Introduction

Circadian clock plays a principal role in orchestrating our daily physiology and metabolism, and desynchrony in clock machinery may result in various lifestyle disorders such as obesity, diabetes mellitus, dyslipidaemia and NAFLD(Doi et al., 2010; Scott, 2015). The molecular clock is operated by set of genes known as the core clock genes (Bmal1, Clock, Per1-2, Cry1-2, Rev-erbα and RORα) which works in a transcriptional-translational feedback loop. The Clock: Bmal1 heterodimer binds to the E-box elements of Per and Cry genes thereby activating their expression and encoding their proteins. Per and Cry proteins return to nucleus and inhibits Clock: Bmal1 transcriptional activity(Buhr & Takahashi, 2013; Mohawk et al., 2012; Partch et al., 2014; Rudic et al., 2004). Reports have shown that Clock gene mutants display impaired glucose and lipid metabolism and are susceptible to diet-induced obesity and metabolic dysfunction, providing strong evidence for a connection between circadian clock and metabolic homeostasis(Doi et al., 2010; Sookoian et al., 2007; Turek et al., 2005). Perturbations in the internal clock system has been shown to cause higher risk for developing obesity, diabetes mellitus, cardiovascular disease, thrombosis and/or inflammation(Mohawk et al., 2012; Panda, 2016). A strong positive correlation exists between aforesaid metabolic syndrome and NAFLD. NAFLD is characterized by the lipid accumulation, oxidative stress and hepatic damage(Friedman et al., 2018). Genomic variants in circadian clock genes are closely associated with hepatic steatosis, with a predisposition to NAFLD development(Shi et al., 2019; Sookoian et al., 2007). In humans, development of obesity, NAFLD and metabolic syndrome have a close association with polymorphisms present in the Clock gene(Shi et al., 2019). In addition to circadian gene polymorphisms, feeding/fasting cycles, feeding time, sleep deprivation, and sleep quality have prominent effects on circadian clocks as they are

known to disrupt circadian rhythms and interfere with intermediary metabolism(Adamovich et al., 2014; Damiola et al., 2000).

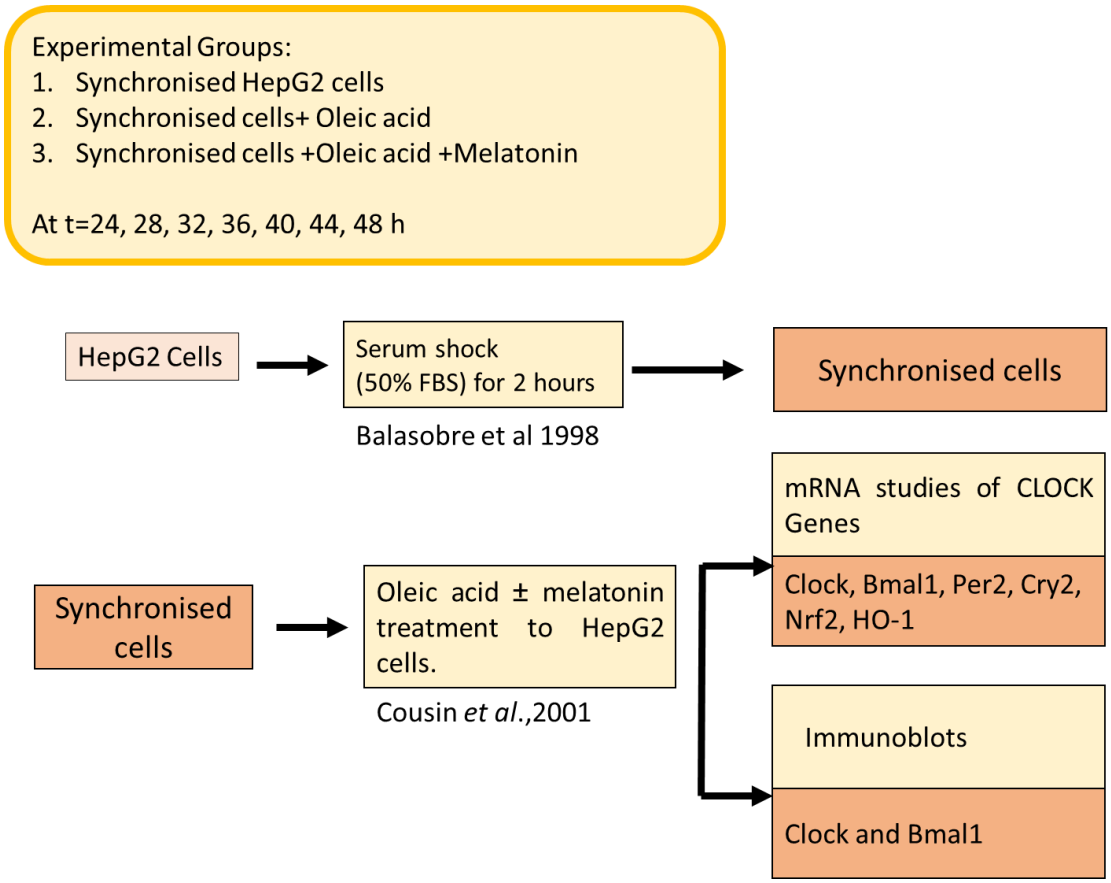
Apart from regulating lipid homeostasis, circadian clock is known to modulate processes that keep reactive oxygen species (ROS) at physiological levels and protect organisms from a built up of oxidative stress(Y.-Q. Xu et al., 2012). Xu et al. (2012) investigated the expression patterns for antioxidant genes in mice liver and found that nuclear factor erythroid-2-related factor 2 (Nrf2) expression was highest during daytime and showed a peak at 18:00, hence proving that these 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 defence have been studied by various research groups wherein they have shown that Bmal1 is a transcriptional regulator of Nrf2(Chambel et al., 2015; Lee et al., 2013; Pekovic-Vaughan et al., 2014).

Melatonin is a neurohormone mainly responsible for regulating circadian rhythms with its levels increasing during the dark phase(Afeche et al., 2008). Melatonin is primarily produced by the pineal gland and various non-pineal sources such as liver, kidney and instestine also synthesize the said hormone(Ralph, 1981). Melatonin is known to bind to G-coupled receptors MT1 and MT2 of hepatic and gastrointestinal cells in humans and rodents(Brzezinski, 1997). Also, melatonin is known to scavenge free radicals and provide protection against ROS followed by inflammation(Ferraro & Lopez-Ortega, 2008; Tan et al., 2015; Zhang & Zhang, 2014). Accumulating evidence has revealed the role of melatonin in reprogramming of the circadian clock in conditions of hepatocellular carcinoma and CCl4 induced liver fibrosis(González-Fernández et al., 2018). There are reports that establish the role of melatonin as potent Nrf2 activator in

ANIT induced cholestasis(Li et al., 2019). Therefore, therapeutic potential of melatonin for treating gamut of liver diseases has been established.

This study showcases the potential of melatonin in making decisive changes towards the correction of circadian clock genes in a steatotic hepatocytes. The study uses OA treated HepG2 cells as an experimental model to decipher the mechanistic details. Further, melatonin mediated correction in Nrf2 and HO-1 genes in a steatotic hepatocytes has also been showcased herein.

Experimental Design



Results

Melatonin increases protein expression Bmal1 in OA treated HepG2 cells.

Protein expression of Bmal1 and Clock was evaluated after HepG2 cells were treated with OA with or without melatonin. Immunoblot data revealed significantly higher expression of Bmal1 protein at 100 µM melatonin concentration. On the other hand, no significant change was observed in Clock protein expression (fig. 2.1).

Melatonin synchronizes core clock gene oscillations in OA treated HepG2 cells.

To study the potential of melatonin in modifying the circadian clock following OA treatment, HepG2 cells were synchronized with serum shock for 2h followed by OA treatment with or without melatonin and subsequently mRNA levels were studied at 4h intervals (24, 28, 32, 36, 40, 44 and 48 h). Synchronized HepG2 cells showed robust oscillatory pattern of core clock genes (*Clock, Bmal1*, *Per2, Cry2*). Presence of OA dampened the oscillations 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* ($P<0.001$). However, expression levels of *Cry2, Nrf2, HO-1* were not adequately corrected (Fig.2.2 and 2.3).

Melatonin improves amplitude and peak time in OA treated HepG2 Cells.

Based on the time course data, the expression values were analyzed for rhythmicity using circwave software. We considered the amplitude and peak time as the major factors for describing the rhythmicity of clock genes in control, OA and OA+ melatonin groups. Circwave analysis also showed a strong positive shift in amplitude of *Bmal1, Clock* and *Per2* following melatonin treatment but the amplitude of *Cry2, Nrf2 and HO-1* were not restored (fig.2.4).

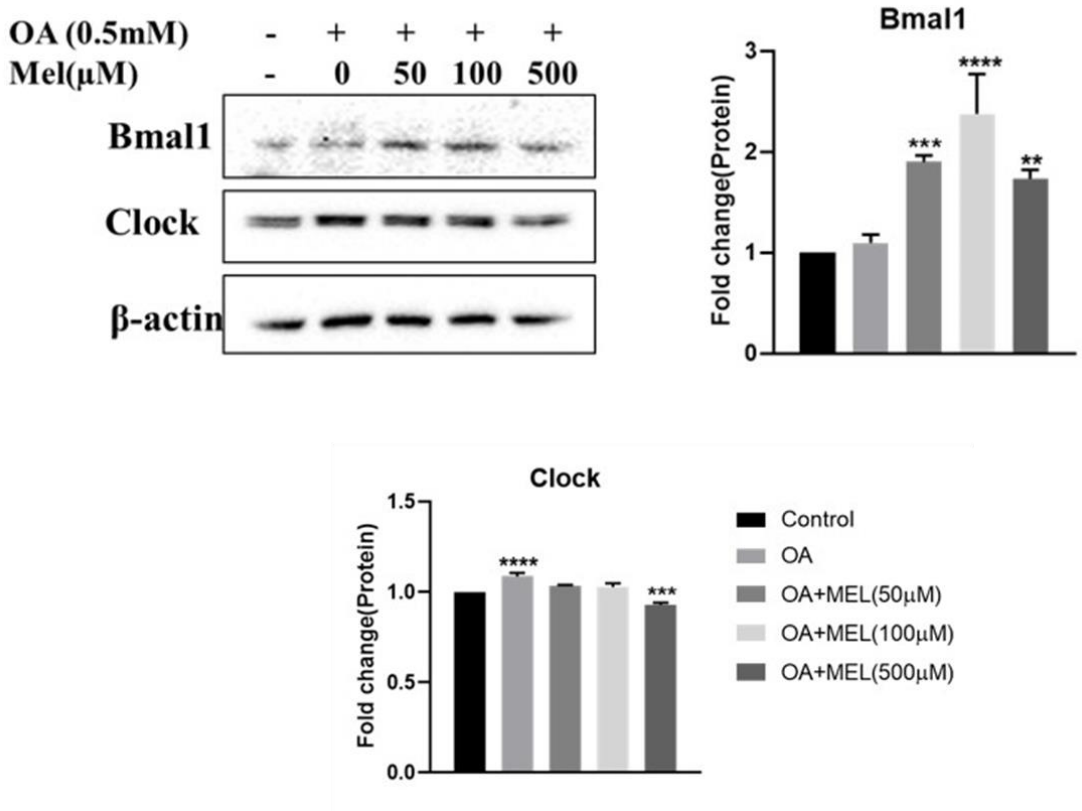


Figure 2.1: Immunoblot analysis of Bmal1 and Clock protein in OA treated HepG2 cells supplemented with different concentrations (0-500μM) of melatonin for 24h. Normalized with the levels is β-actin. Data is represented as mean ± SD. *P<0.05, ***P<0.001 vs control. n=3

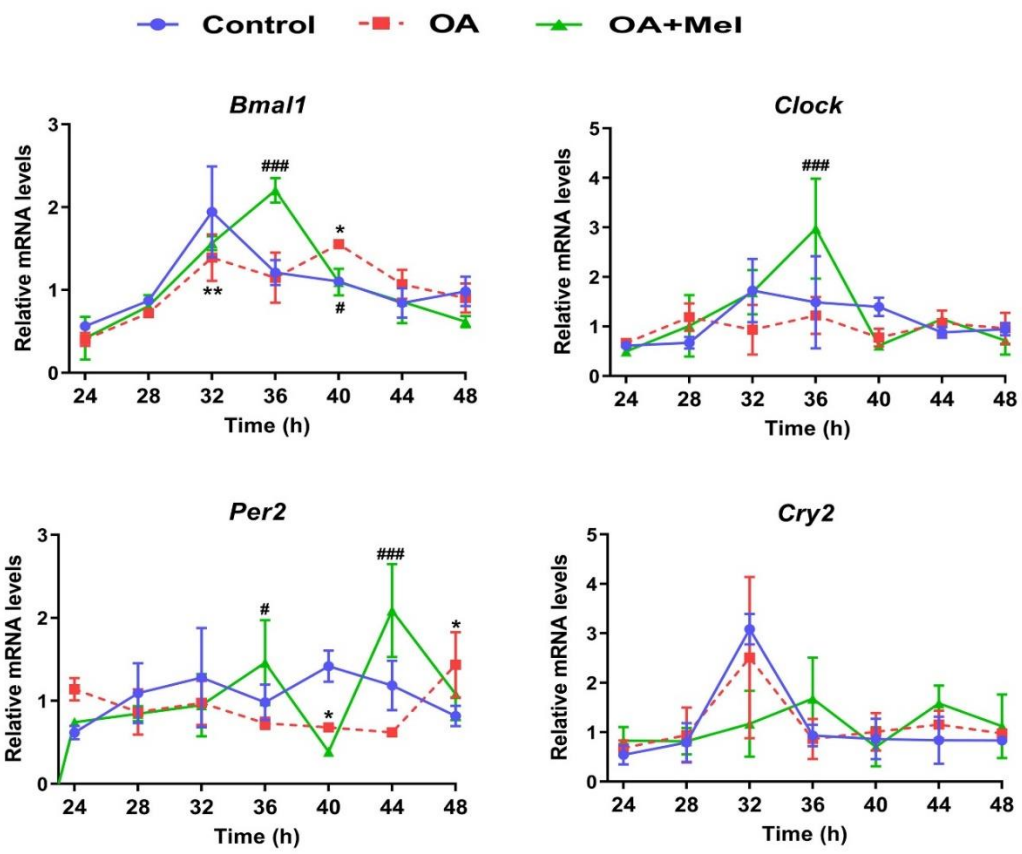


Figure 2.2: Serum Synchronised HepG2 cells were treated with OA in the presence or absence of melatonin. mRNA levels of clock genes were assessed at different time points. Transcription levels were measured by RT-PCR and normalized to GAPDH. Data represented as mean±SD. *P<0.05, ***P<0.001 vs control, #P<0.05, ###P<0.001 vs OA group. n=3

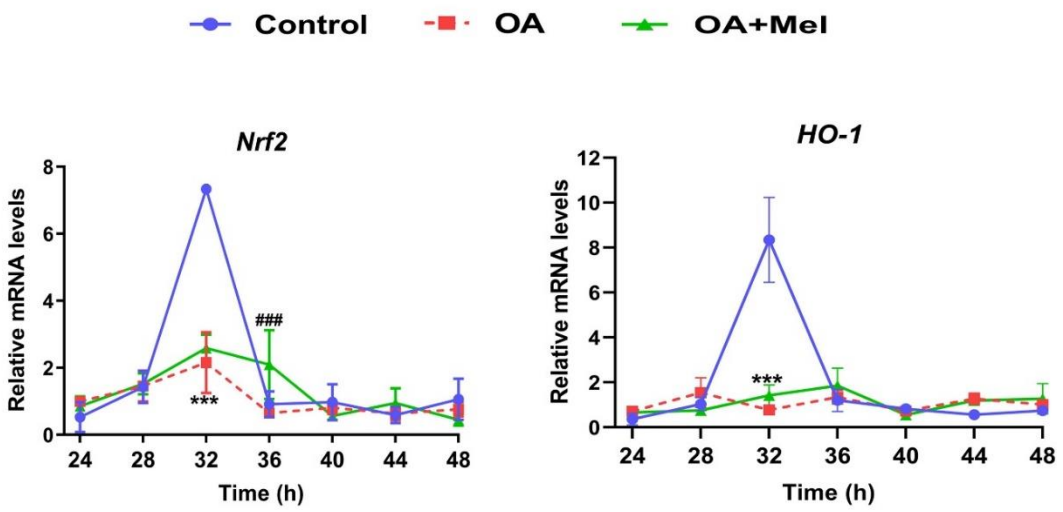


Figure 2.3: Serum Synchronised HepG2 cells were treated with OA in the presence or absence of melatonin. mRNA levels of Nrf2 and HO-1 genes were assessed at different time points. Transcription levels were measured by RT-PCR and normalized to GAPDH. Data represented as mean±SD. *P<0.05, ***P<0.001 vs control, #P<0.05, ###P<0.001 vs OA group. n=3

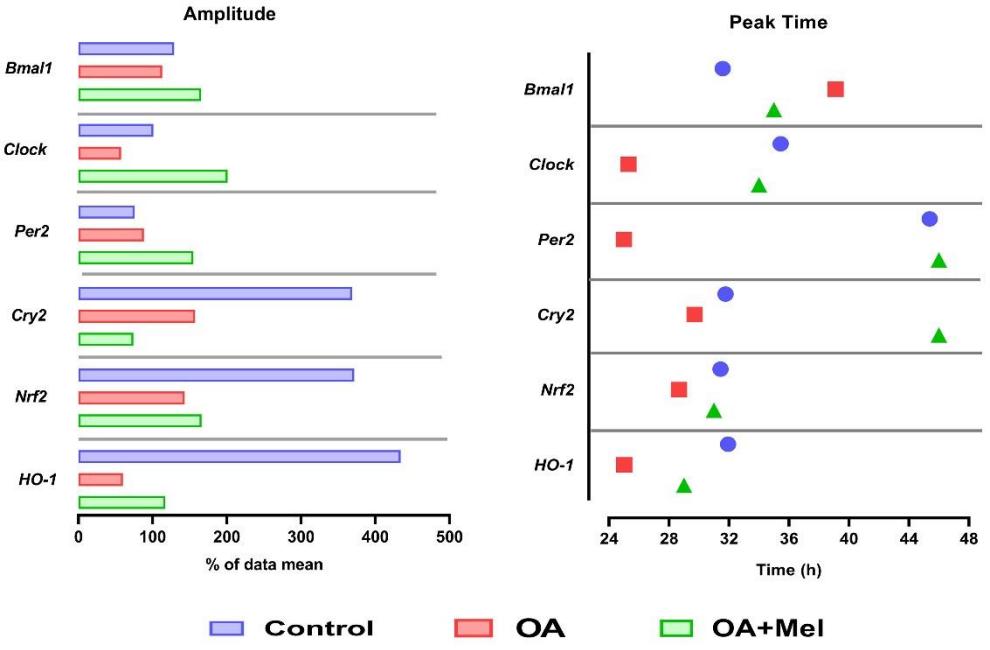


Figure 2.4: Amplitude and Peak time analysis of clock genes and Nrf2 - HO-1 genes in HepG2 cells treated OA with/without melatonin for 24h, using Circwave software.

Discussion

Circadian clock regulates an array of pathophysiological processes in liver wherein epidemiological studies have highlighted the implications of circadian misalignment in metabolic disorders including NAFLD(Shi et al., 2019; K. Xu et al., 2011). In our study, OA induced circadian misalignment and its subsequent impact on antioxidant regulatory genes (Nrf2 and HO-1) has thrown light on the importance of core clock genes in NAFLD. Densitometric analysis of Immunoblots revealed an increased expression of Bmal1 protein at 100 µM and hence the said dose was confirmed for further experiments on circadian gene expression. This result is also in agreement with other report which had shown that 100 µM dose of melatonin had elevated expression of Bmal1, Clock. Per and Cry and lowered Rev-erbα(González-Fernández et al., 2018).

Human diet consists of unsaturated and saturated fatty acids, that majorly include oleic acid (OA) (C18:1) and palmitic acid (PA) (C16:0). Intake of these fatty acids has been reported to significantly disrupt the circadian rhythm and desynchronise the molecular clock function(Tal et al., 2019). The circadian control of clock genes including Period (Per1, Per2 and Per3), Cryptochrome (Cry1 and 2) are regulated by the core clock-Bmal1 proteins. Consistent with the previous studies, we had found that the control HepG2 cells showed rhythmic oscillatory pattern of clock genes. OA treated HepG2 cells showed potentially impaired hepatic clock function as evidenced by mRNA of core clock regulators (Bmal1, Clock, Per2) that is also in agreement with other research groups (Nagura et al., 2018). We report not only a decrement in the transcripts of Clock, Bmal1, Per2 but also moderate to significant shift in the peak time as evidenced by circwave analysis. In contrast, the peak time of Cry2 was oblivious to OA treatment. Zhou et al., (2014) had reported that exogenous PA had reduced protein levels of Clock

and Bmal1 in mouse hepatocytes(Zhou et al., 2014). Compelling evidence demonstrates that resynchronising the circadian rhythm by various compounds is crucial for improving the pathophysiological conditions of metabolic disorders (Qi et al., 2018). Herein, we report that melatonin supplementation to OA treated HepG2 cells had resulted in corrective changes in the oscillatory pattern of core clock genes and a positive shift in amplitude (circwave analysis), that strongly implies towards use of melatonin as a therapeutant in lifestyle disorders. In our study, OA supplementation to HepG2 cells is employed as a model to achieve high fat induced alteration in expression of canonical circadian clock genes and circadian clock-controlled genes as reported by other research groups in mouse models (Hsieh et al., 2010; Kohsaka et al., 2007; Yanagihara et al., 2006).

Our study is possibly the first profound investigation that scrutinizes the nuances of amplitude and peak time in an invitro experimentally simulated condition of NAFLD. Emerging evidence points towards the importance of the cellular antioxidant components and to understand the circadian variations resulting in pathophysiological condition such as cancer (Blask et al., 2002), Pulmonary fibrosis (Pekovic-Vaughan et al., 2014), Pancreatic cell function (Lee et al., 2013) etc. These reports emphasize on the importance of understanding the circadian variations to derive best therapeutic response to drugs or other test compounds. In our study, Peak time of Nrf2 was not affected by OA treatment, but a significant shift was observed in HO-1 that was partially restored by melatonin treatment. Further, the loss of amplitude for Nrf2 and HO-1 following OA treatment could not be restored by melatonin.

In diseases like pulmonary fibrosis and diabetes, circadian control of Nrf2 is well established, but oscillatory pattern of said genes lacks clarity under conditions of

NAFLD (Wible et al., 2018). Findings of the present study had revealed that OA treatment to HepG2 cells led to circadian misalignment in Nrf2 and HO-1, while exogenous administration of melatonin moderately re-entrained circadian oscillations of the said genes. Our previous study had reported perturbations in NRF2-ARE pathway genes in PA induced steatosis (Upadhyay et al., 2020). Various lines of evidence had suggested that melatonin acts as a potent Nrf2 activator(Arioz et al., 2019; Ding et al., 2014; Wang et al., 2012). In our study, melatonin mediated corrective changes in Nrf2, and HO-1 oscillations in OA treated HepG2 cells is reported for the first time.