Summary

Chronodisruption in non-alcoholic fatty liver disease: Role of melatonin

Circadian clock are the endogenous oscillators that regulates numerous physiologic and metabolic processes. In mammals, the master clock is centrally located in the superchiasmatic nucleus of the hypothalamus. Suprachaismic nucleus (SCN) clocks are constantly entrained to the day-night cycle of 24 hours. Rhythmic clock gene expression and protein expression has been identified in the peripheral tissues or non-SCN cells throughout the body. Importantly, circadian rhythmicity persists in isolated tissues in vitro, where no control of the SCN exists, indicating the endogenous nature of the circadian oscillation at the periphery. At the molecular level, circadian clock is regulated by transcriptional-translational feedback loop that consist of core clock components wherein, Clock and Bmal1 are the positive regulators and Per and Cry function as the negative regulators. Additional loop that reinforces the oscillation of primary loop consist of nuclear receptors, where REV-ERBα inhibits transcription of Bmal1 whereas; RORα brings about its activation.

Disruption of circadian clock and development of various health problems is well established. Several studies have shown that knockout rodent models of core clock genes develop lifestyle disorders such as obesity, diabetes and Nonalcoholic Fatty liver disease (NAFLD). In vitro studies have also shown that circadian misalignment results in dysregulation of metabolic homeostasis in various cell types. Epidemiological studies had revealed that liver pathologies are more prevalent in the Western countries due to the modification of eating habits and lifestyle. NAFLD is a multifactorial disease with a broad spectrum of liver damages from steatosis to hepatocellular carcinoma (HCC). Increased oxidative stress is a major contributing factor in progression of

NAFLD. Nrf2-ARE pathway is an antioxidant defence system known to show positive effect in combating oxidative stress and by activating antioxidant enzymes. The ability of Nrf2 to protect against liver damage by activating antioxidant enzymes as well as inhibiting de novo lipogenesis is well established.

In circadian regulation of redox homeostasis, levels of antioxidant enzymes such as glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase are known show rhythmic expression. Reports Nrf2 is reported to be under the transcriptional regulation of bmal1 and hence oscillatory pattern in Nrf2 expression has been reportd in rodent models of pulmonary fibrosis and diabetes.

Melatonin; a neurohormone is secreted by pineal gland is responsible for regulating the circadian rhythm. But, there is an increasing evidence showing its involvement in multiple key physiological functions. Melatonin is known to protect against obesity and hepatic steatosis by improving lipid dysmetabolism and attenuating inflammation in high fat diet fed mice. Dysregulation of the circadian clock pathway in mice treated with CCl_4 induced liver fibrosis is known to show improvement following melatonin treatment. One of the recent findings had shown a regulatory role of melatonin in lipid homeostasis and clock gene regulation in mice exposed to constant light.

In Chapter 1, validation of Oleic acid (OA) treated HepG2-NAFLD model was carried out, wherein we had observed that OA treated to HepG2 cells resulted in decreased cell viability in dose dependent manner. Data obtained by MTT assay was used to standardize the dose of OA and melatonin, that did not result in lipotoxicity. So, 0.5 mM OA and 100 µM melatonin concentration was used for further experiments. OA treatment to HepG2 cells recorded increased lipid accumulation, increased intracellular oxidative stress and altered mitochondrial membrane potential. Melatonin treatment

accounted for improvement in the said parameters. Melatonin had also shown an increment in expression levels of lipolytic genes and decrement in lipogenic genes in OA treated HepG2 cells. The results obtained in chapter 1 forms the basis of our detailed investigation.

Chapter 2 deals with role of melatonin in circadian desynchrony induced by OA treated HepG2 cells. A time point based analysis was carried out of core clock genes (Bma1, Clock, Per2, Cry2) and antioxidant genes (Nrf2, HO-1) in HepG2 cells synchronised by serum shock for 2 h, followed by treatment of OA with or without combination of melatonin. Dampened oscillations of Clock, Bmal1, Per2 and Cry2 were observed in OA treated HepG2 cells. Melatonin treatment had significantly improved oscillation of Bmal1, Clock and Per2, however oscillations of Nrf2 and HO-1 were not adequately corrected. Oscillation of Cry2 were not affected in OA treated HepG2 cells. Rhythmicity of this data was statistically analyzed using circwave software and a strong positive shift in amplitude of Bmal1, Clock and Per2 was observed following melatonin treatment. But the amplitude of Cry2, Nrf2 and HO-1 were not found to be restored in melatonin treated groups. These results had provided compelling evidence that exogenous melatonin can partially restore the core clock genes oscillations in OA induced circadian desynchrony.

Chapter 3 focuses on the extent of pathophysiological changes obtained in our experimental model of NAFLD. In this study, High fat high fructose (HFHF) diet and chronodisruption induced by jetlag protocol (JL) were studied singly or in a combined experimental schedule. In our study, significantly elevated titers of AST and ALT were observed in HFHF and/or JL mice implying towards varying degrees of liver damage. An improvement in key lipid metabolism genes (CPT-1, PPARα, SREBP-1c, FAS and

CD36) following melatonin treatment was also observed. Fatty manifestations and distorted histoarchitecture were observed in HFHF and/or JL groups, with minimal effect recorded in JL group. The observed pathophysiological changes and the extent of damage in HFHF+JL group was visibly more than HFHF group that had also implied towards a possible synergy between HFHF and JL. But, exogenous melatonin treatment recorded improvement in the same. Melatonin had recorded a decrement in hepatic fat accumulation in HFHF and HFHF+JL groups as evidenced by ORO staining. An increment in the mRNA expression of Cyp2e1, Nrf2, HO-1, NQO-1 genes were observed in HFHF, JL and/or HFHF+JL groups, but exogenous melatonin treatment had accounted for a decrement in expression levels of said genes. Also, the mRNA expression of GSS, SOD, GCLC and GCLM had shown a decrement in the HFHF and/or JL groups, but melatonin treatment had accounted for an upregulation of these parameters. These findings are suggestive of an improved antioxidant status. The mRNA levels of pro-inflammatory cytokines IL-1β and TNFα had recorded an increment in HFHF and/or JL groups, but melatonin treatment had resulted in a decrement in the said inflammatory markers. This study is first to investigate a comparative and synergistic effect of dietary and photoperiodic manipulation on NAFLD and hints at a protective role of melatonin in alleviating pathophysiology associated with HFHF and/or JL induced NAFLD.

Chapter 4 circadian desynchrony in HFHF and/or JL induced NAFLD. In this study, HFHF mediated poor circadian oscillation of positive regulators (Bmal1 and Clock) was observed in liver. The oscillations of Per1, Per2 and Cry2 did not show significant variations in HFHF fed mice. Likewise, the observed oscillations of core clock genes (Bmal1 and Clock) were found to be similar and comparable in liver of the two disease

models used herein (HFHF and JL). But, oscillations of Per1, Per2 and Cry2 appeared to be dampened in the JL group. 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. The Nrf2 and Keap1 proteins had shown a peak at ZT6 whereas; the HO-1 had peaked at ZT12 in liver of healthy mice. Feeding of HFHF diet caused flattening of Nrf2 and Keap1at ZT6. Also, ZT12 of HO-1 had shifted to ZT6 in this group. Melatonin treatment did not result in any form of corrections of the oscillations of Nrf2, HO-1 and Keap1 proteins wherein Nrf2 and HO-1 showed further flattening of the peaks. Further, Keap1 underwent a shift from ZT6 to ZT24. Nrf2 and Keap1 oscillations did not undergo significant changes in JL group. Nrf2 and HO-1 proteins witnessed the flattening of the curve in HFHF+JL group whereas Keap1 recorded a shift from ZT6 to ZT12. HO-1 noted a change in peak from ZT12 to ZT6, but Nrf2 protein was oblivious to melatonin treatment.

Based on the findings of the four key studies envisaged herein, it can be concluded that high fat high fructose diet causes desynchrony of clock genes oscillations. Further, exogenous melatonin makes corrective changes in the oscillations of core clock genes that translates into an overall improvement in physiology and histoarchitecture of NAFLD in C57BL6/J mice.

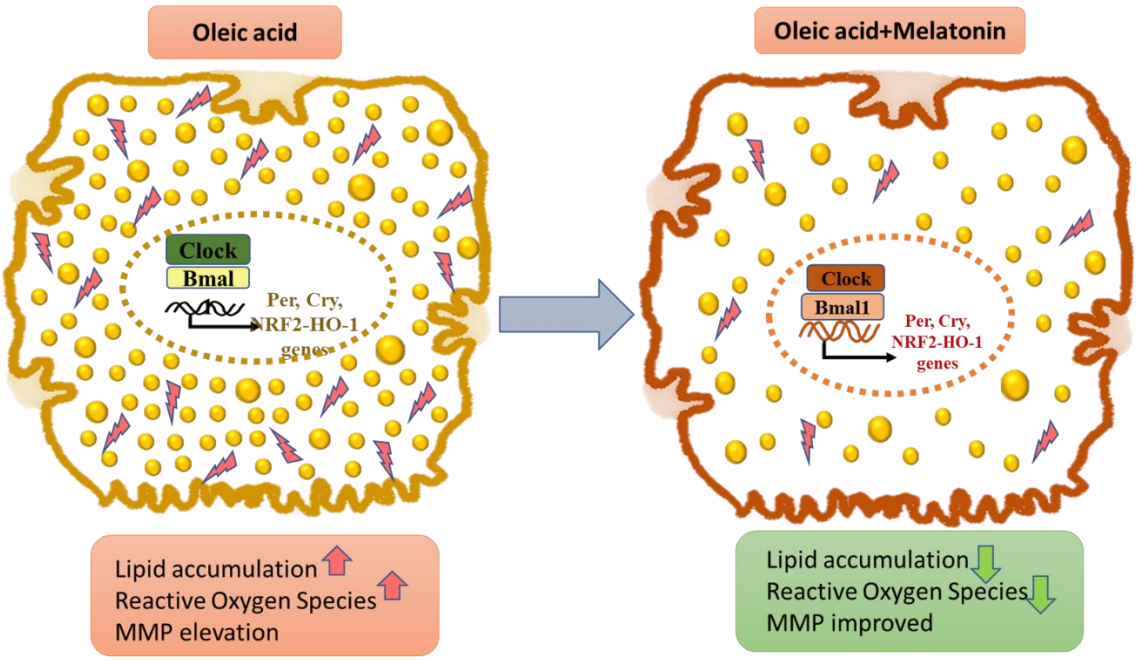

Figure S1: Overview of melatonin mediated corrective changes in clock genes oscillations in OA treated HepG2 cells.

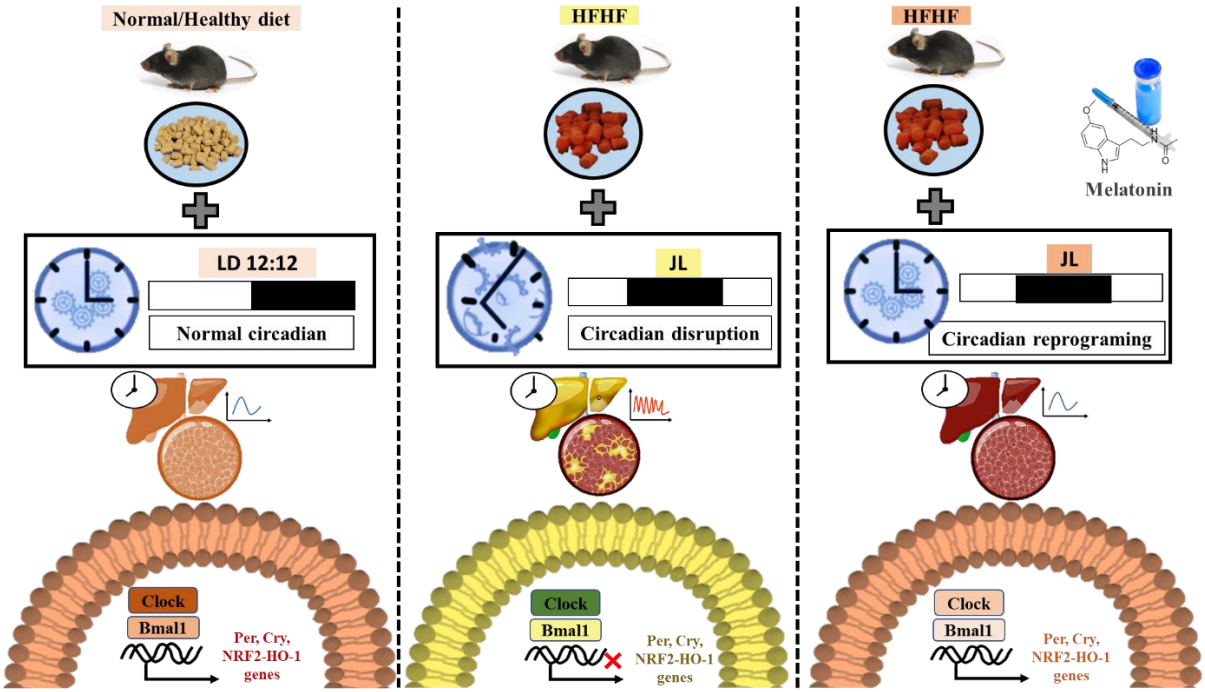

Figure S2: Overview of melatonin mediated reprogramming of clock genes oscillations in HFHF and/or JL induced NAFLD.