Introduction

The function of the circadian clock has been well established now to be beyond a mere regulation of the clock genes(Reinke & Asher, 2019). The biological clock regulates a wide range of physiological activities(K. Xu et al., 2011). Classically, Bmal1 and Clock interact to form a heterodimer which translocates to the nucleus and activates transcription of *cry* and *per* genes. The molecular clock-generated circadian rhythms are controlled by an auto-regulatory transcriptional and translational feedback loops. Transcription factors Bmal1 and clock regulate period and cryptochrome genes with 24 h periodicity(Buhr & Takahashi, 2013). Several studies have revealed a connection between circadian clock and lifestyle disorders(Farhud & Aryan, 2018). High fat diet leads to alterations in the expression and cycling of the canonical circadian clock genes, nuclear receptors and clock-controlled genes(Kohsaka et al., 2007). Also, there are reports of a marked increase in the expression of Per1 and Per2 and suppression of clock expression in chronic jetlagged (Iwamoto et al., 2014). Chronic jetlag has also been reported to aggravate steatohepatitis and even induce hepatocellular carcinoma in $Fxr^{-/-}$ mice(Kettner et al., 2016).

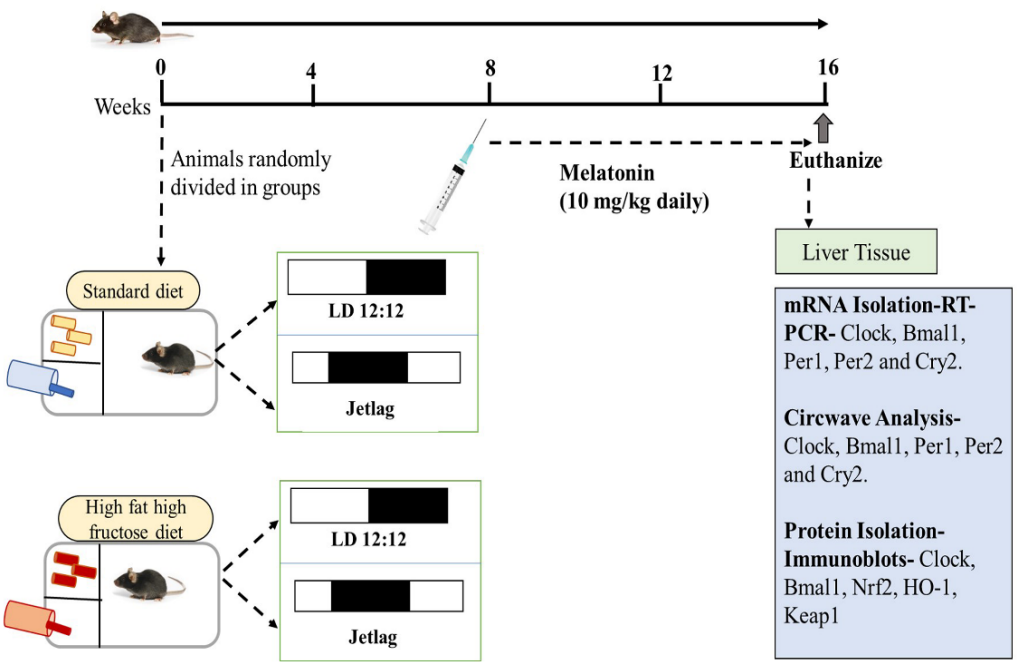
The pathophysiology of NAFLD is best explained by the multiple hit model, wherein oxidative stress plays a primary role in initiating hepatic damage(Buzzetti et al., 2016). The cellular response against oxidative stress is mainly regulated by the Kelch-like ECH-associated protein 1 (Keap1) - nuclear factor erythroid 2-related factor 2 (Nrf2) - antioxidant response elements (ARE) genes(Kaspar et al., 2009). In conditions of NAFLD, Nrf2 has been shown to be downregulated which is accompanied by an increased oxidative stress(Upadhyay et al., 2020). Xu et al. (2012) investigated the expression patterns for antioxidant genes in mice liver and they found that Nrf2

expression pattern was highest during daytime and showed a peak at 18:00, hence proving circadian variations of Nrf2 could modulate cell response to oxidative stress(Y.-Q. Xu et al., 2012). Circadian-clock-dependent regulation of redox status, ROS homeostasis, and antioxidant defense is studied by various research groups wherein they have shown preliminary evidence suggesting Bmal1 as a transcriptional regulator of Nrf2(Early et al., 2018; Pekovic-Vaughan et al., 2014). Also, there are reports which suggest Nrf2 to be under circadian control in diseases like pulmonary fibrosis and diabetes(Pekovic-Vaughan et al., 2014). A recent study has demonstrated that synchronized circadian clock facilitates the protective role of Nrf2 in terms of a faster and more efficient defensive response against environmental insults(Frigato et al., 2020).

Melatonin attenuates dysregulation of the circadian clock pathway in mice with CCl_4 induced liver fibrosis(González-Fernández et al., 2018). One of the recent findings have shown a regulatory role of melatonin in lipid homeostasis and clock gene regulation in mice exposed to constant light(Hong et al., 2020). Melatonin was also shown to be efficiently restore the altered redox homeostasis in rodent model exposed to artificial light(Verma et al., 2020).

This study investigates the oscillatory pattern of hepatic clock genes and antioxidant genes in HFHF and/or JL induced NAFLD. Role of melatonin in synchronising the disturbed oscillations was also studied for its therapeutic potential.

Experimental design



Results

Melatonin resynchronizes hepatic clock gene expression pattern desynchronized by HFHF and/or JL mice.

A time point study (ZT=0, 6, 12, 18, 24 h) was conducted to assess possible alterations in core clock genes (*Clock, Bmal1, Per1, Per2 and Cry2*) in liver of control and experimental groups. Both *Bmal1* and *Clock* mRNA peaked at ZT6 in liver of control mice. HFHF feeding resulted in a flattened peak of *Bmal1* and *Clock* mRNA. Melatonin treatment accounted for improvement in *Bmal1* oscillation at ZT6 whereas the clock showed 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 (Fig. 4.1). A positive shift in amplitude observed in circwave analysis further justified restoration of *Bmal1* oscillation by melatonin treatment (Fig. 4.4 and 4.5). Similarity in oscillations of *Bmal1* and *Clock* genes in 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 (Fig.4.2). Exogenous melatonin appears to restore the oscillations of said genes as evidenced by the circwave analysis (Fig. 4.4 and 4.5). 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 moderate restoration of peak in *Clock* mRNA at ZT12 (Fig. 4.3). Thus, HFHF reported change only in the positive arm of transcriptional-translational feedback loop, whereas JL and HFHF+JL resulted in more prominent disruption of circadian clock. Overall,

melatonin supplementation alleviated 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*.

Melatonin improves amplitude and peak time in hepatic clock gene expression in HFHF and/or JL subjected mice.

Based on the time course data, the expression values were analyzed for rhythmicity using circwave software. Amplitude and peak time were considered as major factors for describing rhythmicity. Bmal1, Clock, Per1 and Cry2 genes peaked at beginning of light phase (ZT=6h), while Per2 peaked at ZT=24h (Fig.4.4). HFHF resulted in variation in peak time of Clock and Per1 while limited variation was observed in Bmal1, Per2 and Cry2. No variation was observed in peak time of mRNA of Bmal1, Clock, Per1 and Cry2 in mice subjected to JL alone, while Per2 mRNA peak showed a shift. However, HFHF and JL group showed greater variation in peak time of Clock, Per1 and Cry2. Exogenous Melatonin treatment modestly restored the peak time of said genes. Further, HFHF resulted in decreased amplitude of Bmal1, Per2 and Cry2 genes(Fig.4.4). However, JL and HFHF+JL resulted in decreased in amplitude of all the core clock genes.

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 liver of control mice showed 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 restoration of ZT6 peak of Bmal1 whereas the peak of ZT12 of Clock remained unchanged (Fig. 4.7). Further, Nrf2 and Keap1 proteins showed peak

at ZT6 and HO-1 peaked at ZT12 in liver of healthy mice. Feeding of HFHF diet caused flattening of ZT6 peak of Nrf2 and Keap1. Also, 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(Fig. 4.7). JL induced significant distortion in 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 (Fig. 4.8). A combination of HFHF+JL caused lack of oscillations in Bmal1 as evidenced by flattened curve. Also, Clock oscillation at ZT6 were unchanged but ZT18 was lacking. Further, Nrf2 and HO-1 proteins witnessed the flattening of the curve in 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 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 (Fig 4.9).

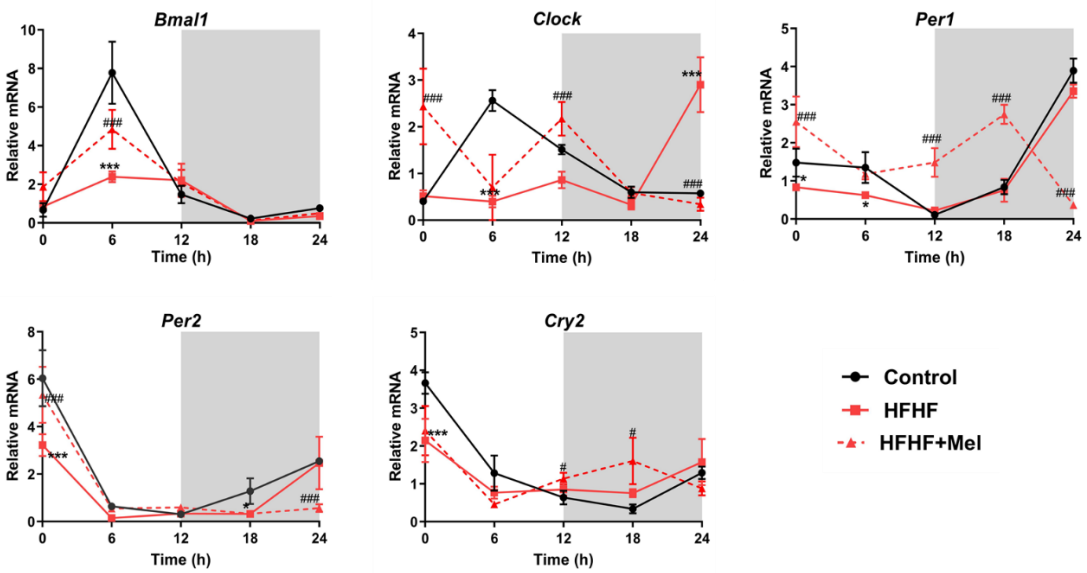


Figure 4.1: Melatonin reprograms the circadian clock gene in liver of HFHF mice as evidenced by their mRNA profiles. Grey shaded area indicates dark phase (ZT12 to ZT24). Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL. n=4 for each time point.

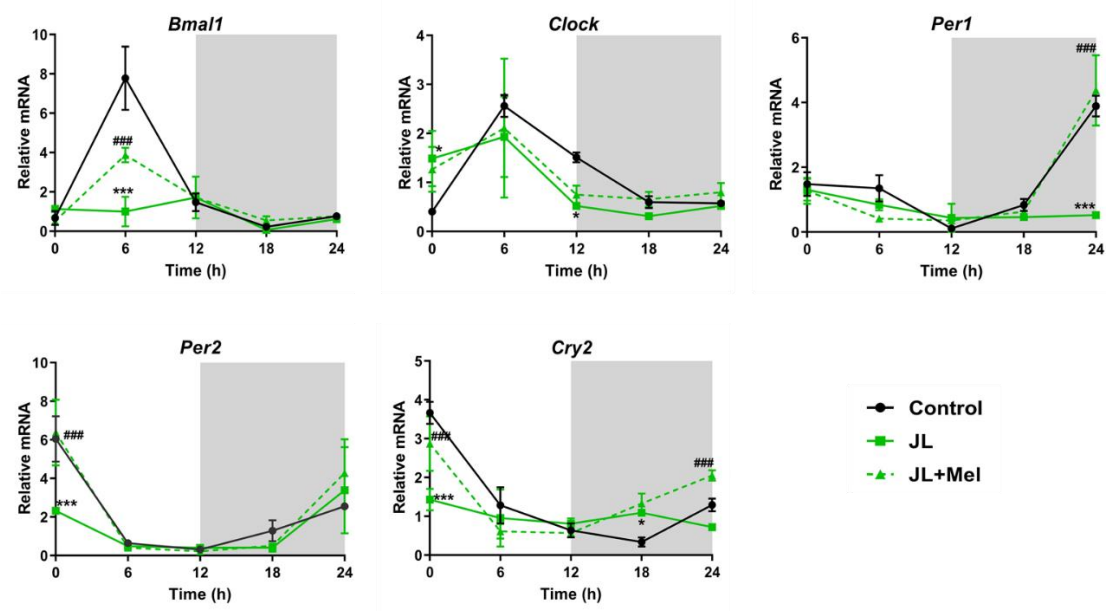


Figure 4.2: Melatonin reprograms the circadian clock gene in liver of JL mice as evidenced by their mRNA profiles. Grey shaded area indicates dark phase (ZT12 to ZT24). Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL. n=4 for each time point.

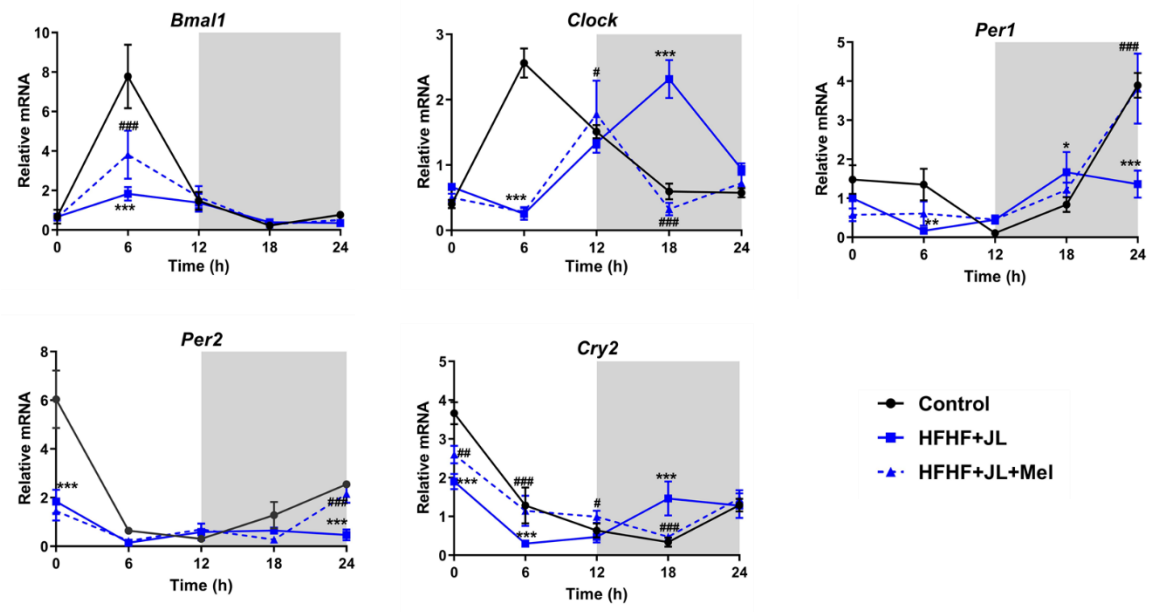


Figure 4.3: Melatonin reprograms the circadian clock gene in liver of HFHF+JL mice as evidenced by their mRNA profiles. Grey shaded area indicates dark phase (ZT12 to ZT24). Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL. n=4 for each time point.

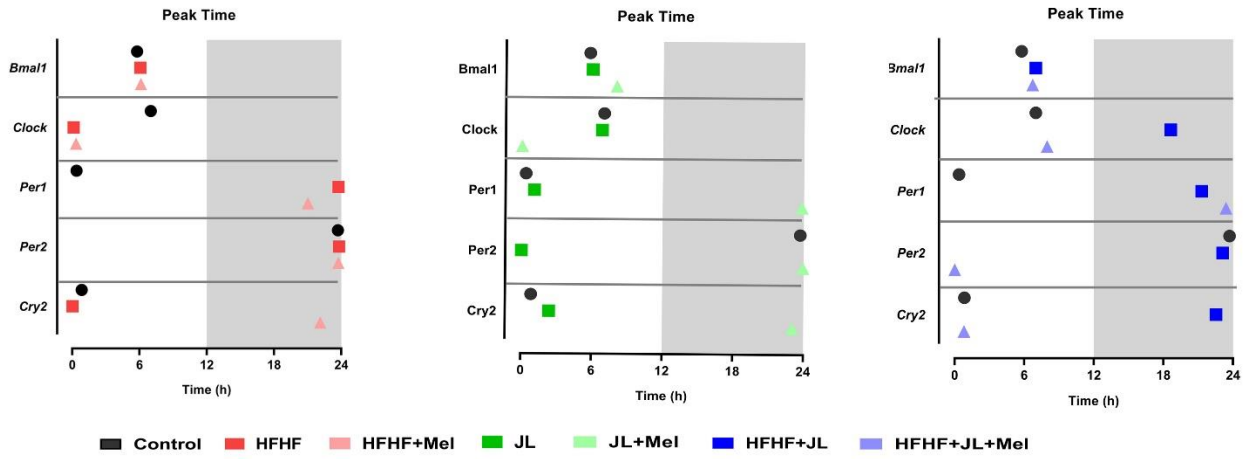


Figure 4.4: Circwave analysis of clock genes in liver of HFHF and/or JL treated mice shows an improvement in peak time following melatonin treatment.

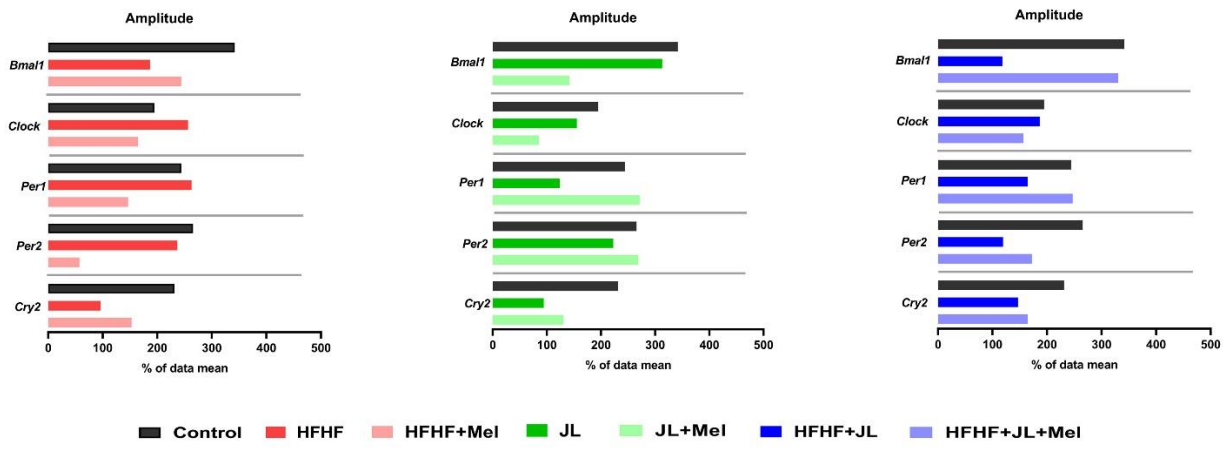


Figure 4.5: Circwave anlaysis of clock genes in liver of HFHF and/or JL treated mice shows an improvement in amplitude following melatonin treatment.

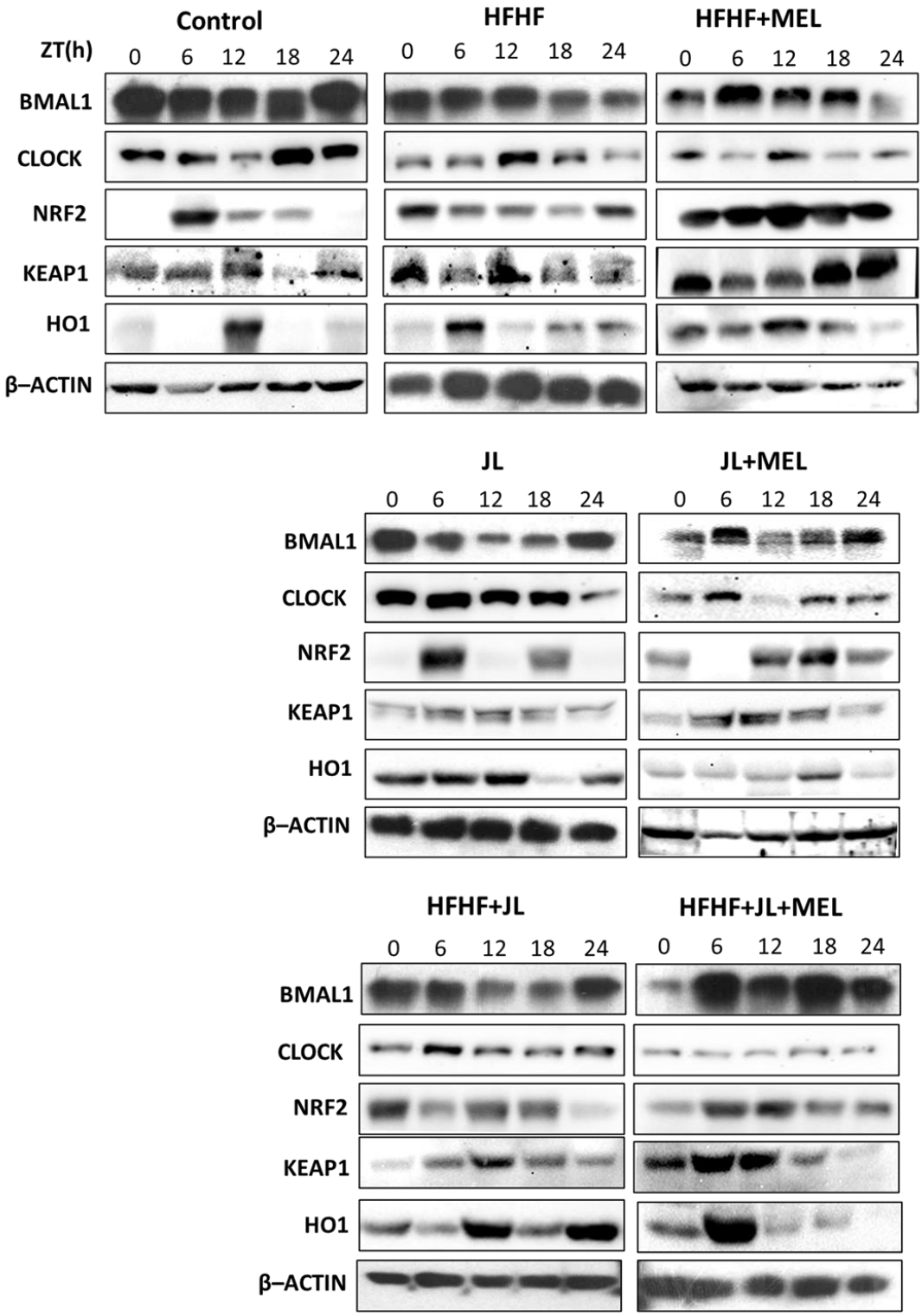


Figure 4.6: Melatonin modulates protein expression pattern of Clock-Bmal1 and NRF2-ARE pathway genes in HFHF and/or JL exposed mice liver. Densitometric analysis of western blot were normalized by β-actin as endogenous control.

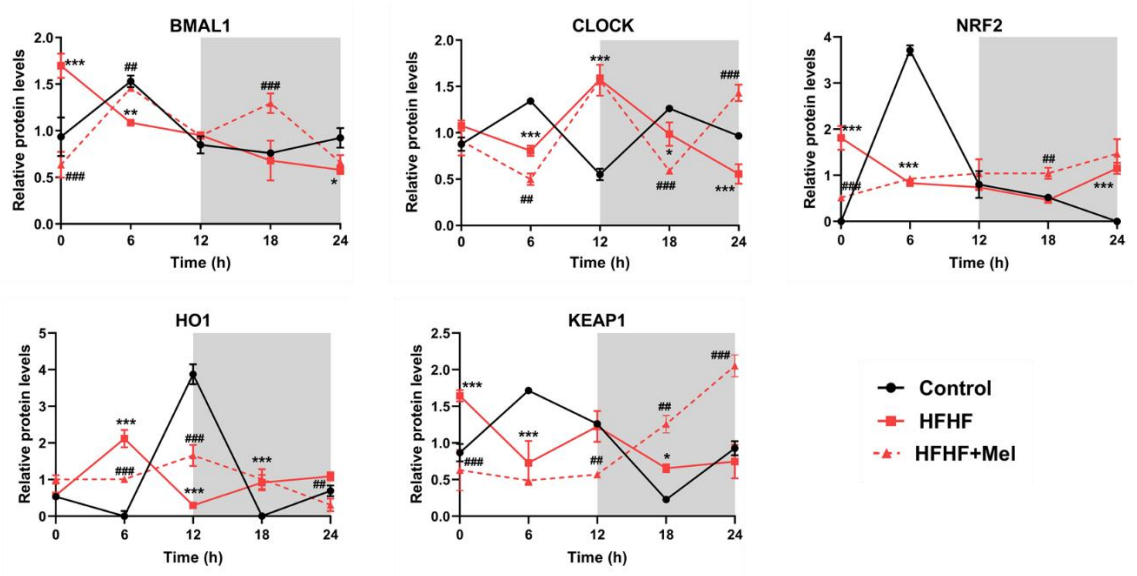


Figure 4.7: Melatonin reprograms protein expression of Clock-Bmal1 and Nrf2-ARE pathway genes in HFHF fed mice. Densitometric analysis of western blot. Blots were normalized by β-actin as endogenous control. Grey shaded area indicates the dark phase (ZT12 to ZT24). Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL.

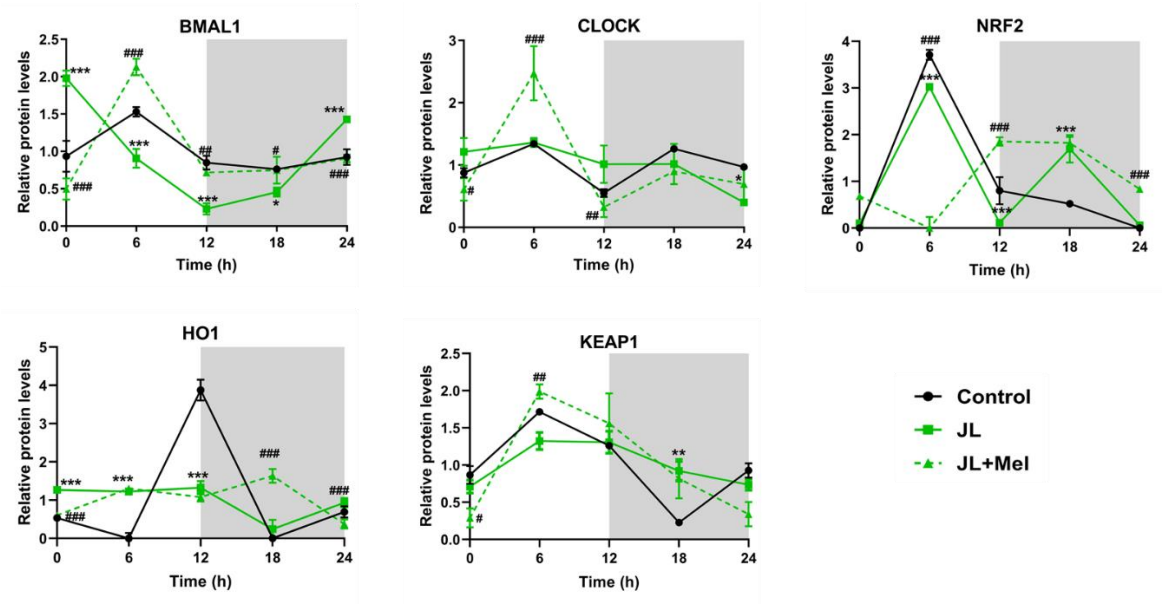


Figure 4.8: Melatonin reprograms protein expression of Clock-Bmal1 and Nrf2-ARE pathway genes in JL subjected mice. Densitometric analysis of western blot. Blots were normalized by β-actin as endogenous control. Grey shaded area indicates the dark phase (ZT12 to ZT24). Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL.

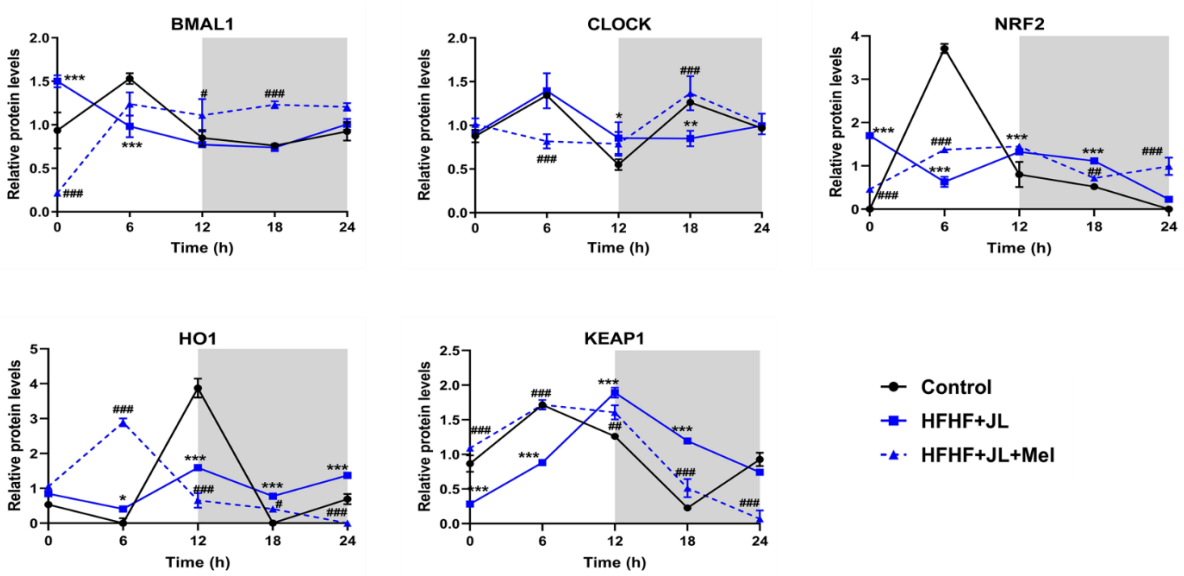


Figure 4.9: Melatonin reprograms protein expression of Clock-Bmal1 and Nrf2-ARE pathway genes in HFHF+JL subjected mice. Densitometric analysis of western blot. Blots were normalized by β-actin as endogenous control. Grey shaded area indicates the dark phase (ZT12 to ZT24). Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL.

Discussion

Suprachiasmatic nuclei (SCN) in the hypothalamus of mammals synchronizes subsidiary peripheral clocks in the body, but liver contains its own clock that regulates fatty acid, glucose, and xenobiotic metabolisms(Adamovich et al., 2014; Genzer et al., 2015; Leclère et al., 2020). Both, food restriction and a high calorie diet are known to entrain hepatic clocks; a physiological response that is independent of SCN (Hara et al., 2001). Recent studies have shown that nutritional challenges including high fat, ketogenic diet and MCD diet resulted in altered oscillations of core clock genes in liver (Hsieh et al., 2010). In the present study, our aim was to validate HFHF/ JL mediated desynchrony. Further, a combination of the two (HFHF+JL) was tried out and each disease control group was treated with melatonin. The resultant perturbations in core clock and peripheral clock genes expression were closely monitored on a time scale (ZT= 0,6,12,18,24 h) and the findings were analysed statistically by circwave tool.

In our study, the HFHF mediated poor circadian oscillation of hepatic core clock genes in liver are in agreement with published studies. HFHF fed mice showed potentially impaired hepatic clock function as evidenced by mRNA of core clock regulators (Bmal1 and Clock) in liver that is also in agreement with other research groups. But the same was not observed in negative regulators (Per and Cry) suggesting that the core feedback loop was markedly reduced by HFHF feeding. On the other hand, a variety of photoperiodic regime have been employed to induce chronodisruption, that amounts to a 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 (Kettner et al., 2016). In our study, melatonin

mediated corrective changes in oscillatory pattern of clock genes in mice subjected to JL was observed. Findings of the other research groups and the data showcased herein establishes the potency of HFHF or JL in manifesting desynchrony of clock genes, that is also a key cause in epidemiology of NAFLD(Gangwisch, 2009). Such a combination of HFHF and JL has never been studied. Based on our findings in 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.

A number of studies have pointed towards the important role of Nrf2 in the pathogenesis of NAFLD(Chambel et al., 2015). Du et al. (2016) explored the therapeutic impact of Nrf2 activation by using osteocalcin and reported that it could improve NAFLD by ameliorating oxidative stress and inhibiting the JNK pathway, which is an important pathway involved in the pathogenesis of NAFLD (Du et al., 2016). Also, Nrf2 has been known to show dual protective role in NAFLD via activating the antioxidant enzymes and inhibiting the enzymes of lipogenesis(Chambel et al., 2015). A previous study from our lab had reported that HFHF induced NAFLD causes perturbation in hepatic antioxidants and Nrf2-ARE pathway. Also, Xu et al (2012) had reported circadian alterations (at various time points) in Nrf2, HO-1 and keap1 proteins in healthy mice. Our findings showed that the time dependent circadian oscillations of said antioxidant proteins were in agreement with the published reports. However, there was a marked difference in the circadian expression of Nrf2, HO-1 and Keap1 proteins in HFHF and/or JL groups. The same can be attributed to a challenged antioxidant defense system in liver and the dynamics of its circadian biology in NAFLD. Disassembly of Nrf2-Keap1 dimer and subsequent nuclear translocation of Nrf2 is a key event in the activation of ARE pathway and positive manifestation of antioxidant

defense system activation(Kaspar et al., 2009). Hence, nuclear translocation of Nrf2 following administration of a test therapeutant is critical for Nrf2 activation and the same has been reported by other research groups (Li et al., 2016; Upadhyay et al., 2018). The observed alterations in pattern of Nrf2 expression observed in our study endorses the above-mentioned mechanism in melatonin treated groups. The re-entrainment of disturbed rhythms of antioxidant genes provides testimony to the efficacy of the therapeutic potential of melatonin.

Perturbations in Nrf2-HO-1 genes in HFHF and/or JL has never been reported and our results throw light on the same. Though 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 fatty liver. Taken together, this study unravels the perturbations in clock gene oscillations and Nrf2-HO-1 in liver in HFHF and/or JL induced NAFLD. The efficacy as well as the limitations of administrating exogenous melatonin are elucidated in detail in this study. The HFHF-JL synergy mimics the actual scenario of a lifestyle disorder and validates our claim of this synergistic effect. Restoration in the oscillatory patterns of core clock genes in this experimental group is an important finding of our study. However, It may be noted that gene mutations, age, sex-specific changes and comorbidities may throw light on some novel findings that needs further scrutiny.