Introduction

Melatonin, a neurohormone produced primarily by the pineal gland in the brain, plays a crucial role in regulating circadian rhythms, particularly the sleep-wake cycle. Known as the “hormone of darkness”, melatonin secretion increases in response to darkness and decreases during daylight (Hardeland, 2008). This rhythmic secretion of melatonin plays a key role in synchronizing the circadian clock with the external environment, promoting overall physiological balance (Cipolla-Neto & Amaral, 2018). Melatonin production begins in evening, peaks in the middle of the night, and gradually tapers off my morning. Melatonin secretion is greatly influenced by light exposure, especially blue light, that can suppress melatonin levels. Thus, artificial light at night (ALAN) and screen use during late hours hinder melatonin release, contributing to sleep disturbances and disruptions in circadian rhythms (Khan et al., 2022; Touitou et al., 2017).

Beyond its role in sleep, melatonin signals are known to regulate the circadian organization of various physiological processes such as antioxidant defence (Reiter et al., 2003), glucose regulation (Watanabe et al., 2020), immune responses (Esquifino et al., 2004; Maestroni & Conti, 2020)and energy homeostasis (Challet & Pévet, 2024). Further, as a potent chronobiotic, melatonin modulates the timing of metabolic processes, aligning them with activity/rest cycles (Baandrup et al., 2016; Rivest et al., 1989)and has been widely investigated for its hepatoprotective properties in NAFLD/NASH (Rezayat et al., 2021a). A significant improvement in plasma liver enzymes was reported in NASH patients treated with exogenous melatonin. NASH patients recorded significantly higher values of leptin and resistin and lower adiponectin levels as compared to healthy individuals and melatonin treatment for 28 days accounted for a significant decrement in leptin levels whereas, adiponectin levels recorded a significant increment (Gonciarz et al., 2013). In another study, melatonin administration suppressed lipid accumulation and peroxidation, improved insulin sensitivity, and attenuated inflammation and fibrogenesis in MCD-diet induced mouse model of NASH (Miguel et al.,

2022). Previous studies in our laboratory had reported that timed exogenous melatonin administration accounted for re-entrainment of hepatic clock genes and improvements in NAFLD via corrections in the Nrf2-HO1 pathway genes (Joshi et al., 2021).

Changes in feeding regimes has been shown to alter oscillations of clock genes in the hypothalamus, liver and skeletal muscles (Saha et al., 2022). In liver, a robust circadian clock regulates key metabolic processes (Mukherji et al., 2019). As a result, circadian desynchrony caused by shift work (Barclay et al., 2012), chronic exposure to artificial light at night (ALAN) (Lei et al., 2024), frequent episodes of jetlag (Escobar et al., 2020; Vosko et al., 2010)or chronic consumption of HFD (Zitting et al., 2022)impacts hepatic physiology, potentially leading to NAFLD that can subsequently progress to NASH.

Nocturnin (Noct) is a metabolically significant hepatic circadian clock output that has emerged as a key regulator of lipid metabolism (Laothamatas et al., 2020). In healthy mice, liver exhibits robust rhythmic Noct expression , with a night-time peak associated with decreased activity of hepatic acetyl CoA, cholesterol, TG synthesis pathways. In contrast, *Noct*$^{-/-}$ mice display increased expression of lipid metabolism genes, thus underscoring Noct's role as a crucial regulator (Stubblefield et al., 2012, 2018). Additionally, global *Noct*$^{-/-}$ mice reported resistance to HFD-induced obesity and hepatic steatosis (Green et al., 2007), suggesting its involvement in the pathogenesis of NAFLD and NASH. In the previous chapter, we had identified potential targets of hepatic Noct and investigated the role of altered hepatic Noct oscillations in diet and/or CD induced NASH. While melatonin shares a similar circadian phase with Noct (Hardeland, 2014), the potential interaction between these two could be a significant factor that warrants further scrutiny. This chapter aims to explore the potential role of melatonin-Nocturnin interaction in NASH pathology.

Methodology

Computational studies

The full-length structure of mouse NOCT (mmu-NOCT) was generated in i-TASSER and validated by Ramachandran Plot analysis in PROCHECK. Molecular docking between melatonin and mmu-NOCT was performed using AutoDock, Pyrx and PyMol.

In vivo studies

C57BL/6J male mice were subjected to high-fat-high-fructose (H) diet alone or in combination with photoperiodic manipulations induced chronodisruption (CD) for 16 weeks. From 9th week till the end of 16 weeks, mice were subjected to exogenous intraperitoneal administration of melatonin (10mg/kg/day).

Experimental Groups:

1. Control (C)
2. High-fat-high-fructose diet (H)
3. Chronodisruption (CD)
4. High-fat-high-fructose diet + Chronodisruption (HCD)
5. High-fat-high-fructose diet + Melatonin (HM)
6. Chronodisruption + Melatonin (CDM)
7. High-fat-high-fructose diet + Chronodisruption + Melatonin (HCDM)

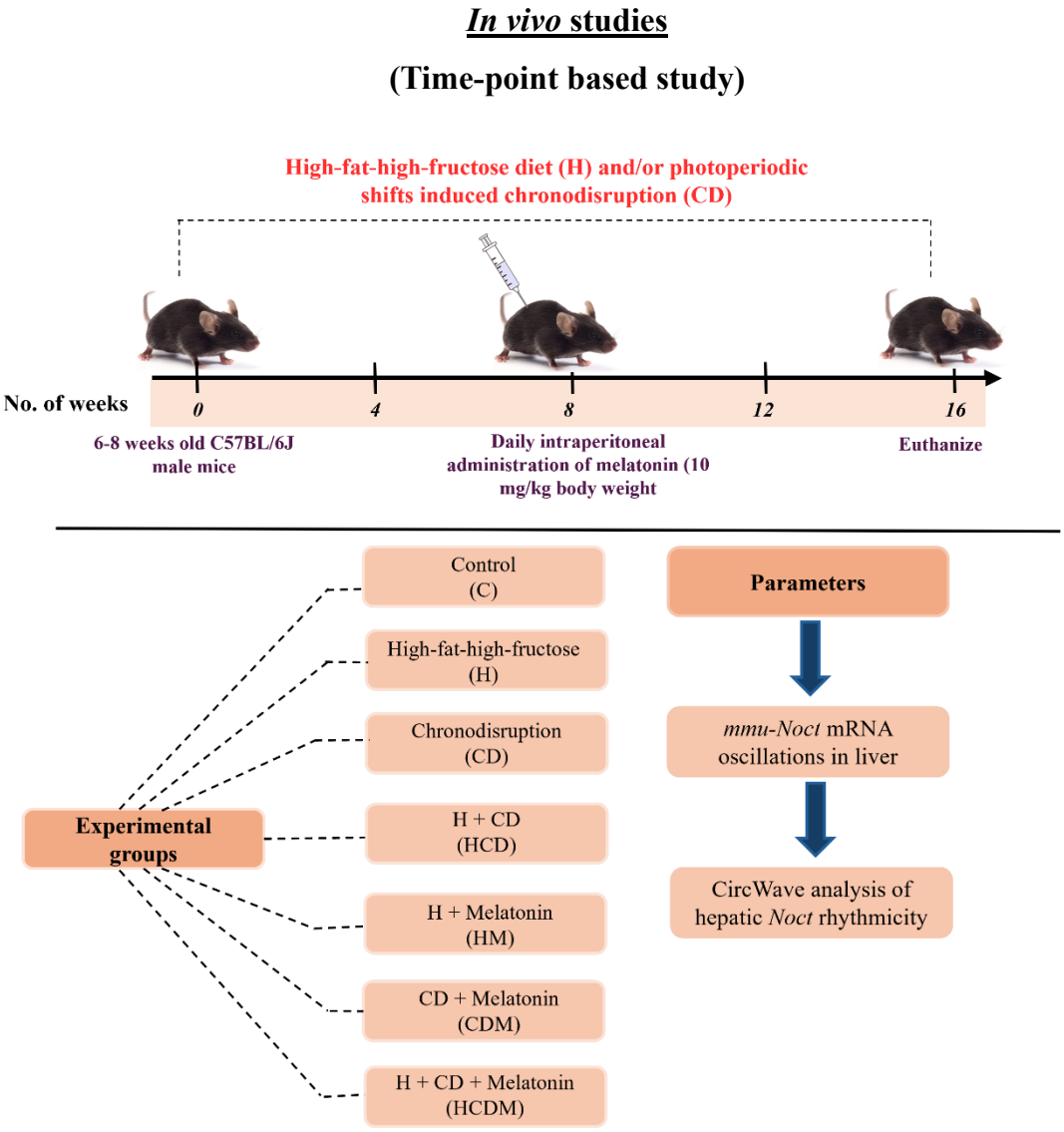
In vitro studies

HepG2 cells were serum-synchronized and subjected to oleic acid (OA) alone or in combination with melatonin for 24 h. Cells were harvested at different time points for gene and protein expression studies.

Parameters:

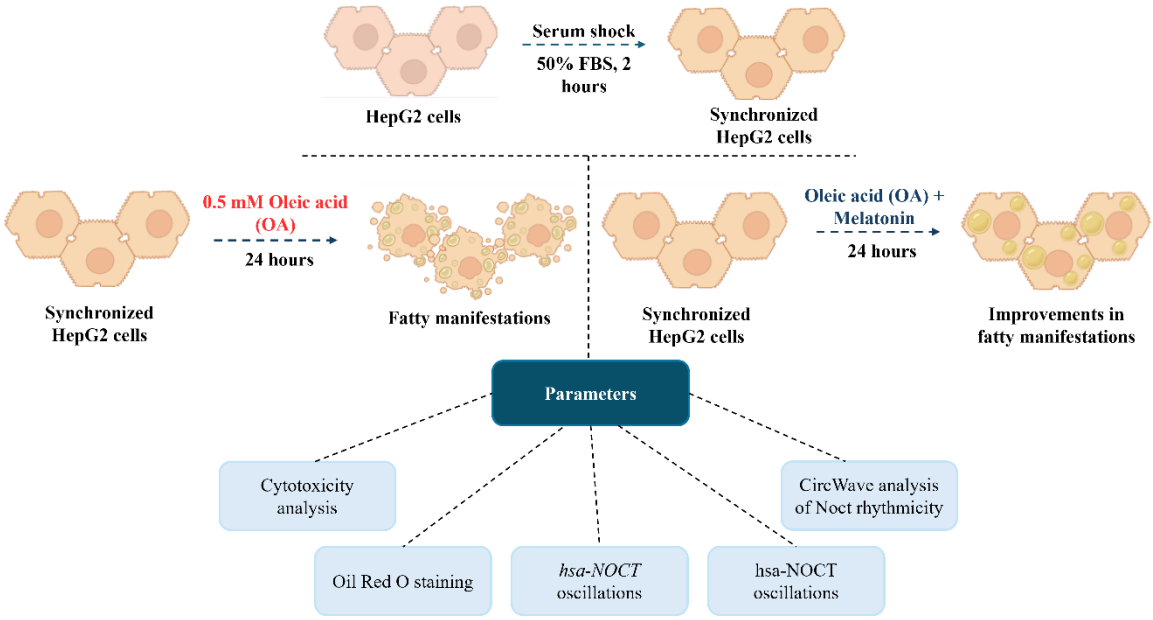
1. Cytotoxicity analysis to find the optimum concentration of Melatonin.
2. ORO staining to assess intracellular lipid accumulation.
3. Gene expression studies by qPCR: *mmu-Noct* and *hsa-NOCT* oscillations.

4. Protein expression studies by Western Blotting: hsa-NOCT oscillations.
5. CircWave analysis to scrutinize Noct rhythmicity.



***In vitro* studies**

(Time-point based study)



Results

Structure generation of mmu-NOCT and its validation by Ramachandran Plot analysis.

Since the full-length structure of mmu-NOCT is not available in protein databases, the iTASSER online tool was used to predict the structure of mmu-NOCT. iTASSER identified templates from a library of known protein structures, then using iterative assembly simulations constructed a complete atomic model, incorporating continuous structural fragments extracted from these template alignments. The resultant model was then compared with a database of proteins with known functions to derive potential functional insights. Herein, the generated structure of mmu-NOCT aligned with the available partial-length protein structures of NOCT (6BT1 and 6MAL.) [Fig. 2.1].

The predicted 3D protein structure was validated using a Ramachandran Plot in PROCHECK. The analysis revealed that 54.4% of the residues were located in the most favoured regions, 36.0% in additional allowed regions, and 19% in generously allowed regions, thereby validating the generated protein structure of mmu-NOCT [Fig. 2.2].

Molecular docking studies between mmu-NOCT and melatonin imply towards melatonin and Nocturnin interaction.

Molecular docking of melatonin (PubChem CID 896) with mmu-NOCT was carried out using AutoDock with default settings, and the results were visualized in PyMol. Melatonin was found to dock at the catalytic site of mmu-NOCT, exhibiting a strong binding affinity (-7 kcal/mol) and a low RMSD value (0), suggesting a potential interaction between melatonin and NOCT [Fig. 2.3].

Exogenous melatonin treatment restores hepatic *Noct* mRNA at ZT12 in high-fat-high-fructose (H) diet fed and/or chronodisrupted (CD) mice.

Disease-control groups (H, CD and HCD) recorded significant increments in hepatic *Noct* mRNA at ZT12. Exogenous intraperitoneal melatonin administration in H group (HM group) recorded significant decrement in hepatic *Noct* mRNA ($p<0.001$). CDM group recorded a non-significant decrement whereas; HCDM group recorded a significant increment ($p<0.05$) that was contradictory to other melatonin-treated groups [Fig. 2.4].

Hepatic *Noct* oscillations are subtly improved by melatonin treatment in H and/or CD mice.

Liver tissues of control and experimental groups were harvested at five different time points (ZT=0,6,12,18 and 24) to assess possible alterations in *Noct* oscillations. Intraperitoneal administration of melatonin in mice subjected to high-fat-high fructose diet (HM), chronodisruption (CDM), or a combination of both (HCDM) resulted in subtle decrements in hepatic *Noct* expression. HM group recorded hepatic *Noct* mRNA expression at ZT12, however, this increment was lower than that observed in the H group [Fig, 2.5 (a)]. This finding suggests that while *Noct* mRNA expression at ZT12 remained unaltered, melatonin (HM) accounted for a moderate improvement towards Control expression. Similarly, in CD and CDM groups, a shift in the *Noct* mRNA peak from ZT12 to ZT6 was observed. Although this peak persisted in CDM group, a significant decrement in the oscillation at ZT6 was recorded [Fig. 2.5 (b)]. In HCDM group, *Noct* mRNA expression showed a corrective shift in peak expression from ZT6 to ZT12, aligning with Control levels [Fig. 2.5 (c)].

Melatonin moderately improves hepatic *Noct* rhythmicity in high-fat-high-fructose diet (H) fed and/or chronodisrupted (CD) C57BL/6J mice.

Hepatic *Noct* rhythmicity was analysed by CircWave software wherein; percentage (%) relative amplitude, peak time (represented in the form of "Centre of Gravity- CoG") and cumulative *Noct* expression was calculated. Further, *Noct* mRNA oscillations were represented in the form of a combinatory sine-cosine waveform for better visualization.

CircWave analysis revealed varying effects of melatonin on hepatic *Noct* mRNA expression across different experimental groups [Fig. 2.6 (a), (b) and (c)]. In both HM [Fig. 2.7 (a)] and HCDM [Fig. 2.7 (c)] groups, a moderate decrement in the % relative amplitude of *Noct* mRNA was recorded. However, CDM group recorded a significant decrement ($p<0.05$) in % relative amplitude [Fig. 2.7 (b)],

Further, shifts in peak time of *Noct* mRNA oscillations were also calculated. In HM group, the CoG remained unchanged at ZT12 [Fig. 2.8 (a)], aligning with control *Noct* oscillations. In contrast, the CDM group exhibited a shift in peak to ZT6, indicating a notable alteration the rhythmicity of *Noct* mRNA [Fig. 2.8 (b)]. Interestingly, HCDM group recorded CoG at approximately ZT12 [Fig. 2.8 (c)].

In terms of cumulative *Noct* expression, HM group recorded a significant decrement ($p<0.05$) [Fig. 2.9 (a)]. However, in both CDM [Fig. 2.9 (b)] and HCDM groups [Fig. 2.9 (c)], the decrements in cumulative *Noct* expression were non-significant.

Melatonin enhanced cell viability and attenuated intracellular lipid accumulation in HepG2 cells subjected to OA treatment.

MTT assay was performed to determine the optimal concentration of melatonin wherein; HepG2 cells were treated with 0.5 mM OA and increasing concentrations of melatonin (5-1000µM) for 24 h. Cytotoxicity analysis revealed that melatonin alone did not exhibit significant toxicity up to 1000µM [Fig. 2.10 (a)]. However, melatonin at concentrations of 100µM and 200 µM accounted for a significant increment 0.5 mM OA-treated HepG2 cells [Fig. 2.10 (b)]. Based on these results, 100 µM concentration of melatonin was chosen for all the further experiments. Furthermore, intracellular lipid accumulation in HepG2 cells treated with a combination of OA and melatonin was assessed by Oil Red O (ORO) staining wherein; OA treated HepG2 cells recorded a significant increment in lipid accumulation and melatonin treatment accounted for a significant decrement ($p<0.05$) [Fig. 2.10 (c)].

Melatonin modulates Noct in HepG2 cells treated with OA.

Serum-synchronized HepG2 cells were subjected to OA alone and/or 100 μM melatonin for 24 h. OA treatment in HepG2 cells significantly upregulated Noct mRNA and protein expression ($p<0.01$) [Fig. 2.11 (b) and (c)]. Melatonin treatment accounted for a significant decrement in Noct mRNA ($p<0.01$) [Fig. 2.11 (a)] and protein expression ($p<0.05$) [Fig. 2.11 (b) and (c)], bringing their expression closer to those observed in the control group.

Melatonin modulates *NOCT* mRNA oscillations in OA-treated HepG2 cells.

Melatonin (100μM) along with OA (0.5 mM) for 24 h demonstrated a notable effect on *NOCT* mRNA expression over time. At 28 h and 32 h, there was a significant decrement in *NOCT* mRNA when compared to OA-treated cells, indicating that melatonin may counteract the OA-induced upregulation of *NOCT.* In addition to these decrements, moderate decrements in *NOCT* mRNA expression were recorded at 24 h, 36 h, 44 h and 48 h, suggesting that melatonin has a consistent dampening effect on *NOCT* expression, though not as pronounced at these intervals [Fig. 2.12].

CircWave analysis revealed a significant decrement ($p<0.05$) in the cumulative *NOCT* mRNA expression in OA + Mel. group compared to both control and OA-treated groups [Fig. 2.13 (d)]. Interestingly, the % relative amplitude of *NOCT* mRNA oscillations recorded a significant increment ($p<0.01$) in the OA + Mel. group [Fig. 2.13 (b)], contrary to the *in vivo* observations. However, the CoG shifted to 36 h (from 32 h in OA group), thus bringing the peak time comparable to control [Fig. 2.13 (c)].

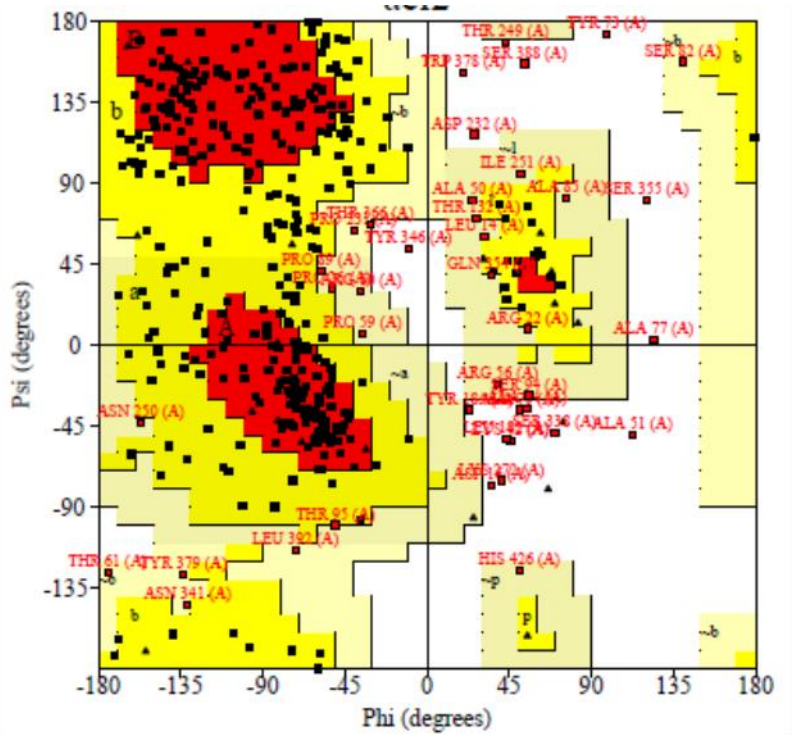
Melatonin alters NOCT rhythmicity in HepG2 cells subjected to OA.

Serum-synchronized HepG2 cells subjected to OA (0.5 mM) alone or in combination with melatonin (100μM) were harvested at different time points (24 h, 28 h, 32 h, 36 h, 40 h, 44 h and 48 h) for assessing possible changes in NOCT protein oscillations. Immunoblots of OA + Mel. treated group revealed

significant increment in NOCT expression at 28 h ($p<0.001$) and 32 h ($p<0.01$) when compared to OA group. Further, a significant decrement was recorded at 24 h ($p<0.05$), however the expression was higher than the OA group [Fig. 2.14 (a) and (b)].

Rhythmicity of NOCT protein was analysed by CircWave analysis wherein; significant alterations were observed [Fig. 2.15 (a)]. Melatonin treatment in OA-treated cells accounted for a significant decrement ($p<0.001$) in the % relative amplitude as compared to OA-treated cells [Fig. 2.15 (b)] . However, the CoG shifted to ~32 h in melatonin-treated group from ~48 h (in control) and ~36 h (in OA-treated group) [Fig. 2.15 (c)]. Interestingly, cumulative NOCT expression over the period of 24 h was significantly increased ($p<0.01$) in OA+Mel. group when compared to OA group [Fig. 2.15 (d)].

Figure 2.1 Full-length protein structure of mmu-NOCT generated by i-TASSER using the amino-acid sequence from NCBI.



Plot statistics

Residues in most favoured regions [A,B,L]	204	54.4%
Residues in additional allowed regions [a,b,l,p]	135	36.0%
Residues in generously allowed regions [~a,~b,~l,~p]	19	5.1%
Residues in disallowed regions	17	4.5%
Number of non-glycine and non-proline residues	375	100.0%

Figure 2.2. Ramachandran Plot analysis of the i-TASSER generated mmu-NOCT protein structure.

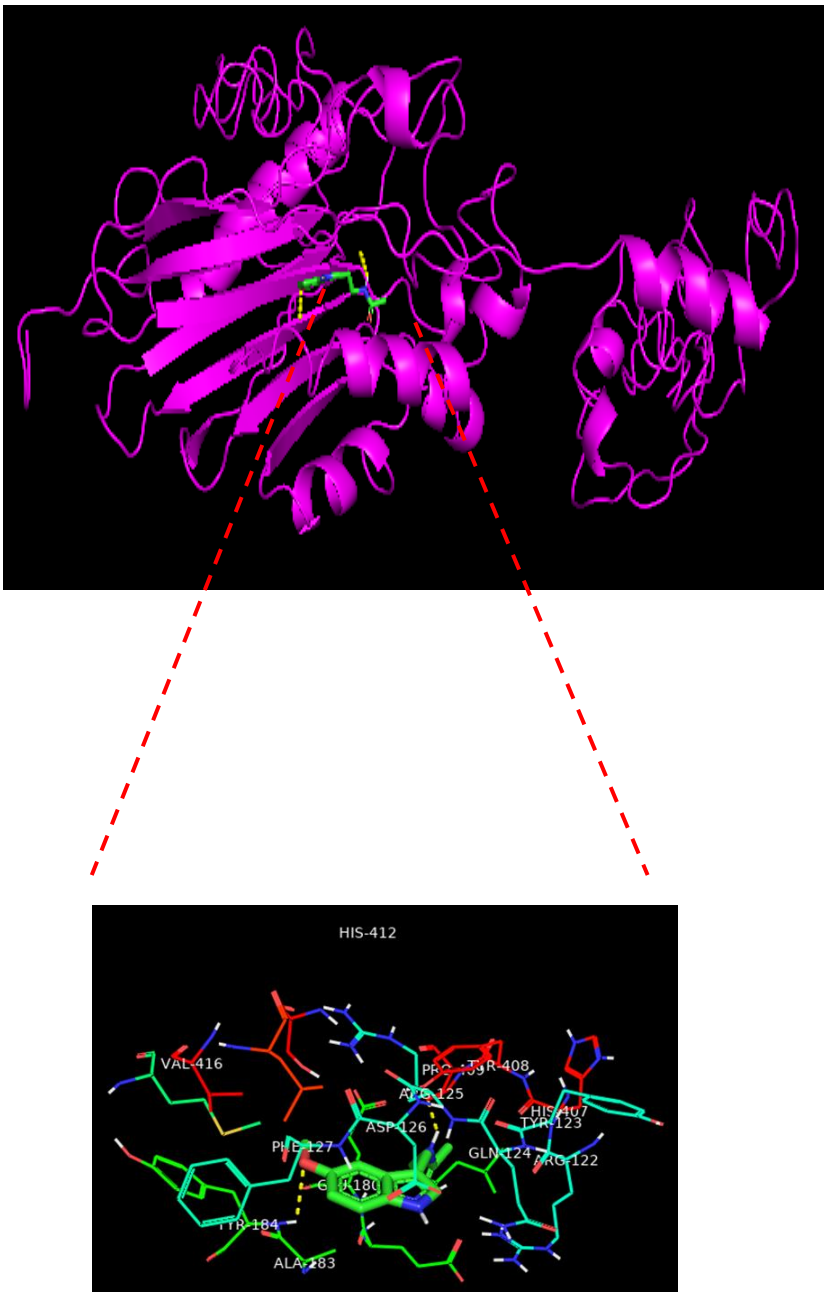


Figure 2.3. Molecular docking between mmu-NOCT and melatonin using AutoDock, Pyrx and PyMol.

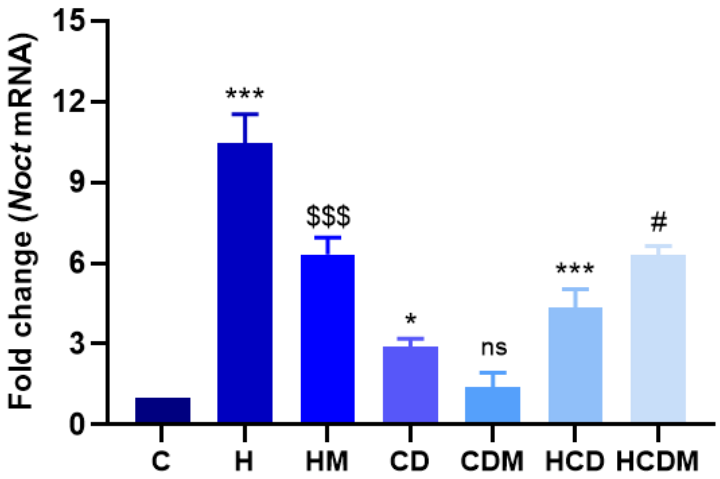


Figure 2.4. Melatonin mediates corrective changes in hepatic *Noct* mRNA at ZT12 in mice subjected to H and/or CD regime for 16 weeks. Melatonin (10mg/kg body weight) was administered intraperitoneally from 9th week till the end of 16 weeks. Results are represented as mean ± SD. *p<0.05, ***p<0.001 when H, CD and HCD are compared with Control; $$$p<0.001 when HM is compared with H group and #p<0.05 when HCDM is compared with HCD group.

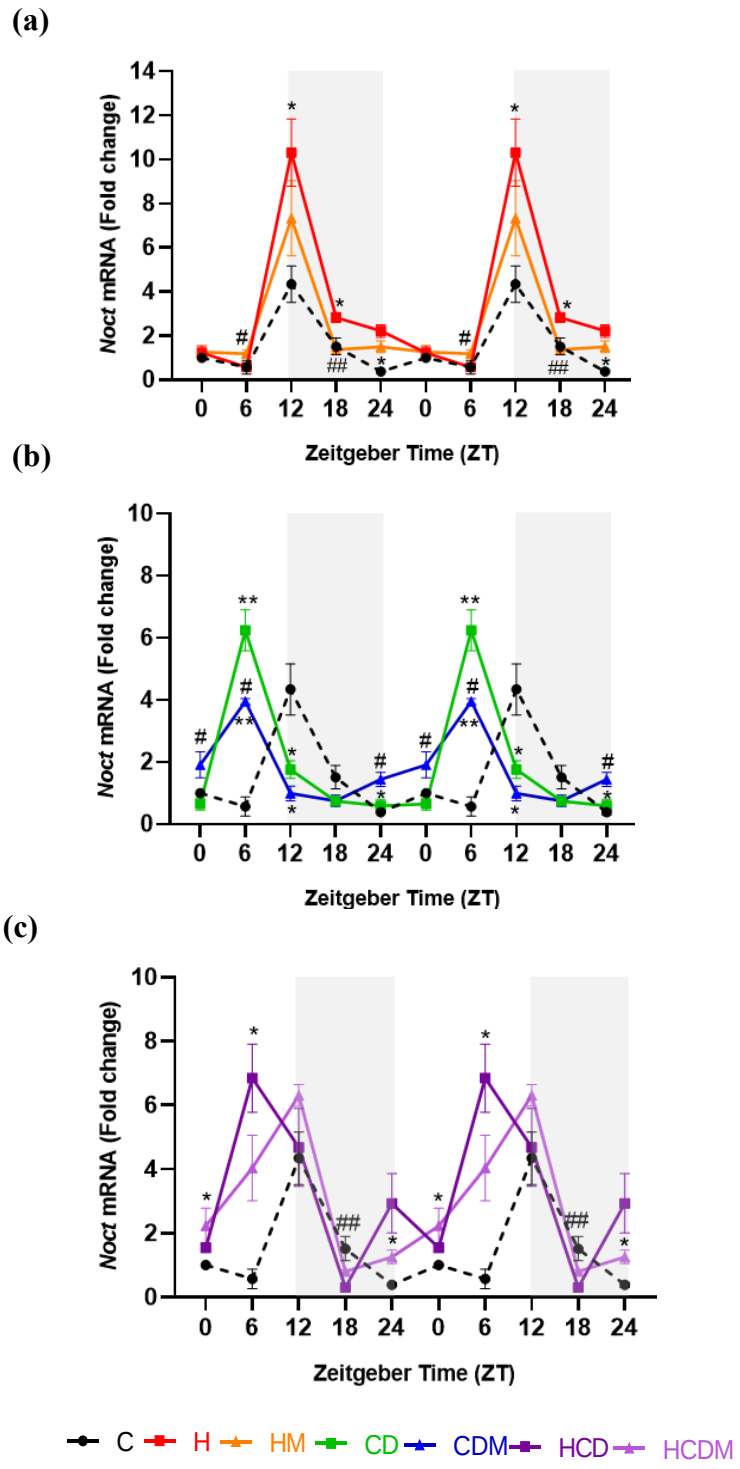


Figure 2.5. Melatonin subtly hepatic *Noct* mRNA oscillations in: (a) H mice; (b) CD mice and (c) HCD mice. Results are expressed as mean ± SD. *p<0.05, **p<0.01 when disease-control groups (H, CD and HCD) are compared with Control and #p<0.05, ##p<0.01 when melatonin-treated groups (HM, CDM and HCDM) are compared with H, CD and HCD respectively.

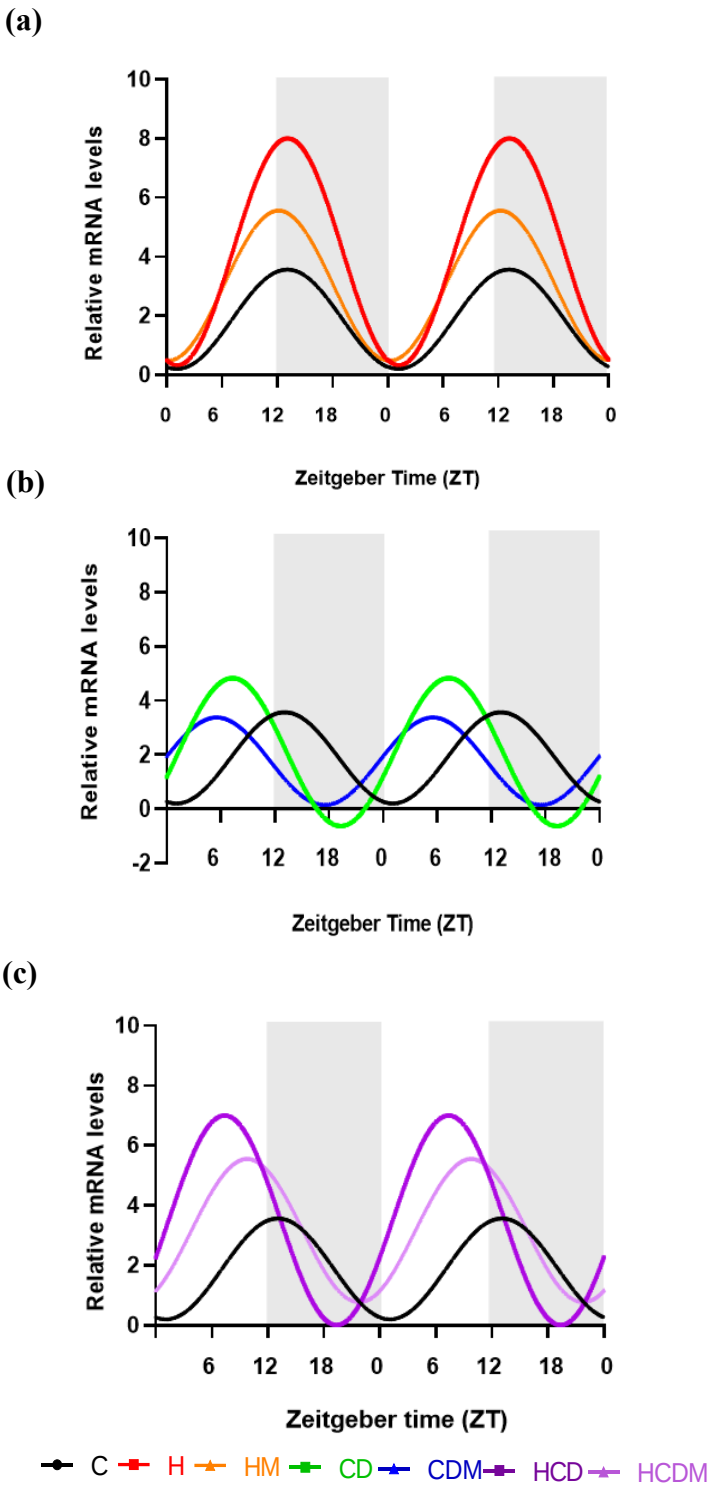


Figure 2.6. Hepatic *Noct* oscillations visualized in the form of a combinatory sine-cosine waveform, as calculated by CircWave software. Note the subtle modulations in *Noct* mRNA oscillations imparted by exogenous melatonin administration.

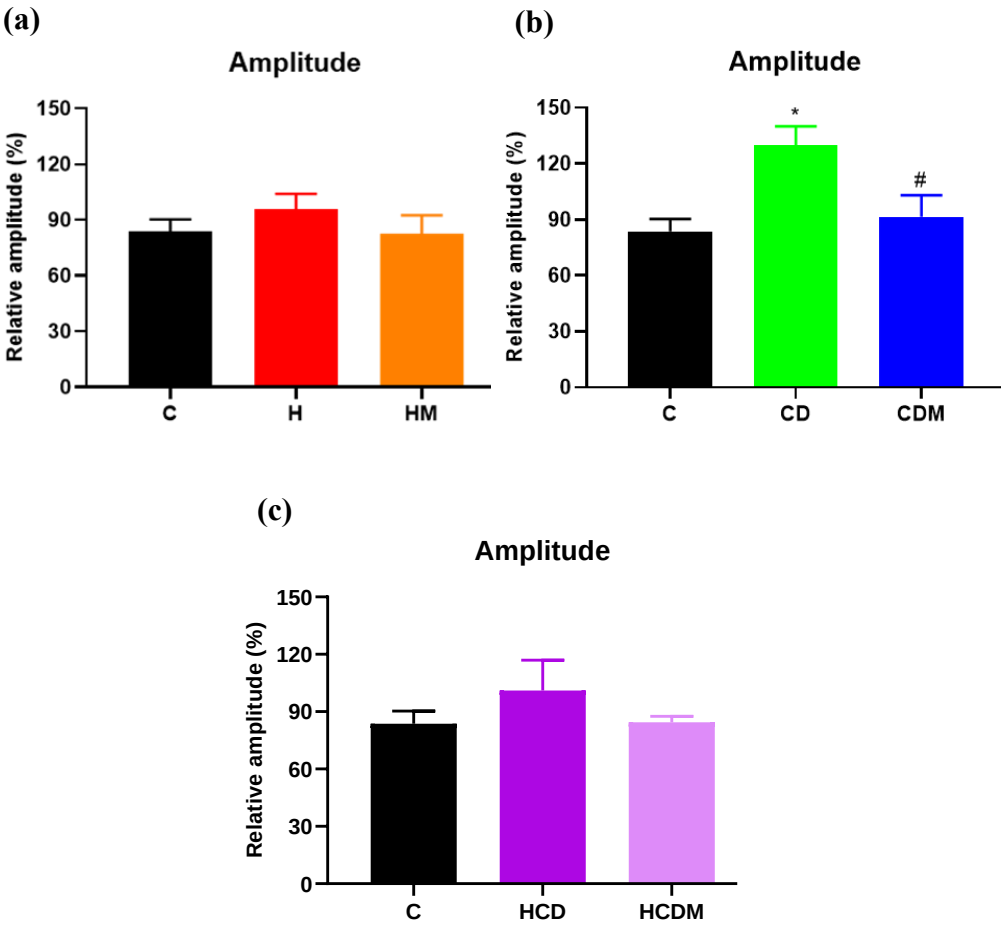


Figure 2.7 Percentage (%) relative amplitude of hepatic *Noct* oscillations in melatonin-treated H and/or CD mice, deduced from CircWave analysis. Results are expressed as mean ± SD. *p<0.05 when CD group are compared with Control and #p<0.05 when CDM is compared with CD group.

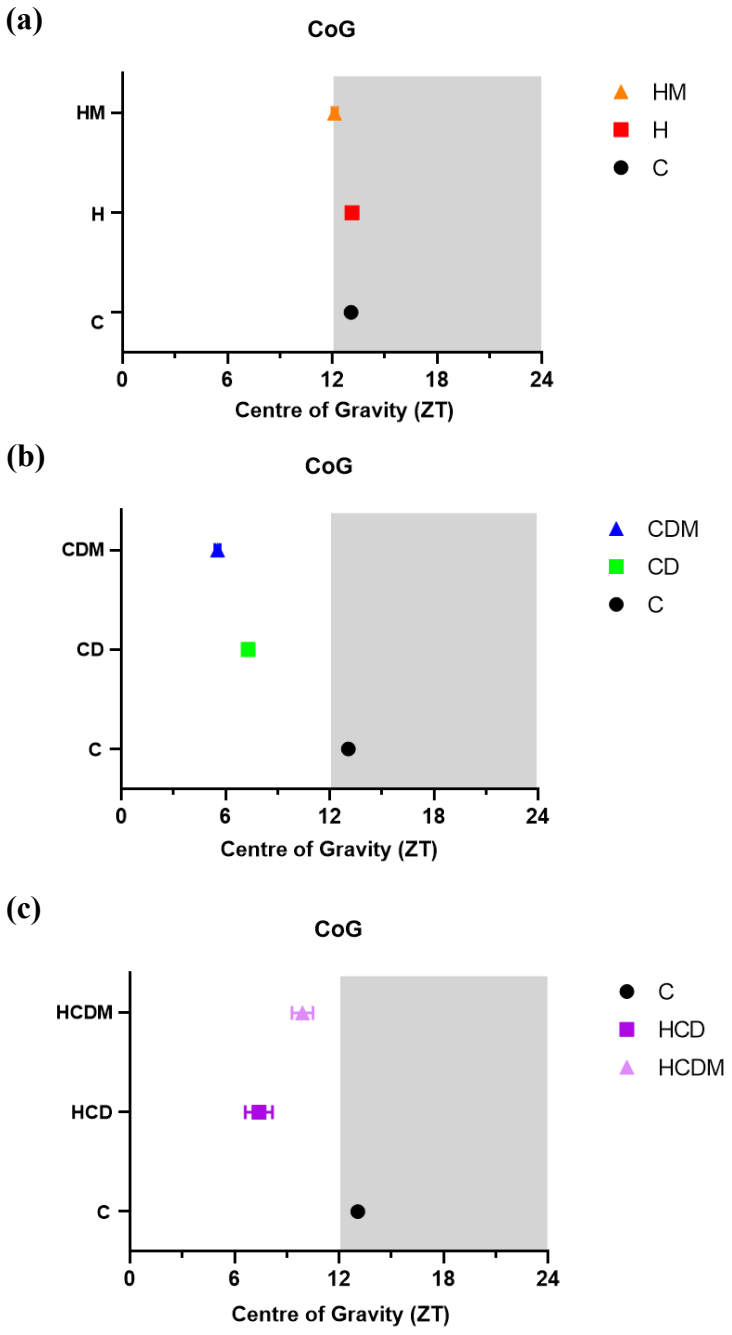


Figure 2.8. Peak time (represented as Centre of Gravity, CoG) of hepatic *Noct* mRNA oscillations in melatonin-treated H and/or CD mice. Grey shaded region represents dark phase (ZT12 to ZT24). Note the moderate improvements in the peak time of *Noct* imparted by exogenous melatonin treatment.

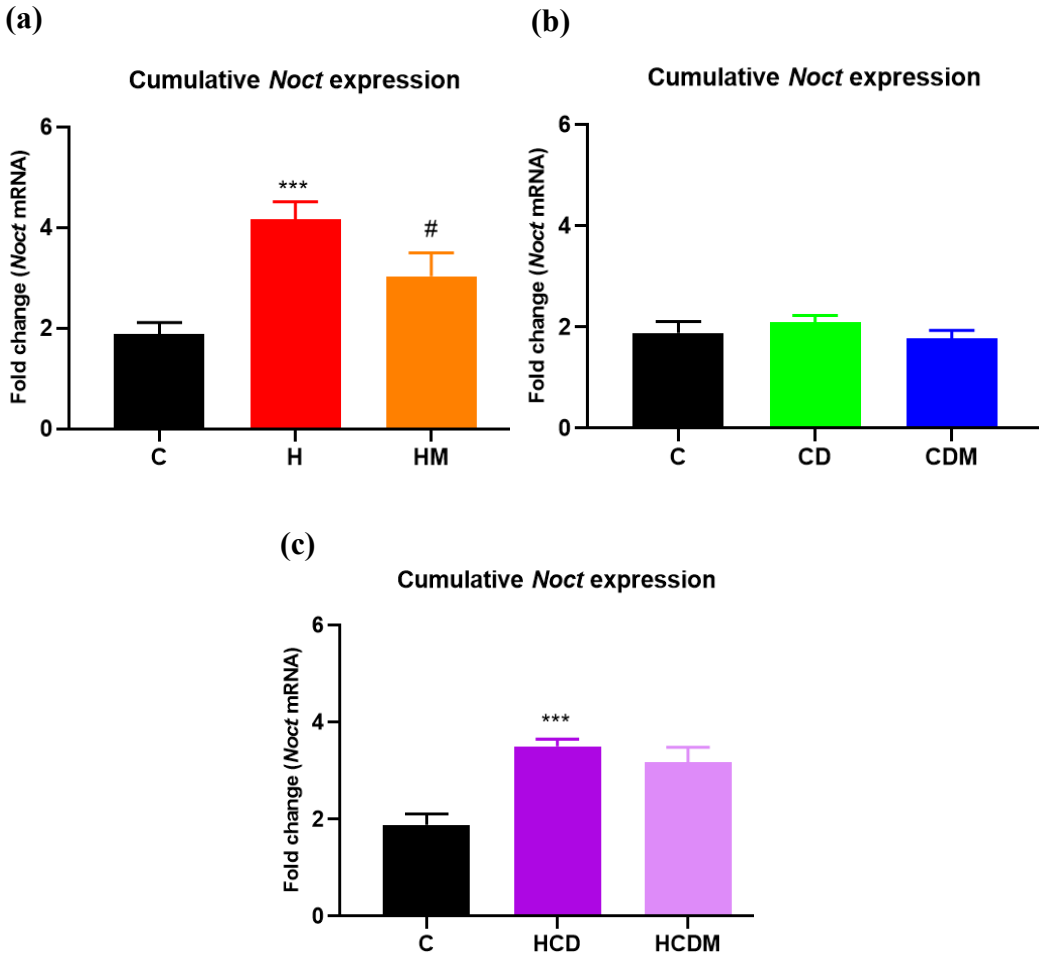


Figure 2.9. Alterations in cumulative *Noct* mRNA expression in the liver tissues of mice subjected to H and/or CD regime for 16 weeks and melatonin-mediated improvements as analysed by CircWave software. Results are expressed as mean ± SD. ***p<0.001 when H and HCD groups are compared with Control and #p<0.05 when HM group is compared with H group,

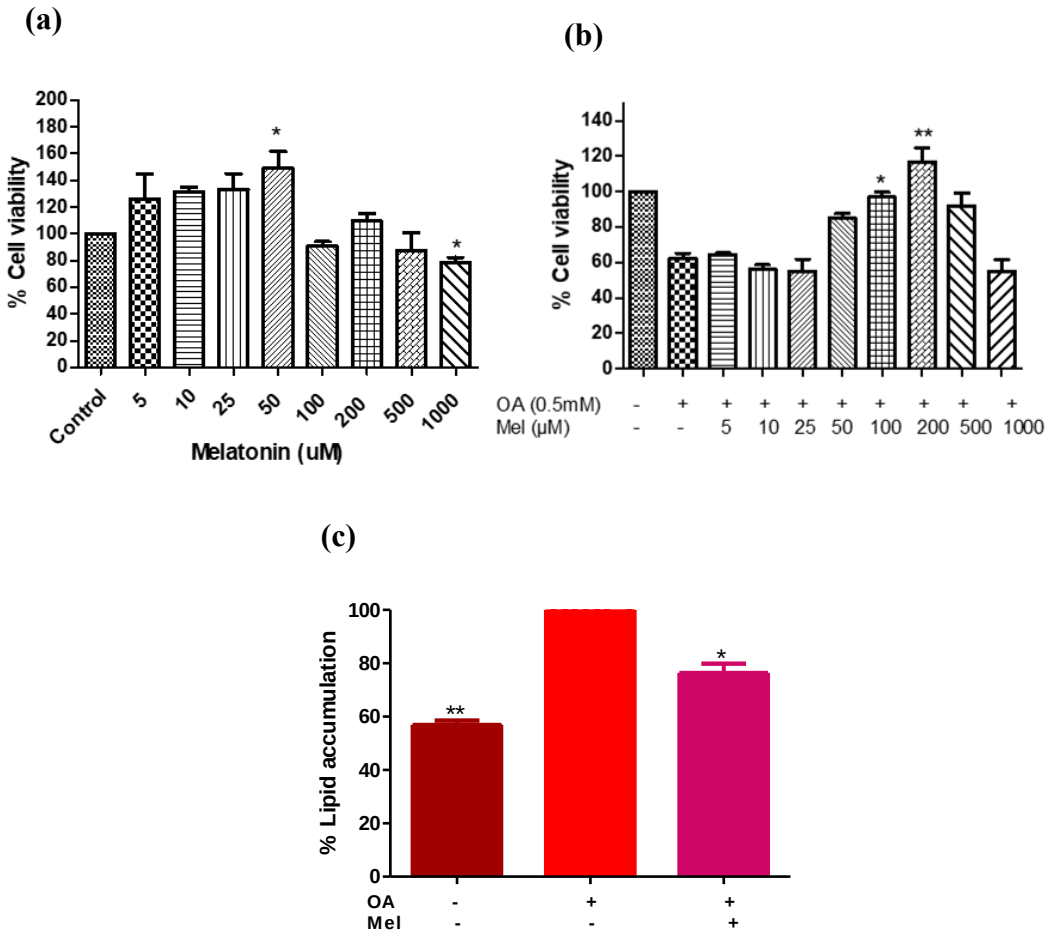


Figure 2.10. (a) and (b) Dose standardization of melatonin in HepG2 cells by MTT assay wherein; HepG2 cells were treated with increasing concentrations of melatonin (5-1000μM) alone or in combination with 0.5 mM OA for 24 h; (c) Lipid droplet accumulation in OA alone and/or 100 μM melatonin assessed by Oil Red O (ORO) staining. Results are expressed as mean ± SD. *p<0.05 and **p<0.01 when treatment groups are compared with Control.

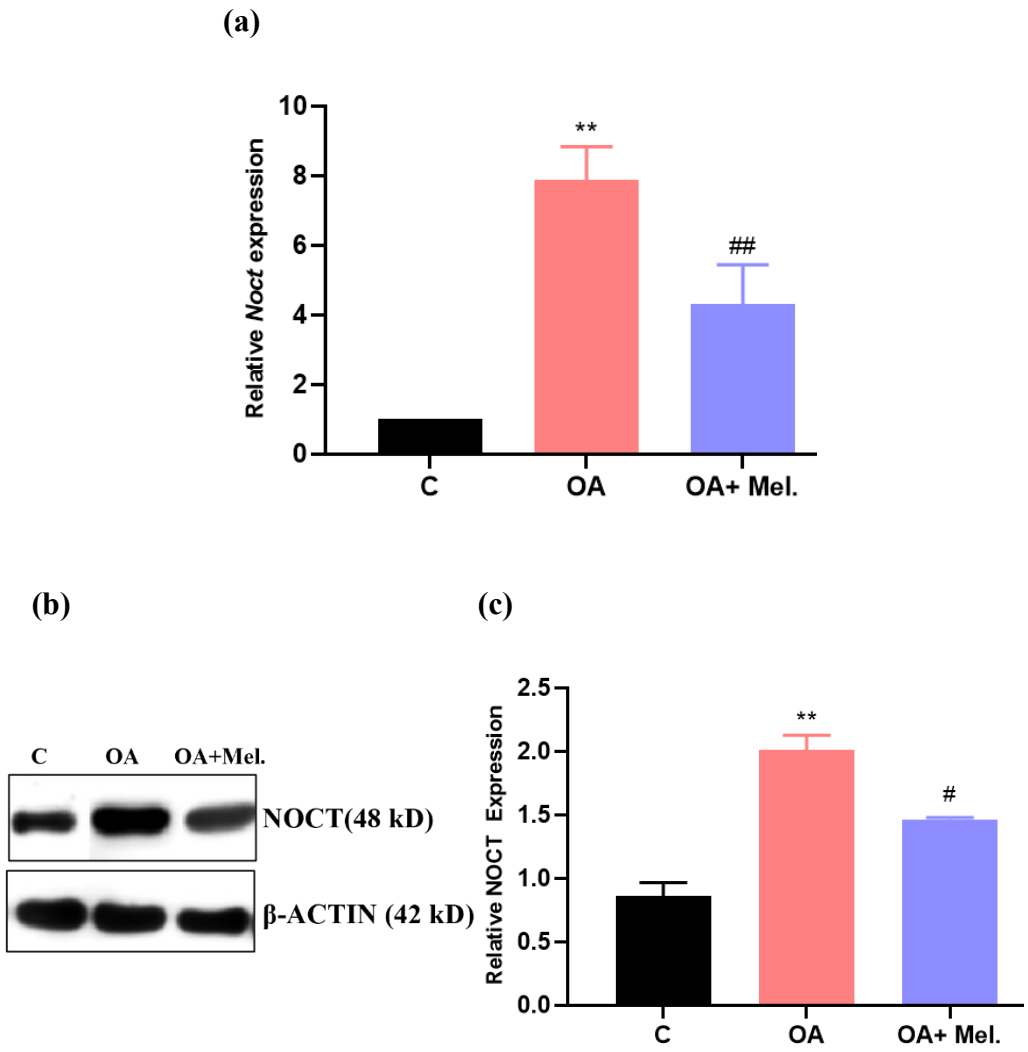


Figure 2.11. Melatonin improves Noct (mRNA and protein) expression in OA-treated HepG2 cells: (a) *NOCT* mRNA expression in HepG2 cells treated with OA and/or Melatonin for 24 h; (b) and (c) NOCT immunoblots and its quantification. Results are expressed as mean ± SD. **p<0.01 when OA group is compared with Control; #p<0.05 and ##p<0.01 when OA + Mel. group is compared with OA group (n=3).

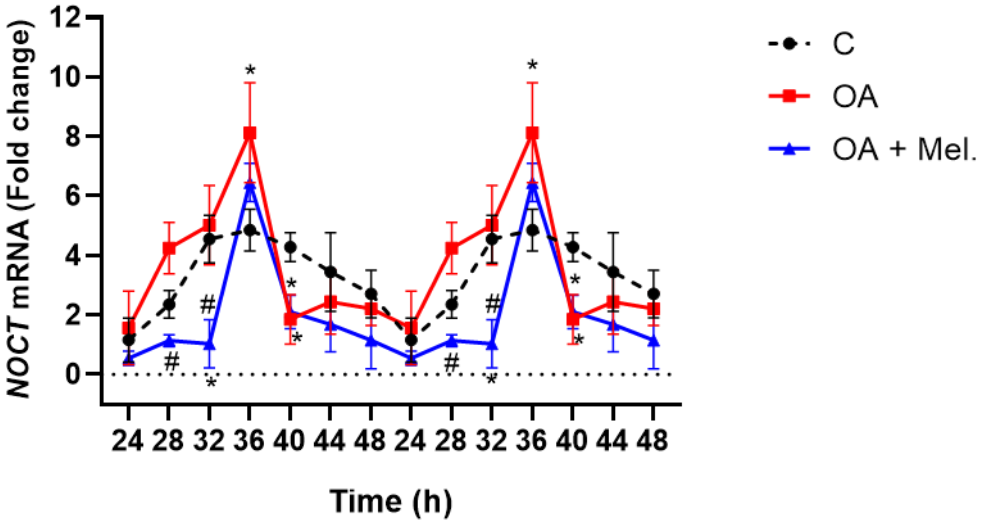


Figure 2.12. Serum-synchronized HepG2 cells were subjected to 0.5 mM OA alone or in combination with 100µM Melatonin for 24 h and subsequently harvested at different time intervals (24 h, 28 h, 32 h, 36 h, 40 h, 44 h and 48 h) for assessing possible changes in *NOCT* mRNA oscillations. Results are expressed as mean ± SD. *p<0.05 when OA group is compared with Control and #p<0.05 when OA + Mel. group is compared with OA group (n=3 at each time point).

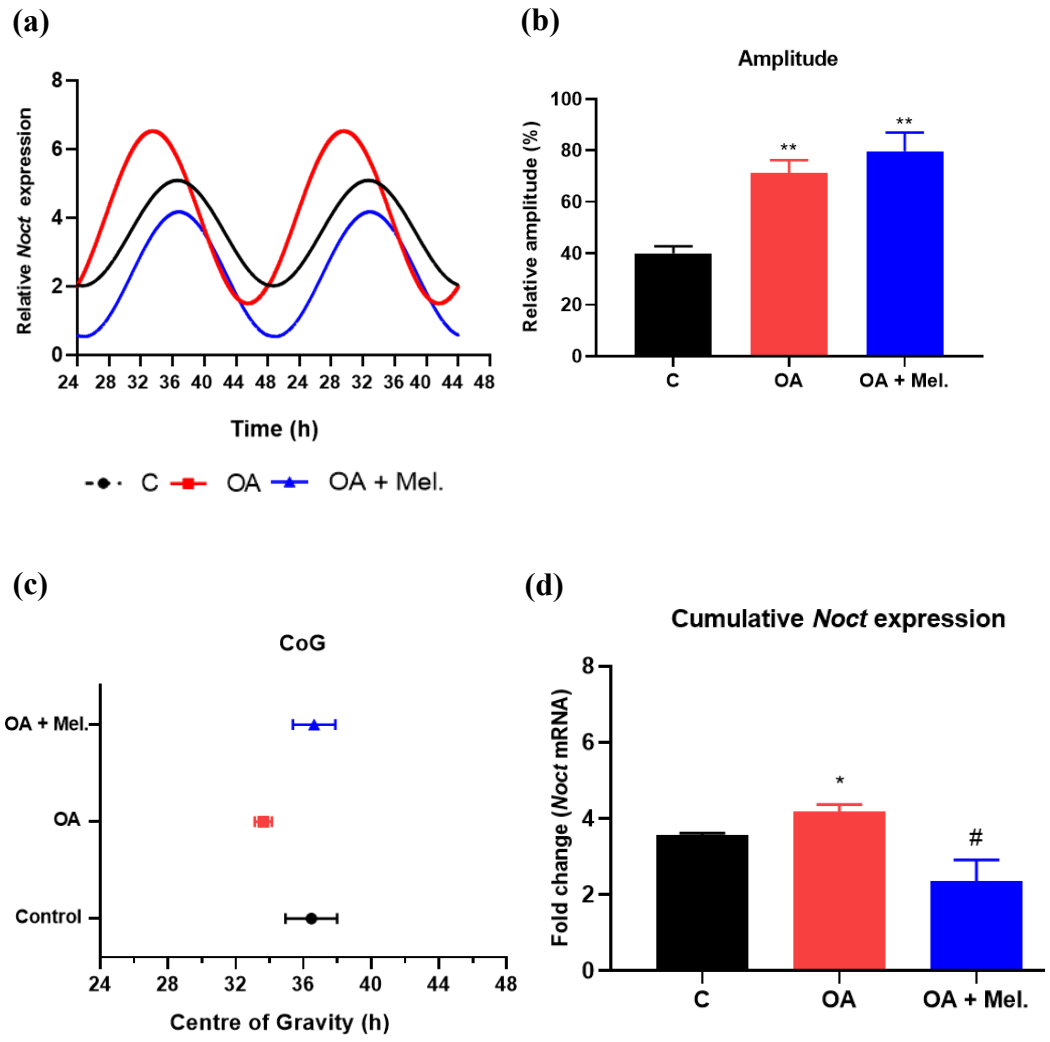


Figure 2.13. CircWave analysis of *NOCT* rhythmicity in OA and/or melatonin treated HepG2 cells: (a) *NOCT* mRNA oscillations in the form of a sine-cosine wavefunction; (b) % relative amplitude; (c) Peak time represented as "Centre of Gravity (CoG) and (d) Cumulative *NOCT* expression. Results are expressed as mean ± SD. *p<0.05, **p<0.01 when OA group is compared with Control and #p<0.05 when OA+ Mel. group is compared with OA group.

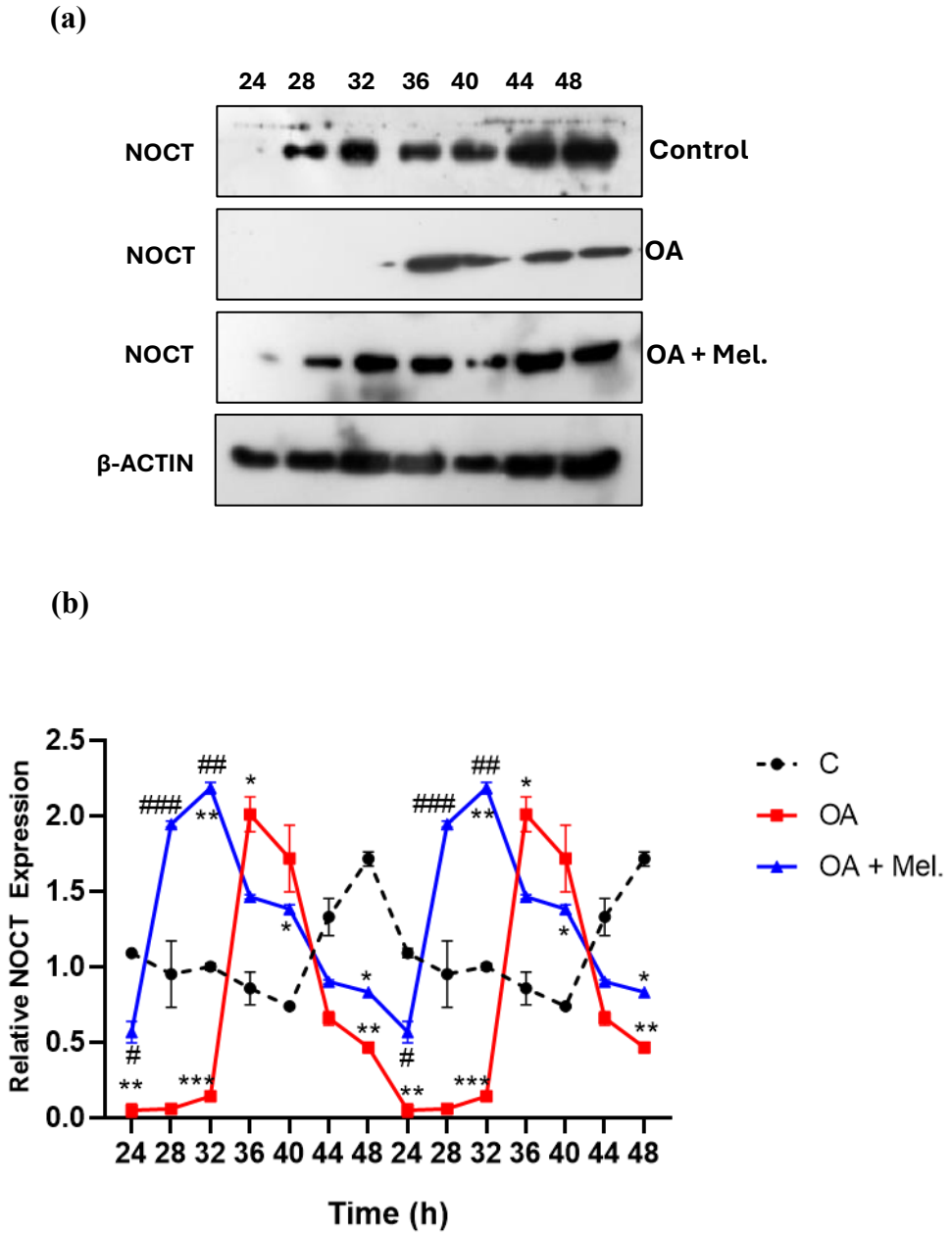


Figure 2.14. Serum-synchronized HepG2 cells were treated with 0.5 mM OA and/or 100 µM Melatonin for 24 h and were subsequently collected to investigate possible alterations in NOCT protein oscillations by Westen Blotting. (a) Immunoblots of NOCT in different experimental groups (b) Quantification of NOCT was performed by normalizing the data with β-ACTIN. Results are expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001 when OA group is compared with Control and #p<0.05, ##p<0.01 and ###p<0.001 when OA + Mel. group is compared with OA group. (n=3 at each time point).

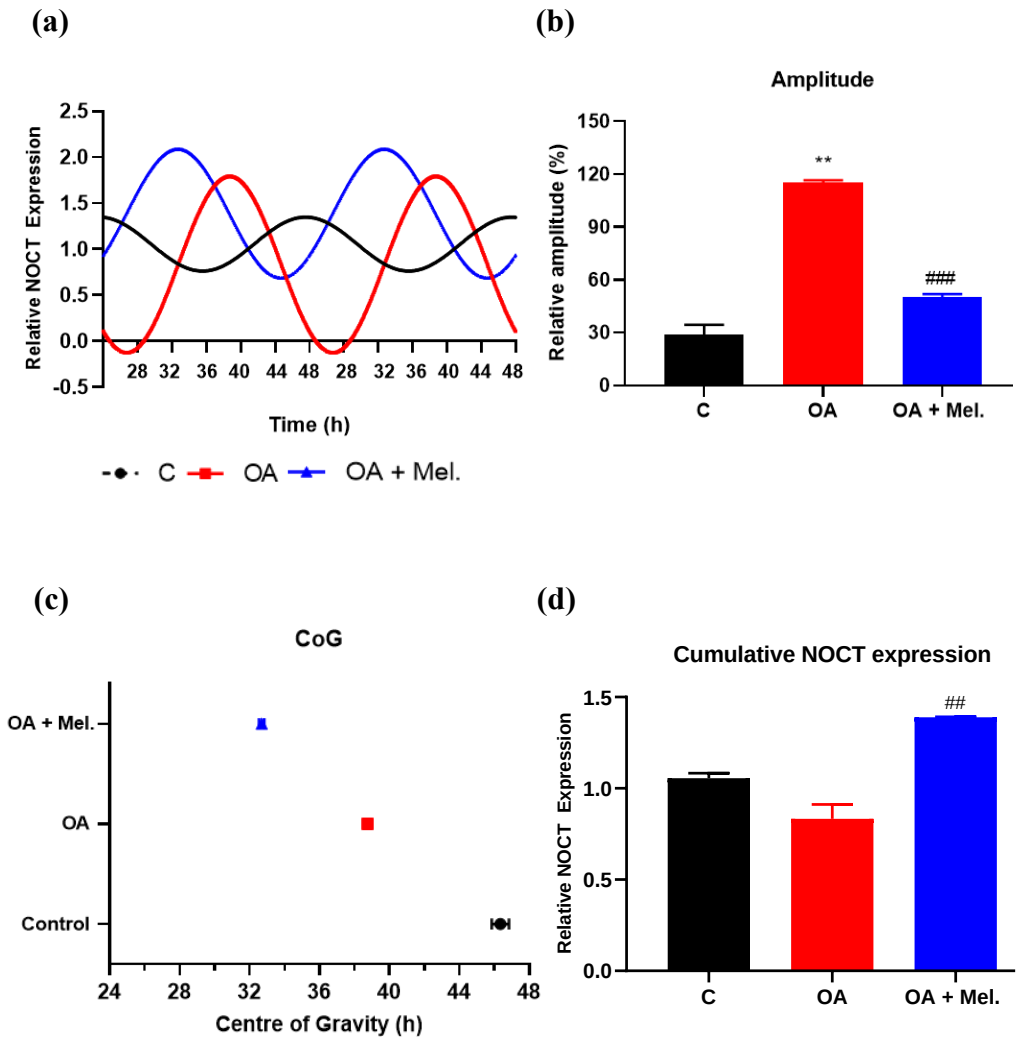


Figure 2.15. NOCT protein oscillations in HepG2 cells subjected to 0.5 mM OA alone or in combination with 100µM Melatonin were analysed by CircWave software to assess the potential changes in NOCT rhythmicity: (a) NOCT protein oscillations visualized in the form of a sine-cosine wavefunction; (b) % relative amplitude; (c) Peak time represented in the form of CoG and (d) Cumulative NOCT expression. Results are expressed as mean ± SD. **p<0.01 when OA group is compared with Control and ##p<0.01, ###p<0.001 when OA + Mel. group is compared with OA group.

Discussion

Melatonin is a pleiotropic neurohormone that is an important component of the circadian clock machinery (Mahmood, 2019). Since the past decade, melatonin has been extensively investigated for its hepatoprotective effects in lifestyle disorders such as NAFLD and NASH (Rezayat et al., 2021b; Terziev & Terzieva, 2023). Previous studies from our laboratory had reported that exogenous melatonin improved fatty manifestations in liver caused due to HFD alone or in combination with photoperiodic shifts induced CD via re-entrainment of the hepatic core clock genes and corrections in the Nrf2-HO1 pathway intermediates (Joshi et al., 2021). Further, the effects of melatonin are also based either on its ability to act as a free radical scavenger or its binding to specific protein targets. Till date, more than 15 proteins, such as enzymes, pores and transporters have been suggested to interact with melatonin at sub-nanomolar to millimolar melatonin concentrations (Liu et al., 2019).

Various studies and the findings of the previous chapter have implied that Noct plays a key role in hepatic lipid metabolism (Douris et al., 2011; Stubblefield et al., 2012, 2018)and alterations in hepatic Noct oscillations contribute to NASH pathology. Further, similar to melatonin, Noct is robustly rhythmic and shares a similar phasing (Hardeland, 2014b), thus, a melatonin-Nocturnin interplay can possibly be an important player in NASH pathology that calls for a detailed study. This chapter aimed to decipher the possible melatonin-Noct interplay in NASH. In the present study, molecular docking studies had revealed that melatonin showed high binding affinity with NOCT as evidenced by high binding affinity score and low RMSD. Further, exogenous melatonin administration had accounted for a significant improvement in liver function markers, lipid profile as well as histopathological observations that were in accordance with our previously published reports. In another study, exogenous melatonin has been shown to alleviate HFD-induced steatotic changes in liver by inhibition of the NLRP3 inflammation in db/db mice (Yu et al., 2021). Melatonin was also reported to improve NAFLD through MAPK/JNK/P38 signalling pathway in diet-induced obese mice (Sun et al., 2016). In the present study, melatonin-induced improvements in serum parameters and histopathology were

associated with a significant decrement in hepatic *Noct* mRNA expression in the HM group, whereas the CDM group showed a non-significant decrement. Interestingly, the HCDM group exhibited a significant increment in hepatic *Noct* mRNA.

To fully understand the extent of melatonin-Noct interactions, hepatic *Noct* mRNA was assessed at five time points and our findings revealed subtle decrements in *Noct* expression with moderate decrements in % relative amplitude in HM and HCDM groups, indicating that melatonin had a corrective, though not drastic effect on the rhythmicity of *Noct.* However, significant decrement in % relative amplitude in CDM group implies that the effect of melatonin on *Noct* rhythmicity was more pronounced in the said experimental group. Further, exogenous melatonin treatment accounted for moderate (HM group) to significant peak shifts (CDM and HCDM groups) in *Noct* expression, implying that melatonin partially restores the temporal misalignment caused due to H and/or CD. Additionally, a significant decrement in the cumulative *Noct* expression was recorded in the HM group, suggesting that melatonin had a marked impact on the overall *Noct* mRNA expression in the context of diet-induced metabolic alterations. However, the non-significant decrements in the said parameter in the CDM and HCDM groups suggest that while melatonin modulated hepatic *Noct* oscillations, its effects on overall *Noct* expression were less pronounced under conditions of CD.

Several studies have highlighted different mechanisms through which melatonin improves OA/palmitic acid (PA)-induced HepG2 model of NASH. Melatonin pre-treatment significantly decreased intracellular TG and cholesterol accumulation by promoting phosphorylation of AMPK and ACC in OA-treated HepG2 cells. Additionally, melatonin increased expression of lipolytic genes such as *PPARα* and *CPT-1*, while decreasing the expression of lipogenic genes including *SREBP1-c, FAS* and *SCD-1* (Mi et al., 2018). Another study had demonstrated that melatonin improved insulin resistance and hepatic steatosis in PA-treated HepG2 cells by downregulating ER stress and alpha-2-HS-glycoprotein levels (Heo et al., 2018).

Findings of the current study revealed that 100 µM concentration of melatonin along with 0.5 mM of OA accounted for a significant decrement in intracellular lipid accumulation that was evident through ORO staining and in accordance with our previously published reports. Additionally, melatonin treatment significantly decreased Noct mRNA and protein expression as compared to OA-treated HepG2 cells, thus offering new insights into the modulatory effects of melatonin in NASH, particularly through regulation of hepatic *Noct* expression.

In this study, melatonin treatment of HepG2 cells resulted in a significant reduction in intracellular lipid accumulation, as observed through ORO staining. Additionally, melatonin treatment significantly decreased *Noct* mRNA and protein levels compared to OA-treated HepG2 cells. These findings offer new insights into the modulatory effects of melatonin in MASLD, particularly through the regulation of hepatic *Noct* expression.

To gain an in-depth understanding of melatonin-mediated corrections in Noct, Noct mRNA and protein oscillations were assessed at different timepoints and analysed using CircWave software. A significant decrement in *NOCT* mRNA was recorded at all time points along with significant decrements in cumulative *NOCT* expression, implying that over time, melatonin effectively downregulates the overall *NOCT* expression induced by OA, thereby highlighting its potential as a modulator of *NOCT* expression under conditions of metabolic dysfunction. However, the % relative amplitude was significantly increased in OA + Mel. group as compared to OA group and this increment suggests that melatonin may enhance the rhythmic alterations in *NOCT* expression in the presence of OA, potentially stabilizing or strengthening the circadian rhythmicity of *NOCT* expression, though it dampens the cumulative expression. Contrarily, melatonin treatment in HepG2 cells accounted for only a moderate decrement in NOCT protein and recorded a higher cumulative expression. Findings showcased herein justify the time-point based assessment of hepatic Noct oscillations as the same were found to be only partially restored by exogenous melatonin treatment and further provide prima-facie evidence of melatonin-mediated alterations in hepatic Noct oscillations in NASH pathology.