, School of Medicine, Washington University, St. Louis, MO, USA

Abstract

Melatonin, a neurohormone, improves hepatic function in diet and/or chronodisruption in Metabolic dysfunction Associated with Steatotic Liver Disease (MASLD). Nocturnin (Noct), a circadian clock output gene, putatively regulates hepatic lipid metabolism but the underlying mechanisms related to its regulation remain largely unknown. Herein, we hypothesise that melatonin-mediated improvement in liver function in MASLD is regulated via Noct and this study delves into Noct as a putative target of melatonin. Molecular docking studies (Autodock, Pyrx and PyMol) confirmed interactions between melatonin and mouse Noct (Binding affinity: -7kcal/mol; RMSD: 0). Further, studies on C57BL/6J mice comprised of experimental groups viz. high-fat-high-fructose (H) diet fed, photoperiodic shifts-induced chronodisruption (CD) or a combination of the two (HCD) wherein melatonin-mediated improvements in serum lipid profile (TGs, total lipids, VLDL-chol., LDL-chol. and total cholesterol) and liver function markers (ALT and AST) were recorded. Further, the fatty manifestations, hepatocyte ballooning, and steatotic score were significantly improved following exogenous melatonin. Likewise, the liver samples of H, CD and HCD mice recorded a marked increment in hepatic Noct mRNA expression whereas melatonin administration accounted for a significant improvement in the said expression. These findings were further validated *in vitro* in HepG2 cells treated with Oleic Acid (OA) cells wherein, melatonin supplementation improved Noct mRNA and protein expressions compared to the disease control. Taken together, this study provides insight into melatonin-mediated modulation in hepatic Noct that correlates with improved hepatic health in experimental models of MASLD.

Keywords: Chronodisruption, High Fat High Fructose Diet, MASLD, Melatonin, Nocturnin

1. Introduction

Biological clocks are innate timekeeping systems that synchronize with the light-dark cycles to maintain homeostasis[1]. The master circadian clock in the suprachiasmatic nucleus synchronizes with the tissue-specific peripheral clocks to maintain physiological homeostasis[1,2]. Melatonin, a pleiotropic neurohormone secreted by the pineal gland is an important component of the circadian clock machinery. Melatonin signals regulate the circadian organization of a plethora of physiological processes such as immune responses, antioxidant defence, glucose regulation and energy homeostasis[3,4]. As a potent chronobiotic, melatonin influences the circadian timing of metabolic processes, aligning them with the activity/rest cycles[5]. Over the years, several studies have reported the hepatoprotective effects of melatonin in lifestyle disorders such as metabolic dysfunction-associated steatotic liver disease (MASLD) that progresses to an irreversible condition called metabolic dysfunction-associated steatohepatitis[6-9] (MASH; formerly known as NAFLD and NASH, respectively). The therapeutic role of melatonin has been documented in an experimental model of MASH wherein mice fed with a high-fat diet (HFD) had shown significant improvements in steatotic score and related parameters such as inflammation and

*Author for correspondence

Article Received: 02.07.2024 Revised on: 11.09.2024 Accepted on: 21.09.2024

hepatocyte ballooning[10]. Studies from our laboratory had established an association between hepatic clock gene oscillations and the Nrf2-HO1 pathway in experimental models of MASLD wherein exogenous melatonin had accounted for corrective changes in core clock gene oscillations resulting in metabolic reprogramming of liver[11].

Oscillations of clock genes in the hypothalamus, liver or skeletal muscles have been reported to undergo shifts due to changes in feeding regimens. A robust circadian clock is functional in the liver and drives the majority of the key metabolic processes[10]. Changes/shifts in oscillations of core circadian clock genes and nuclear receptors can impact the functioning of clock-controlled genes in mice fed with HFD[12]. Thus, circadian desynchrony arising from shiftwork[13], heightened exposure to artificial light at night[14], frequent episodes of jetlag[15,16] or a chronic consumption of HFD[11,17] can impact hepatic physiology that may culminate in MASLD.

Nocturnin (Noct) or Ccrn4l is one of the metabolically relevant hepatic circadian clock outputs[18] that belongs to the exonuclease, Endonuclease and Phosphatase (EEP) family[19,20] and has emerged as one of the key regulators of lipid metabolism[21,22]. The action of Noct is manifested via its deadenylase activity that removes poly (A) tail from its target mRNAs, thereby directing them towards degradation[23,24]. The liver displays a robust rhythmicity of Noct in healthy mice wherein; a night-time peak corresponds with a lowered expression of hepatic acetyl CoA, cholesterol and triglyceride (TG) synthesis pathways. In contrast, *Noct*$^{-/-}$ mice show a higher expression of genes regulating lipid metabolism thus underlining the importance of Noct as a key regulator[22]. Further, resistance against HFD was recorded in global Noct (*Noct*$^{-/-}$) knockout mice which implies its role in the pathogenesis of MASLD or MASH[25]. Taken together, Noct plays a role in regulating lipid metabolism, but some questions related to its function in a steatotic liver are unanswered. The night-time peak of Noct in a healthy liver implies its subtle role which needs profound scientific scrutiny on the regulation of Noct in the said disease condition. Though melatonin shares a similar phasing as Noct, a melatonin-nocturnin interplay can be an important player that calls for a detailed study. Alterations in Noct levels under experimentally induced MASLD and the role of melatonin-Noct interplay are the crux of this study.

2. Materials and Methods

2.1 Chemicals and Reagents

Cell culture media (DMEM), Trypsin (TPVG), Fetal Bovine Serum (FBS), antibiotic-antimycotic and bovine serum albumin (BSA) were procured from HiMedia Laboratories, Mumbai, India. SYBR select master mix and TRIzol were purchased from Invitrogen, USA. RNA-Later solution, iScript cDNA synthesis kit and Clarity Western ECL substrate were procured from Ambion Inc., USA and Bio-Rad Laboratories, USA, respectively. Primary antibodies against Nocturnin (SC-376584) and β-actin (E-AB-40338) were purchased from Santa Cruz Biotechnology and E-labscience, USA, respectively. Secondary antibodies (SC-516102; 32469) were procured from Santa Cruz Biotechnology and Invitrogen, respectively. Hematoxylin, eosin, Oleic Acid (OA) (O1008), Oil Red O (ORO) stain (O0625), and melatonin (444300) were procured from Sigma Aldrich (MO, USA). Methanol, MTT dye and DMSO were purchased from SRL Pvt. Ltd., Mumbai, India.

2.2 *In Silico* Studies

The protein structure of full-length mouse NOCT (mmu-NOCT) was generated using the iTASSER server (https://zhanggroup.org/I-TASSER/). The structure thus generated was confirmed by performing Ramachandran Plot analysis in PROCHECK (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/). Further, molecular docking between mmu-NOCT and melatonin was performed in Autodock using the default parameters of AutoDock. PyRx and PyMol were used for visualization.

2.3 *In Vivo* Studies

Six to eight-week-old male C57BL/6J mice (20-22g) were procured from ACTREC, Navi Mumbai, and housed according to CCSEA guidelines. Before initiating the experimental regime, mice were allowed to acclimatize for 7 days. The experimental design was approved by the Institutional Animal Ethics Committee (IAEC, MSU-Z/IAEC06/01-2022). All the *in vivo* investigations were performed in the CCSEA-approved animal house facility of the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. After completion of the acclimatization period, mice were randomly divided into 7 experimental

groups and subjected to a high-fat-high-fructose (H) diet alone or along with photoperiodic shifts-induced chronodisruption (CD) for 16 weeks.

2.4 Photoperiodic Manipulations and Experimental Design

Mice were randomly divided into 7 experimental groups: (i) Control, (ii) H (high-fat- high-fructose diet + 20% fructose water), (iii) CD (standard chow diet + CD), and (iv) H+ CD, (v) H+ Mel (HM), (vi) CD + Mel (CDM) and (vii) HCD + Mel (HCDM), and housed for 16 weeks. From the 9th week till the end of 16 weeks, groups (v), (vi), and (vii) received daily intraperitoneal injections of melatonin (10mg/kg, at ZT10). Chronodisruption (CD) was induced using a standardized protocol that involved phase advance-phase delay photoperiodic manipulations. Mice were kept in two different rooms: Room 1 (light period from 7:00 am to 7:00 pm, dark period from 7:00 pm to 7:00 am) and Room 2 (dark period from 11:00 am to 11:00 pm, light period from 11:00 pm to 11:00 am). Mice were transferred from Room 1 to Room 2 on Mondays (lights off at 11:00 am; phase advance of 8 hours) and back to Room 1 on Thursdays (lights off at 7:00 pm, phase delay of 8 hrs) (Figure 1). All the transfers occurred at 10:55 am. Morphometric parameters such as body weight, food and water intake were noted on alternate days.

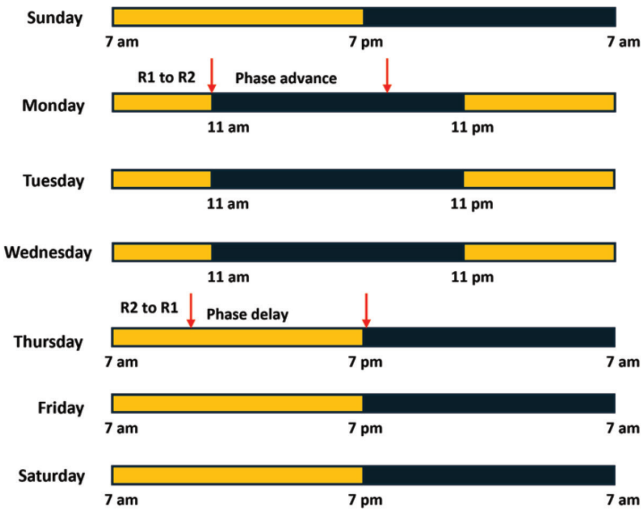


Figure 1. Schematic representation of photoperiodic shifts induced chronodisruption (CD) regime that was followed in the study. Experimental groups (C, H and HM) were housed in L:D 12:12 throughout the protocol whereas, CD, CDM, HCD and HCDM groups were subjected to eight hours of phase-advance and eight-hour delay regime for `16 weeks (11,17).

Melatonin was freshly dissolved in 1% EtOH, diluted with 0.9% saline and subsequently stored in tubes covered with aluminium foils. Melatonin (10 mg/kg B.W.) was intraperitoneally injected daily at ZT10.

After 16 weeks, mice were euthanized using mild isoflurane at ZT12. The serum was isolated by collecting whole blood and centrifuging at 3000rpm for 10 minutes at 4°C and was subsequently stored at -80°C. Liver tissue samples were processed in different ways: stored in 4% Paraformaldehyde (PFA) for histopathology and preserved in RNA later for gene expression studies.

2.5 Serum Biochemical Parameters

Levels of liver function markers (ALT and AST), as well as serum lipid profile (total lipids-TL, LDL-Chol., VLDL, HDL chol., total cholesterol-TC and triglycerides-TG), were quantified using commercially available kits (Reckon Diagnostic kits, Vadodara, Gujrat, India).

2.6 Histopathological Analysis

4% PFA was used to fix liver tissue samples that were subsequently dehydrated, set in paraffin wax blocks and sliced into sections of 5 µm thickness. Sections were then stained with hematoxylin and eosin (H and E), mounted on glass slides and photographed using a bright-field microscope (Leica DM 750 microscope). Investigators blinded to the study performed scoring of steatosis and ballooning hepatocytes of control and experimental mice.

2.7 Cell Culture

Human Hepatoma (HepG2) cells were obtained from NCCS, Pune, India, and cultured in DMEM that comprised 10% FBS and 1% antibiotic-antimycotic solution, in a humidified atmosphere with 5% CO_2. The cells were passaged using 1X TPVG when they reached approximately 80% confluency.

2.8 Treatment of HepG2 Cells

Stock solution of OA was prepared as described earlier (11). Further, 10% BSA was used to conjugate with 100 mM OA to form a 10 mM BSA-conjugated OA sub-stock. Melatonin was dissolved in 100% ethanol to prepare a 1 mM stock solution. Subsequent dilutions of both OA and melatonin were prepared in incomplete cell culture media to obtain relevant concentrations of working solutions. HepG2 cells were treated with OA and/or melatonin

for 24 h and harvested for gene and protein expression studies.

2.9 Cytotoxicity Analysis

HepG2 cells were seeded in a 96-well plate (cell seeding density:1×10^4 cells/per well)in DMEM and treated with increasing concentrations of OA (conc. range: 0.5mM to 2 mM), either alone or in combination with melatonin (conc. range: 5 µM-1000 µM), for 24 hours. After treatment, the cells were incubated with MTT dye (0.5 mg/mL) for four hours, and the resulting formazan crystals were dissolved in 100 µL of DMSO solution. Absorbance was then measured at 540 nm using a Synergy HTX multimode reader, USA.

2.10 Intracellular Lipid Accumulation

Intracellular lipid droplet accumulation in HepG2 cells was evaluated using ORO staining as previously described (11). Briefly, HepG2 cells were cultured in 6-well plates and subjected to OA (0.5 mM) and/or melatonin (100 µM) treatment for 24 hrs. Following treatment, 4% PFA was used to fix the cells that were subsequently rinsed with 1X phosphate-buffered saline. The cells were then stained with ORO, and the ORO levels were quantified by adding 100% isopropanol to each well. Absorbance was determined at 510 nm in a Multi-mode Microplate Reader (Synergy HTX, Biotek Instruments, USA)

2.11 Gene Expression Studies

Total RNA from HepG2 cells/hepatic tissue were isolated using TRIzol reagent and subsequently, cDNA was synthesized using iScript from Bio-Rad Laboratories, USA. (iScript; Bio-Rad Laboratories, USA). *Noct* mRNA expression was studied using qPCR (Quantstudio 5 Real-Time PCR system, ThermoFisher Scientific, USA) with primers specific to relevant genes (Table 1) and SYBR Select Master mix. Data was normalized using a housekeeping gene (18S rRNA) and analysed with the $2^{-\Delta\Delta Ct}$ method.

2.12 Protein Expression Studies

HepG2 cells were lysed in pre-chilled 1X RIPA buffer supplemented with a protease inhibitor cocktail (Sigma Aldrich, USA), and total protein levels were determined using the Bradford method. An equal quantity of protein was loaded onto a 10% polyacrylamide gel for SDS-PAGE, which was subsequently transferred to a PVDF membrane (Bio-Rad, USA). The membrane was incubated overnight at 4°C with a primary antibody against Noct (1:500). Following this, the blots were treated with a secondary anti-mouse horseradish peroxidase antibody for 1.5 hours at room temperature. After stripping the blots with stripping buffer, they were re-probed with a primary antibody against β-actin (1:1000). Finally, the blots were developed using ECL reagent (Bio-Rad, USA) and visualized on X-ray films.

2.13 Data Analysis and Statistics

All the datasets are presented as mean ± SD. The data were analysed using one-way ANOVA and plotted using GraphPad Prism 8.1.1. software.

3. Results

3.1 Structure Generation of Mouse NOCT

iTASSER online tool was used to predict mmu-NOCT protein structure based on the amino acid sequence from NCBI (Figure 2(A)). iTASSER starts by identifying templates from a library of known structures. It then constructs a complete atomic model through iterative assembly simulations using continuous structural fragments extracted from these template alignments. Afterwards, the generated model was compared with a database of proteins with known functions to gain functional insights[26].

The generated 3D protein structure was further validated by Ramachandran Plot in PROCHECK wherein

Table 1. List of primers

Target	Primer	Sequence (5' to 3')
mmu-Noct	Forward primer (FP)	ACCAGCCAGACATACTGTGC
	Reverse primer (RP)	CTTGGGGAAAAACGTGCCT
mmu-18S rRNA	FP	GCAATTATTCCCCATGAACG
	RP	GGCCTCACTAAACCATCCAA
hsa-NOCT	FP	TGTTCCCCGACTTGCCTGGG
	RP	TGTAACCAACATAGTAACTCGCGG
hsa-18S rRNA	FP	CGTTCAGCCACCCGAGATT
	RP	GACCCGCACTTACTGGGAATT

54.4% of residues were in the most favoured regions, while 36.0% of the total residues fell in additional allowed regions and 19% fell in generously allowed regions (Figure 2(B)).

3.2 Molecular Docking Studies

Molecular docking of melatonin (PubChem CID 896) and mmu-NOCT was performed in Auto Dock using default parameters and the results were visualized in

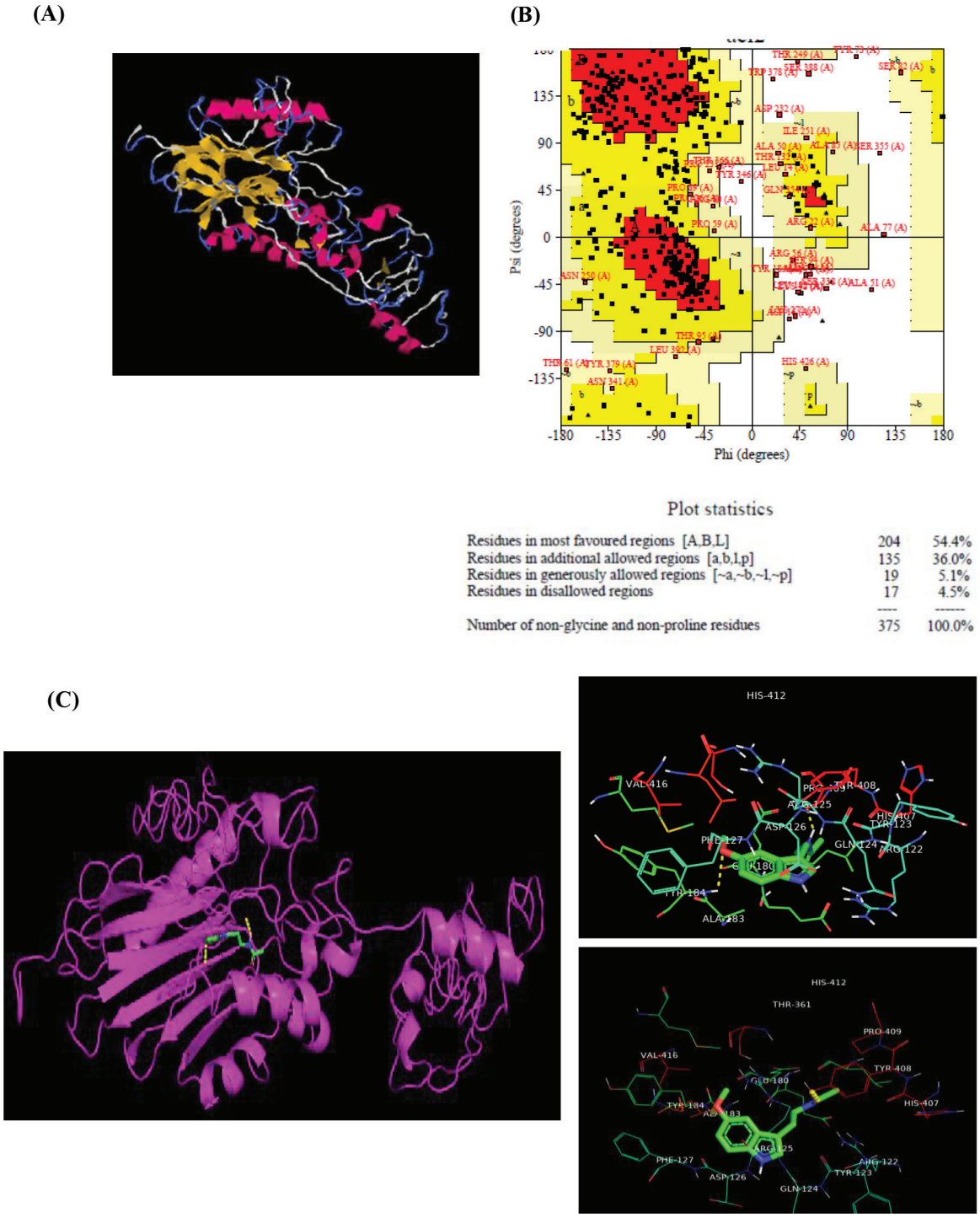


Figure 2. Computational analysis of NOCT and melatonin: **(A)** structure generation of mmu-NOCT by i-TASSER, **(B)** NOCT structure validation by Ramachandran Plot analysis, and **(C)** Molecular docking of mmu-NOCT and melatonin.

PyMol. Melatonin docked at the catalytic site of mmu-NOCT with a high binding affinity (-7 kcal/mol) and low RMSD value (0), thus providing preliminary evidence on melatonin-Noct interaction (Figure 2(C)).

3.3 Melatonin-Mediated Improvements in MASLD Pathophysiology

C57BL/6J mice subjected to H and/or CD regime (for 16 weeks) recorded higher body weights (Figure 3(A)), liver weights (Figure 3(B)) and liver-to-body weight ratios (Figure 3(C)) as compared to control mice. Exogenous melatonin administration recorded a significant decrement in the aforementioned indices in HM and HCDM groups.

Disease control groups (H, CD and HCD) recorded significant elevation in circulating titres of liver function markers (ALT and AST) (Figure 4) and serum lipid profile (total lipids - TL, total cholesterol- TC, TGs, LDL-c and VLDL-c) along with a decrement in HDL-c (Figure 5). Melatonin administration (HM, CDM and HCDM groups) produced significantly low ALT and AST levels and lipid profiles (Figures 4, 5). Microscopic evaluation of liver tissue samples of H, CD and HCD mice showed varying degrees of hepatocyte ballooning, cellular derangement and hepatic cords (Figure 6(A)). Random scoring of hepatic tissue sections showed significantly higher indices of hepatocyte ballooning and steatotic scores in H, CD and HCD groups (Figures 6(B, C)). Melatonin treatment led to a significant decrease in the above parameters in HM, CDM and HCDM groups (Figures 6(B, C)).

3.4 Melatonin Restores Hepatic Noct mRNA in H and/or CD Mice

H, CD and HCD groups recorded significant increments in hepatic *Noct* mRNA. The melatonin-treated group (HM) recorded a significant improvement whereas the CDM group had a nonsignificant improvement in *Noct* mRNA. However, HCDM had recorded a significant increment contrary to the other melatonin-treated groups (Figure 7).

3.5 Melatonin Reduces Lipid AccumulationI by Oleic Acid (OA) in HepG2 Cells

OA and melatonin toxicity were assessed by performing an MTT assay. HepG2 cells were subjected to increasing doses

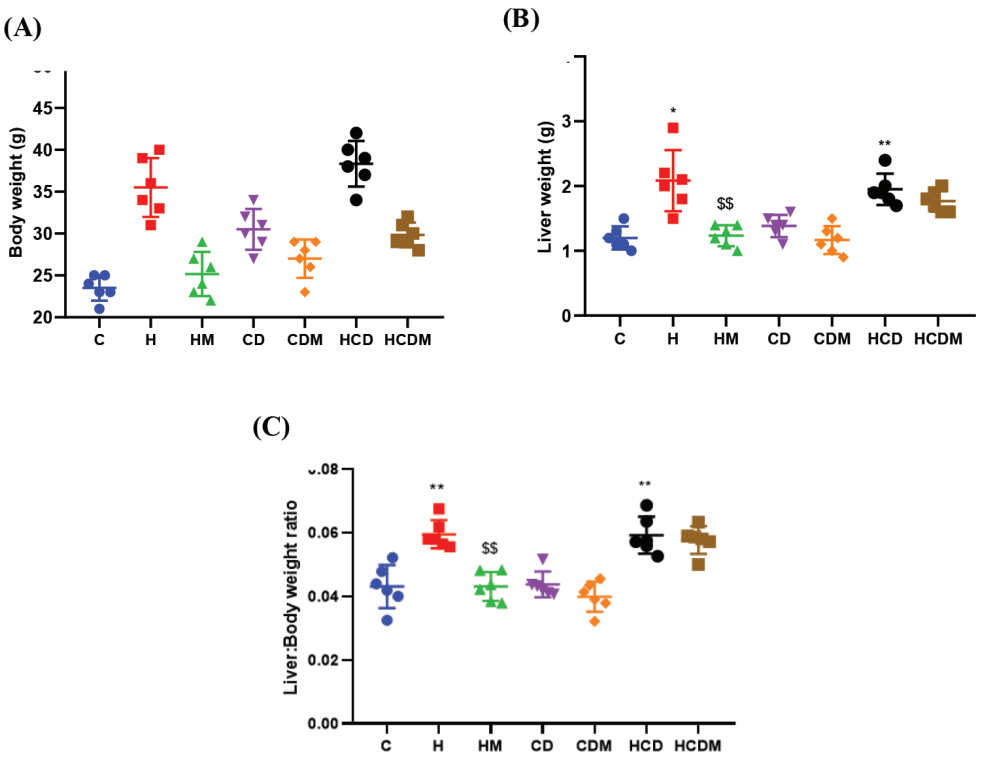


Figure 3. Melatonin-mediated alterations in **(A)** body weight, **(B)** liver weight, and **(C)** liver: body weight ratios of C57BL/6J male mice subjected to high-fat-high-fructose (H) diet and/or photoperiodic shifts induced chronodisruption (CD). *p<0.05, **p<0.01 when H, CD, HCD is compared with control (C). $$<0.01 when HM is compared with H group.

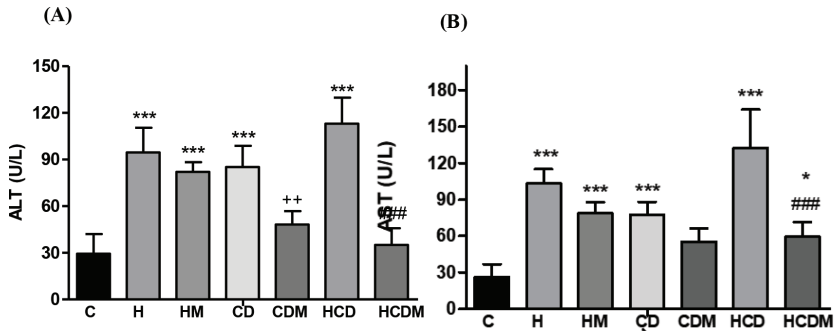


Figure 4. Changes in serum liver function markers: **(A)** ALT and **(B)** AST in C57BL/6J mice subjected to H and/or CD regime for 16 weeks and corrections following exogenous melatonin treatment. ***p<0.001 when H, CD and HCD are compared with control (C); ++p<0.01 when CDM is compared to CD and ###p<0.001 when HCDM is compared to HCD.

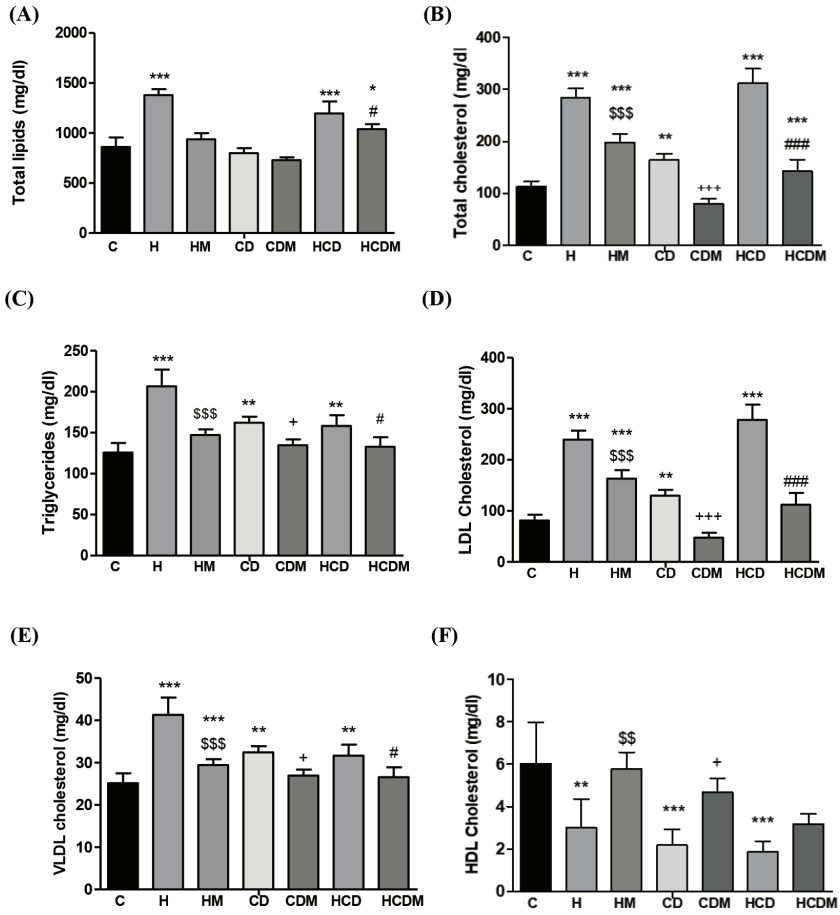


Figure 5. Alterations in serum lipid profile: **(A)** TL, **(B)** total cholesterol, **(C)** TGs, **(D)** LDL-chol, **(E)** VLDL-chol. and **(F)** HDL-chol. **p<0.01, ***p<0.001 when H, CD and HCD compared to control. $$ p<0.01, $$$ p<0.001 when HM is compared to H group; +p< 0.05, +++ p<0.001 when CDM is compared to CD group and # p<0.05, ###p<0.001 when HCDM is compared to HCD group.

of OA (0.5mM-2.0 mM) and/or melatonin (5μM-1000μM) for 24 hours. 0.5 mM concentration of OA recorded ~60% cell viability and 100 μM concentration of melatonin recorded ~90% cell viability and were therefore used for further experiments (Figure 8). The OA-treated HepG2 cells showed significant accumulation of intracellular lipid droplets. Melatonin-treated HepG2 cells accounted for lowered intracellular lipid accumulation (Figure 9).

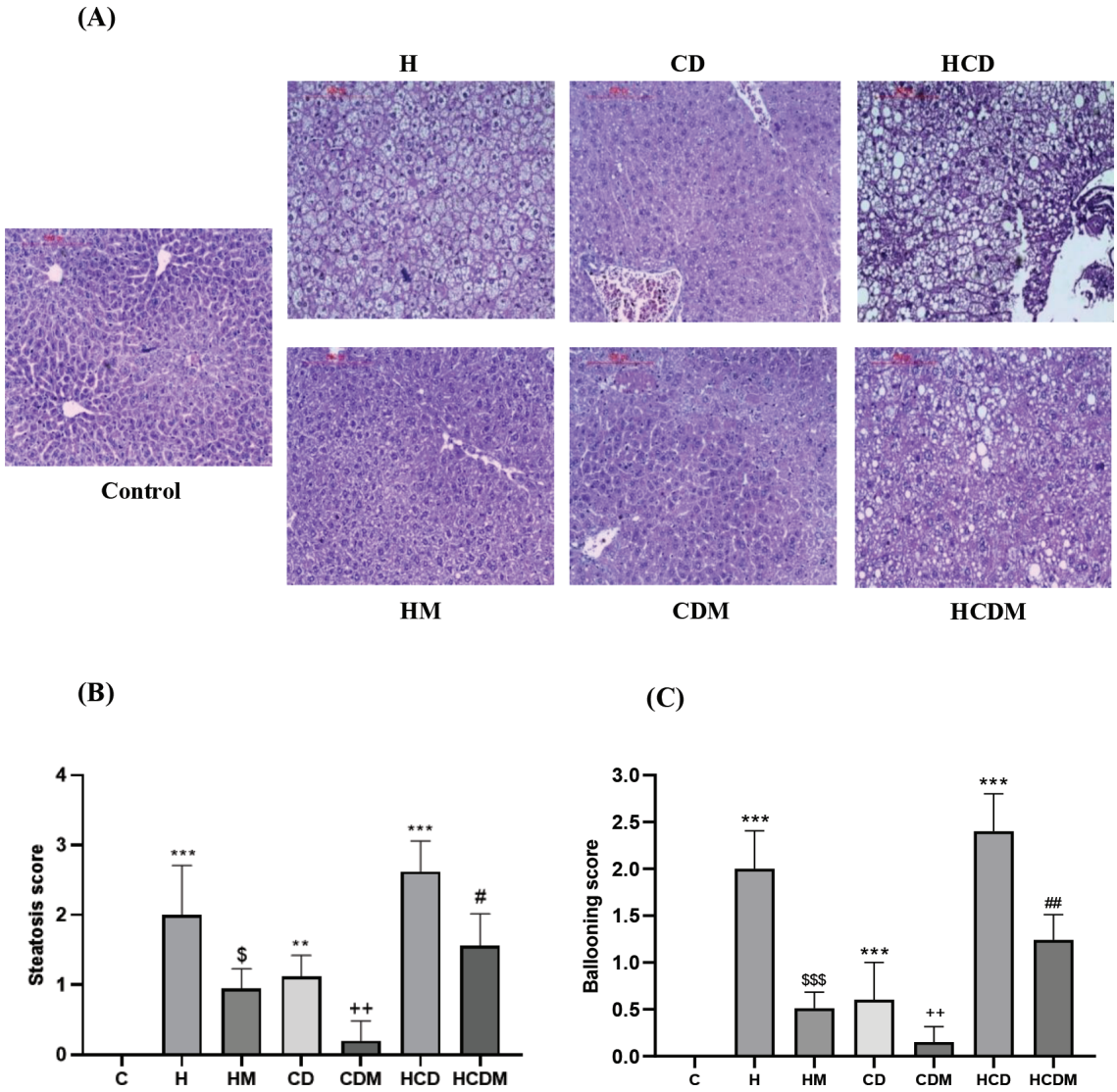


Figure 6. Histopathological analysis of liver sections of C57BL/6J **(A)** male mice subjected to H and/or CD for 16 weeks, **(B)** melatonin-mediated corrections in steatosis scores and (**C**) ballooning scores. **p<0.01, ***p<0.001 when H, CD and HCD are compared to control. $ p<0.05, $$$ p<0.001 when HM is compared to H group; ++ p<0.01 s when CDM is compared to CD group and # p<0.05, ##p<0.01 when HCDM is compared to HCD group.

3.6 Melatonin Restores NOCT in OA-treated HepG2 Cells

HepG2 cells were subjected to serum shock treatment for 2 hr to synchronize individual cellular circadian clocks and subsequently subjected to OA alone and/or 100 μM melatonin. OA-treated HepG2 cells significantly upregulated NoctmRNA and protein levels. Melatonin treatment accounted for a significant decrement in Noct-mRNA (Figure 10(A)) and protein expression (Figure 10(B, C)) and was comparable to that of control.

4. Discussion

Cellular and organismal level circadian clocks act as an immediate sensor of the environmental changes[27,28]. Although Nocturnin (Noct) has to date not been reported to directly regulate the expression of core clock genes, it plays a role in mediating its rhythmic output(38). The circadian deadenylase, Noct, has a crucial role in the post-transcriptional regulation of metabolic genes[29]. Resistance towards HFD-induced obesity and hepatic steatosis was reported in *Noct* knock-out (*Noct* $^{-/-}$) mice wherein lower expression of lipid metabolism genes (*Srebp1-c*, *Scd-1* and

L-Fabp) is speculated to be regulated via Noct[25]. Another study revealed increased expression of metabolic genes (*Acaca, Fasn, Pnpla3, Acss2, Gpam, Pmvk, Sqle, Fdps, Lss, Dhcr7, Acly, Hmgcs1* and *Mvd*) in *Noct*$^{-/-}$ mice, highlighting the importance of Noct as a possible regulator of hepatic metabolism[22]. These studies indicate that an altered hepatic Noct expression might have significant implications in the pathogenesis of lifestyle disorders and, hence, calls for a detailed investigation. In this study, C57BL/6J mice fed with a high-fat-high-fructose (H) diet, either alone or combined with chronodisruption (CD) induced due to photoperiodic manipulations, along with HepG2 subjected to OA served as experimental models of MASLD.

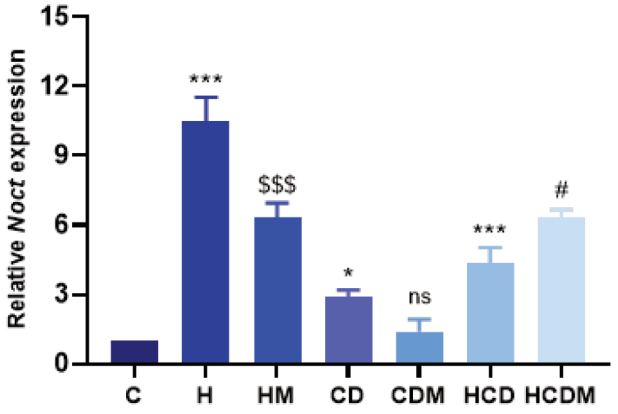


Figure 7. Melatonin-mediated changes in hepatic *Noct* mRNA (at ZT12) of C57BL/6J mice subjected to H and/or CD regime. *p<0.05, ***p<0.001 when H, CD and HCD are compared to the C group. $$$ p<0.001 when HM is compared with H and # p<0.05 when HCDM is compared with HCD.

In our study, significantly higher titres of TL, TC, TGs, LDL-c, and VLDL-c in H, CD and HCD groups were in agreement with other published reports from our laboratory (11,17) as well as reports published by other research groups[30]. The H, CD and HCD mice recorded significantly increased titres of the liver function markers (ALT and AST) that imply liver damage. Microscopic evaluation of hepatic tissue samples of H, CD and HCD mice revealed significant intrahepatic lipid accumulation as well as hepatocyte ballooning that validated the development of MASLD. Various studies had reported that hepatic Noct expression peaks at ZT12 [22] and this

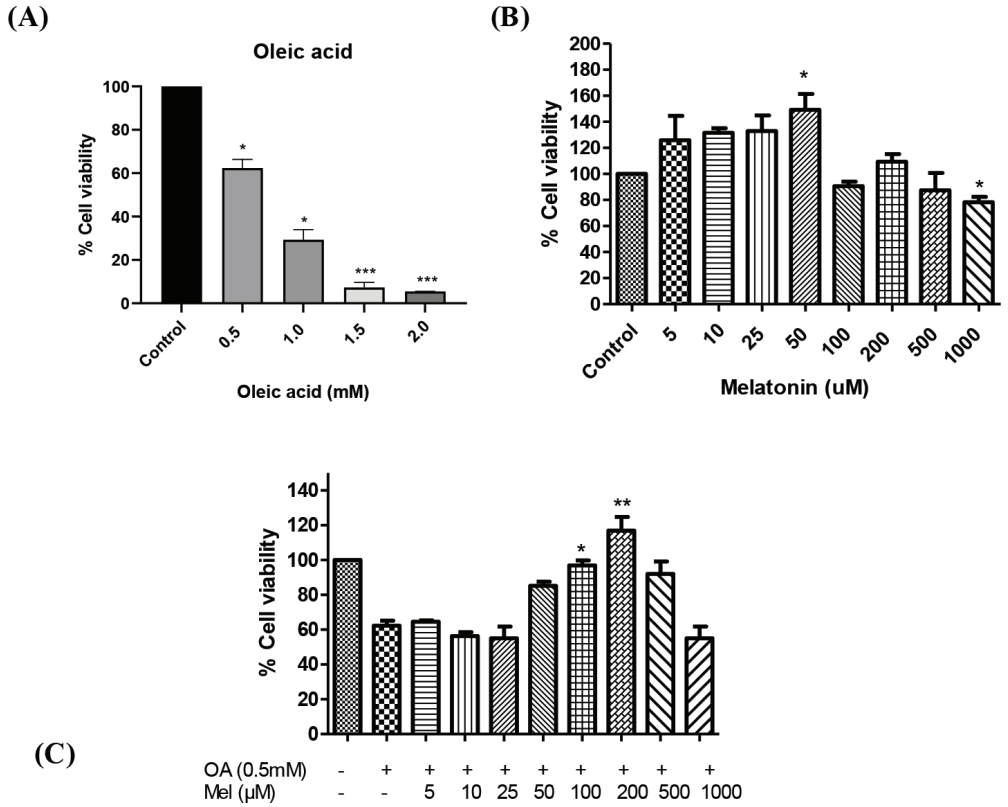


Figure 8. Dose standardization of Oleic Acid (OA) and/or melatonin by MTT assay. *p<0.05, **p<0.01, ***p<0.001 when the various concentrations are compared with untreated (Control) HepG2 cells.

formed the basis for sample collection at ZT12. In the H, CD and HCD groups, a significant increase in *Noct* mRNA agrees with the elevated serum lipid profile and liver function markers in the said groups. Furthermore, the significant increase in *Noct* mRNA at ZT12 in our study aligns with a report of a 3-week-long protocol in HFD-fed mice[22]. This inventory is the first to report higher levels of hepatic *Noct* due to photoperiodic shifts and is a novelty of this study.

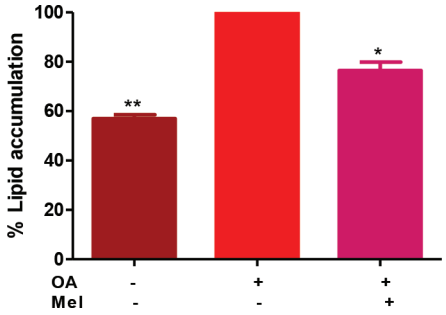


Figure 9. Percentage intracellular lipid accumulation in HepG2 cells subjected to 0.5 mM OA and/or 100μM for 24 hours, as assessed by Oil Red O staining. *p<0.05 and **p<0.01 when C and OA+ Mel. is compared to OA group.

The *in vivo* findings were subsequently validated in HepG2 cells subjected to OA treatment. The serum shock treatment is widely used as a mode for synchronizing cellular clocks in an *in vitro* scenario. Hence, HepG2 cells were serum-synchronized for two hours and then treated with OA for 24 hours[31,32]. Consistent with the *in vivo* findings, a significant increase in Noct mRNA and protein was recorded in OA-treated cells. Noct has been researched in HepG2 cells, but its role in the pathophysiology of hepatic disorders lacks a detailed investigation. This study is the first to report higher levels of Noct in the OA-treated HepG2 model of MASLD.

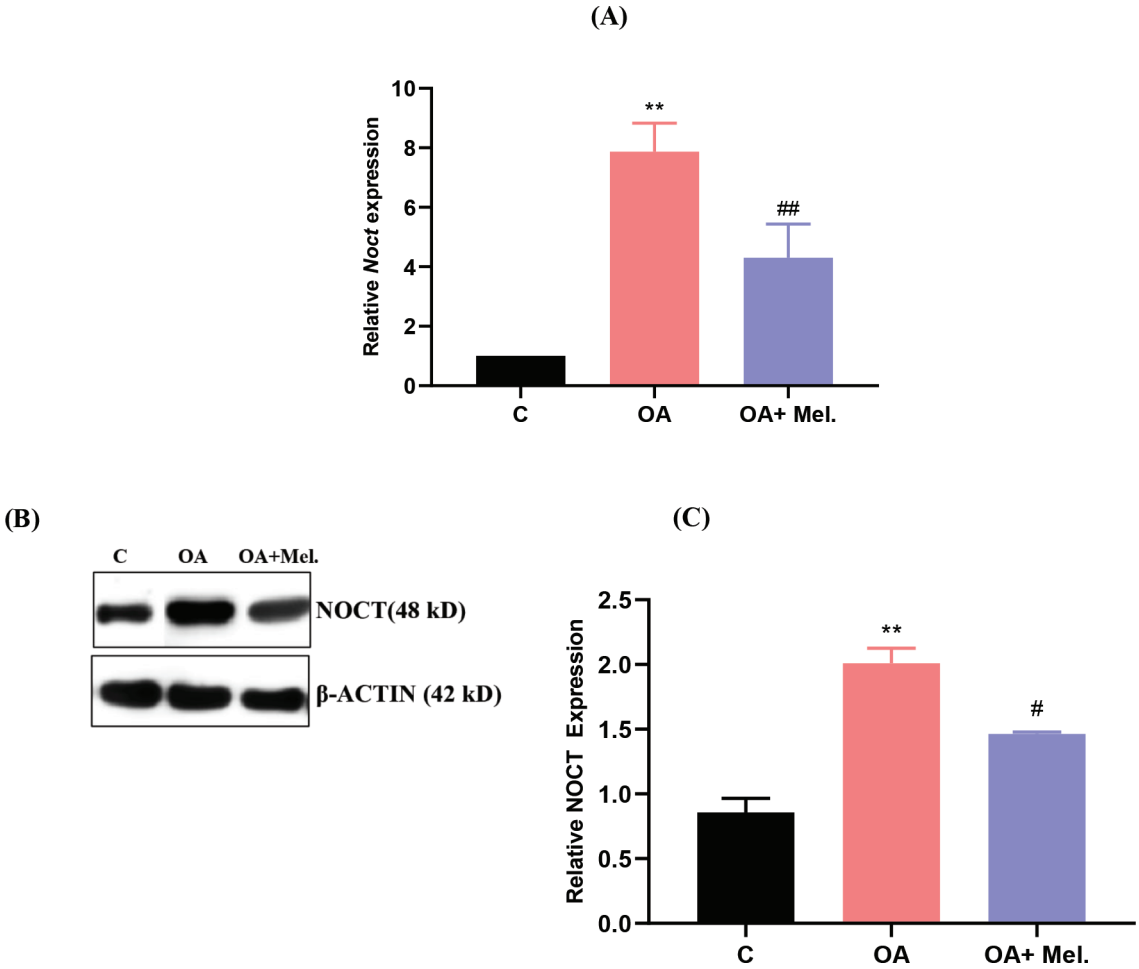


Figure 10. **(A)** Alterations in Noct mRNA, **(B)** and **(C)** protein in HepG2 cells subjected to OA and/or melatonin treatment. **p<0.01 when OA is compared to Control (C) and # p<0.05, ## p<0.01 when OA + Mel is compared to the OA group.

The hepatoprotective effects of melatonin have been widely reported by several researchers[33-35]. It is interesting to note that, Noct is robustly rhythmic, a trait comparable to Melatonin[36]. Moreover, studies have shown that melatonin and Noct have a similar phasing, thus implying their possible interaction[37]. After generating the protein structure of NOCT and its validation by the Ramachandran Plot, molecular docking studies revealed that melatonin showed a high binding affinity with NOCT as evidenced by a high binding affinity score and low RMSD (root mean square deviation). In our study, exogenous melatonin administration accounted for a significant improvement in liver function markers (ALT and AST), lipid profile (TLs, TCs, TGs, LDL-c, VLDL-c) as well as histopathological observations, and agree with our published reports[11,17]. Further, exogenous melatonin was also reported to improve HFD-induced steatotic changes in the liver by suppressing NLRP3 inflammasome in db/db mice[33]. In another study, melatonin improved MASLD via MAPK-JNK/P38 signalling in diet-induced obese mice[38] In the present study, melatonin-mediated improvements in serum parameters and histopathology coincided with a significantly lowered hepatic *Noct* mRNA in the HM group whereas, a non-significant decrease was observed in CDM group. Interestingly, the HCDM group recorded a significant increase in hepatic *Noct* mRNA that could not be explained and may need further investigation(s).

Melatonin pre-treatment in OA fed HepG2 cells inhibited TG and cholesterol accumulation to significant levels and the same was attributed to acetyl-CoA carboxylase and AMP-activated kinase phosphorylation. Further, melatonin upregulates lipolysis genes (*PPAR-α* and *CPT-1*) whereas expression of lipogenic genes (*SREBP-1c*, *SCD-1*, and *FAS*) were downregulated[40]. In another study, palmitic acid-treated HepG2 cells had recorded an increase in expression of the α2-HS-glycoprotein gene (AHSG) and its protein, fetuin-A (FETUA), whereas phosphorylated AKT (pAKT) was downregulated. Melatonin was instrumental in reversing the changes along with a decrement in ER stress markers (*XBP-1, ATF-4, Bip, CHOP, PERK, ATF-6*)[41]. In the present study, HepG2 cells subjected to melatonin recorded a significant decrease in intracellular lipid accumulation, which was evident from ORO staining. Further, melatonin treatment led to a significant lowering of Noct mRNA and protein as compared to OA-treated HepG2 cells. Thus, our studies provide novel insights into the modulatory action of melatonin in MASLD via corrections in hepatic Noct expression. Further, assessment of possible alterations in Noct rhythmicity may provide deeper insight into melatonin-mediated effects and shall help to decipher the mechanism for melatonin-Noct interactions.

5. Conclusion

Our studies provide *prima facie* evidence of melatonin-NOCT interactions and their possible role in regulating liver functions.

6. Acknowledgement

This work was supported by the Department of Biotechnology (DBT) [BT/PR36169/MED/30/2197/2020] and [BT/PR42042/MED/30/2323/2021] grants. S.K. acknowledges the Indian Council of Medical Research (ICMR) for providing financial assistance in the form of a Senior Research Fellowship (SRF) [3/1/2(15)OBS/2022-NCD-II].

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