Synopsis of the thesis entitled

Chronodisruption in Nonalcoholic fatty liver disease: Role of Melatonin

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Introduction

Light is the most important external stimuli which keeps body synchronized and any disturbance in this can disrupt the biological rhythm which is important for maintenance of normal physiology. In today's scenario, working hours of people are often just the reverse to the one set by the nature. Doctors, nurses, call center executives, shift job workers and transcontinental travelers are subjected to drastic changes in their biological clock. Such changes in life style has been attributed to cause various health complications primarily due to disturbances in Circadian Rhythm (Scheer *et al*., 2009). Malfunctioning of circadian or biological clock causes circadian rhythm disorders often culminating in metabolic syndromes (Maury *et al*., 2010). Reports on perturbations of internal clock system have shown increased risk for disorders including obesity, diabetes mellitus, cardiovascular disease, thrombosis and inflammation (Mazzoccoli *et al*., 2012, Nohara *et al*., 2015). Strong positive correlation exists between aforesaid metabolic syndromes and development of non-alcoholic steatohepatitis (NASH).

NASH is associated with hepatic dysregulation of energy metabolism, lipid accumulation, oxidative stress and inflammation (Rolo *et. al.,* 2012). The pathophysiology of NASH is best explained by multiple hit model, wherein oxidative stress plays a primary role in initiating hepatic damage. The homeostasis of fat and energy is maintained by mitochondria through β-oxidation, electron transport and production of ATP and reactive oxygen species. Increase in intracellular lipid flux results into mitochondrial dysfunction and subsequent induction of ROS production. All these factors result in activation of inflammatory pathways leading to hepatocyte necroinflammation and worsening the condition of NASH. Various studies with rodent models have shown a strong correlation of circadian disruption with liver metabolism and energy homeostasis. In this context, Bmal1 mutant mice were found to develop hepatic steatosis even when fed with standard laboratory chow (Shimba *et al*., 2011). Further, melatonin, a neurohormone, apart from its involvement in regulation of circadian rhythm, has been reported to be beneficial in regulating lipid metabolism and reducing liver fat accumulation and insulin resistance in NASH (Bass *et al*., 2010).

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. In conditions of NASH, Nrf2 has been shown to be downregulated which is accompanied by an increased oxidative stress (Upadhyay et al., 2020). The classical understanding is that Nrf2 coordinates the elimination of ROS and electrophiles

derived from lipid peroxidation, thus preventing hepatocellular oxidative stress and mitochondrial dysfunction. In addition, there is growing evidence in the literature that Nrf2 regulates fatty acid metabolism by repressing genes that promote lipid accumulation in hepatocytes. In this way, Nrf2 shows dual protective role in progression of NASH (Chambel et al., 2015). Further, 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 that circadian variations of Nrf2 could modulate cell response to oxidative stress. Circadian-clock-dependent regulation of redox status, ROS homeostasis, and antioxidant defense is studied by various research groups wherein they have shown preliminary evidences suggesting Bmal1 as a transcriptional regulator of Nrf2 (Lee *et al*., 2013, Pekovic *et al*., 2014)

Despite of various evidences showing correlation of circadian clock with NASH in clock gene ablation models, Jetlag and/or High fat high fructose model which truly mimics chronodisruption in shift workers and transcontinental travellers needs detailed investigation on rhythmic expression of clock genes and its association with antioxidant defence genes further implicating towards condition of NASH.

Objectives

The study is divided into following objectives:

Objective 1: Modulatory role of melatonin in circadian desynchrony and NASH.

This objective shall be achieved through following studies:

Study 1: Validation of invitro hepatoprotective role of melatonin in OA treated HepG2 cells.

Study 2: Alterations in clock genes in OA treated HepG2 cells: Role of Melatonin

Study 3: Alterations in NRF-ARE pathway in OA treated HepG2 cells: Role of Melatonin.

Objective 2: Deciphering role of melatonin in a jet-lagged model of NASH.

This objective shall be achieved through following studies:

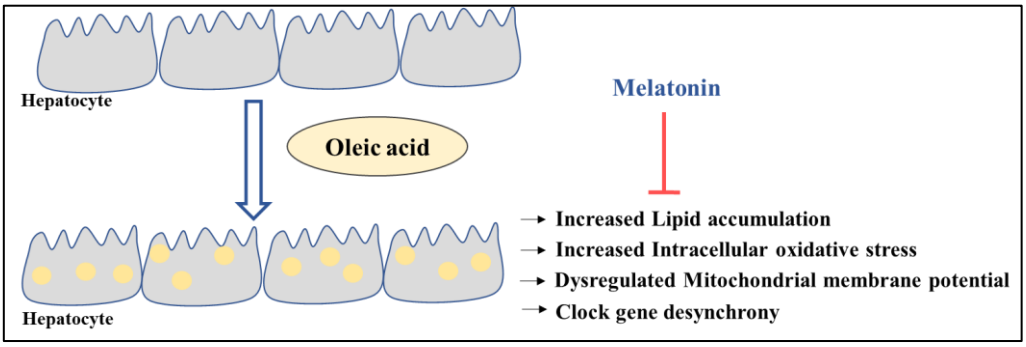
Study 1: Effect of Jetlag, HFFD and exogenous melatonin on physical parameters, Lipid profile and status of liver: Improved status of experimentally induced NASH.

Study 2: Jet lag / HFFD induced chronodisruption in Nonalcoholic fatty liver disease: Role of exogenous melatonin.

Observations

Objective 1: Modulatory role of melatonin in circadian desynchrony and NASH.

Study 1: Validation of invitro hepatoprotective role of melatonin in OA treated HepG2 cells.



Oleic acid (OA) induced HepG2-NASH model was used in this study. Treatment of OA (0.5-2 mM) recorded a dose dependent decrement in % cell viability as assessed by cytotoxicity (MTT) assay. However, melatonin treatment in HepG2 cells showed cytotoxicity not more than ~20% at highest dose (1000 µM). Also, Melatonin reduced OA induced cytotoxicity in HepG2 cells in dose dependent manner. So, 0.5 mM OA and 100 µM Melatonin doses was standardized for further experiments. OA treatment showed increased lipid accumulation and melatonin reduced the same as assessed by Oil Red O staining. Also, OA treatment showed increased green fluorescence indicating heighted intracellular oxidative stress, while melatonin co-supplementation resulted in relatively lower green fluorescence. mRNA expression of lipid metabolism governing genes was examined by RT-PCR. OA treatment recorded significant decrement in lipolytic genes (CPT-1 and PPARα) and significant increment in lipogenic gene (SREBP1c). Melatonin treatment resulted in significant increase in lipolytic genes and decrease in lipogenic gene.

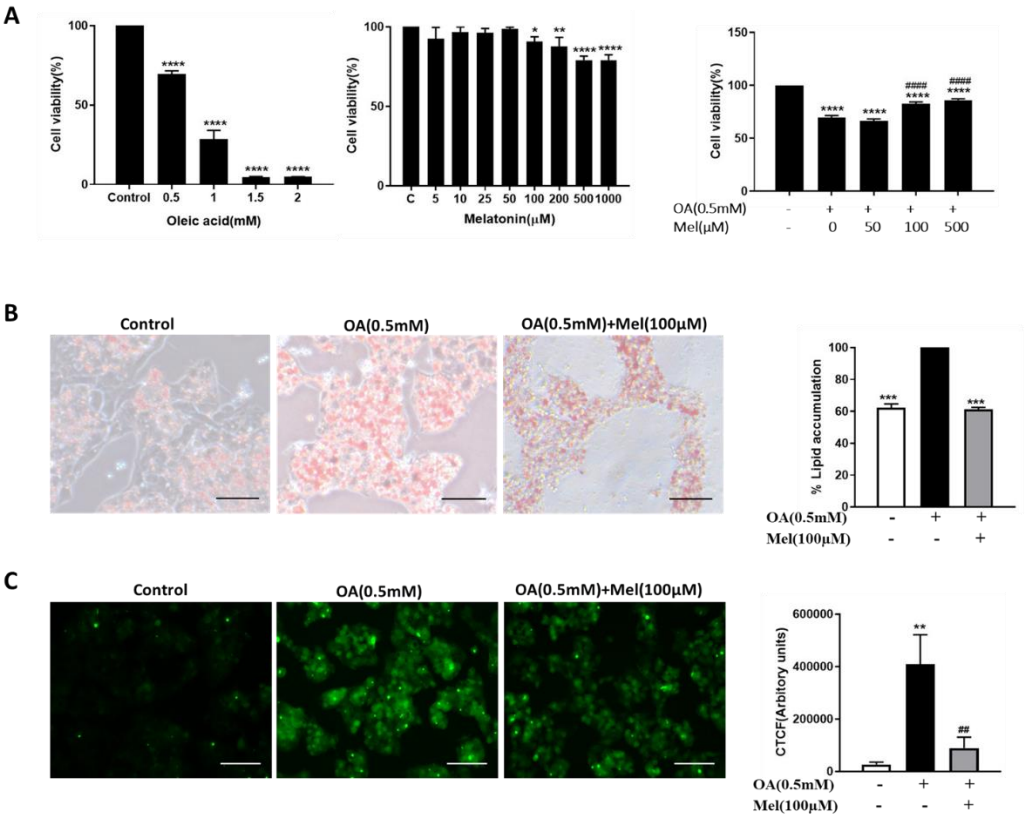


Fig 1: Effect of melatonin on OA treated HepG2 cells. (A) Cell viability assessed by MTT assay (B) Lipid accumulation assessed by Oil Red O staining (C) Intracellular oxidative stress as assessed by DCFDA staining. Data represented as mean±SD. *P<0.05, ***P<0.001 vs control, #P<0.05, ###P<0.001 vs OA group. N=3

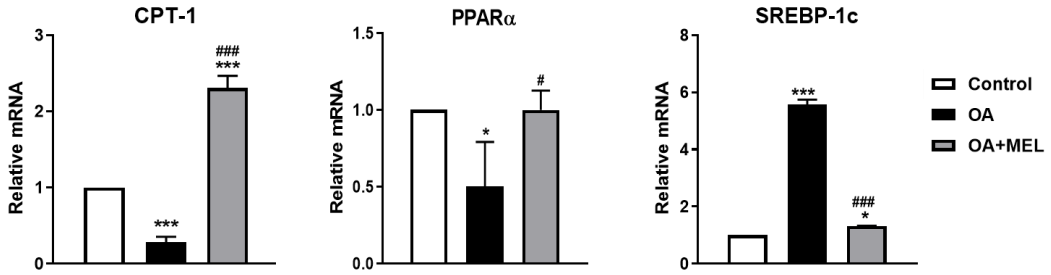


Fig 2: Melatonin improves lipid metabolism in OA induced NASH in HepG2 cells. HepG2 cells were treated with BSA conjugated OA for 24h in presence or absence of melatonin. mRNA expression values were normalized to GAPDH. Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs OA group. N=3

Study 2: Alterations in clock genes in OA treated HepG2 cells: Role of Melatonin.

In this study, HepG2 cells were serum synchronized for 2h, followed by OA and/or melatonin treatment and mRNA was harvested at time interval of 4h. Synchronized HepG2 cells showed robust oscillatory pattern of core clock genes (Clock, Bmal1, Per2, Cry2). Treatment of OA severely dampened the oscillation of Clock, Bmal1 and Per2 while oscillation of Cry2 was not affected. Intriguingly, Melatonin co-treatment reversed the shallow expression of Bmal1, Clock and Per2. Collectively, these results indicate that Melatonin treatment improved the expression of core clock gene expression in conditions of desynchrony induced by OA treatment. Rhythmicity of clock genes oscillation is under analysis by cosinor software.

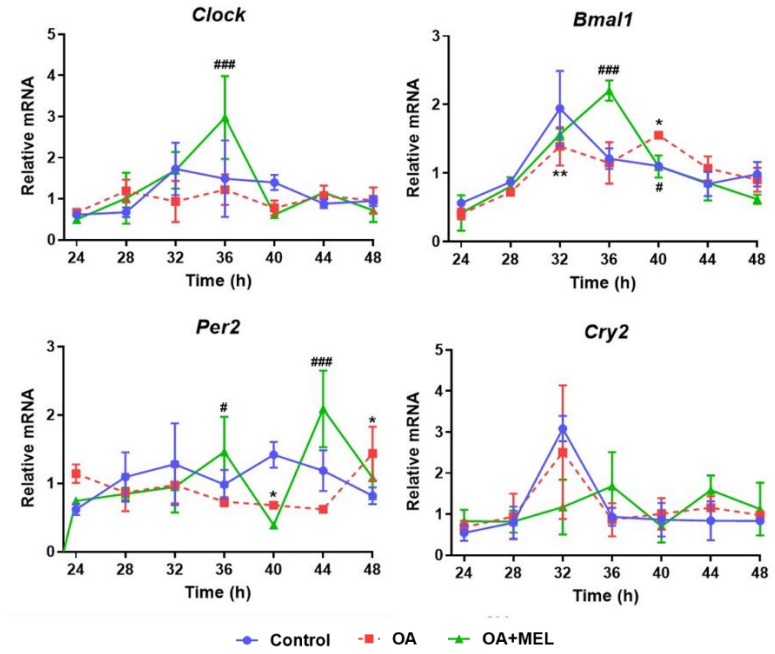


Fig 3: Melatonin modulates oscillation of clock genes in OA induced desynchrony. After 2h of serum shock, HepG2 cells were treated with OA and/or melatonin for 24 h. Cells were collected for mRNA analysis at an interval for 4h between 24 to 48 h. transcription levels were measured by RT-PCR and normalized 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

Study 3: Alterations in NRF-ARE pathway in OA treated HepG2 cells: Role of Melatonin.

Serum synchronized HepG2 cells showed strong oscillatory pattern of NRF2 and HO-1, while diminished oscillation was observed after OA treatment. Melatonin was inefficient in restoring the oscillations of NRF2 and HO-1 expression.

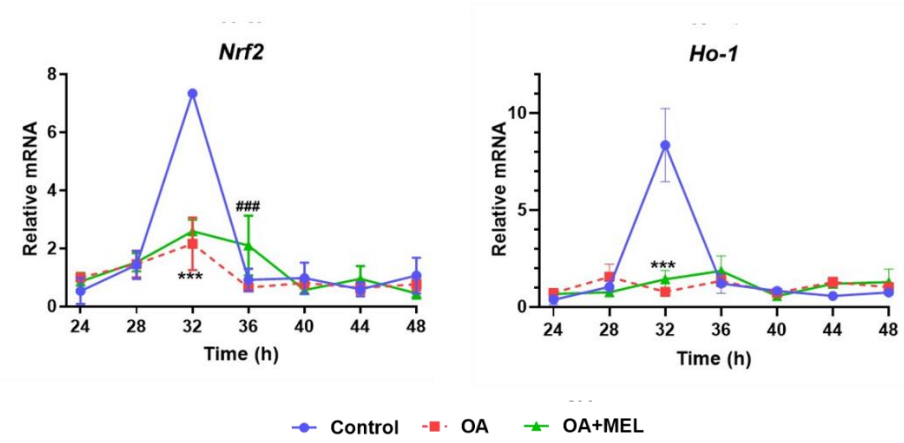
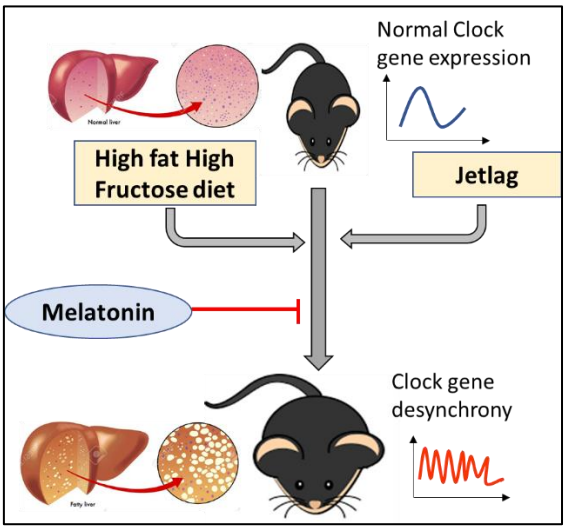


Fig 4: Melatonin modulates oscillations of Nrf2 and HO-1 genes in OA treated HepG2 cells. Data represented as mean±SD. ***P<0.001 vs control, ###P<0.001 vs OA group. N=3

Objective 2: Deciphering role of melatonin in a jet-lagged model of NASH.



Study 1: Effect of Jetlag, HFHF and exogenous melatonin on physical parameters, Lipid profile and status of liver: Improved status of experimentally induced NASH.

C57BL/6J mice were subjected to jetlag, High fat high fructose diet (HFHF) or combination of both. A significant increase in body weight was noted in HFHF and HFHF+Jetlag group, while Jetlag showed no change as compared to control. Further, melatonin treatment suppressed the increase in body weight in HFHF and Jetlag group. Melatonin treatment to HFHF+Jetlag group showed no significant change in body weight. Increased enzyme activity (AST and ALT levels) was observed in all the three disease control groups. However, Melatonin supplementation showed decrease in these liver injury markers. The mRNA levels of CPT-1 showed a decrease in HFHF and Jetlag group, in contrast, it increased in HFHF+Jetlag group. Melatonin treatment

resulted in significant decrease in the CPT-1 mRNA levels, suggesting decreased lipid load in melatonin treated groups. Additionally, expression of PPARα and SREBP1c reported increment in all the three disease control group, wherein increment was maximum in HFHF+Jetlag group. Moreover, melatonin supplementation reported significant decrease in the said genes. These results indicate that melatonin showed protective role against HFHF and/or Jetlag subjected mice induced NASH.

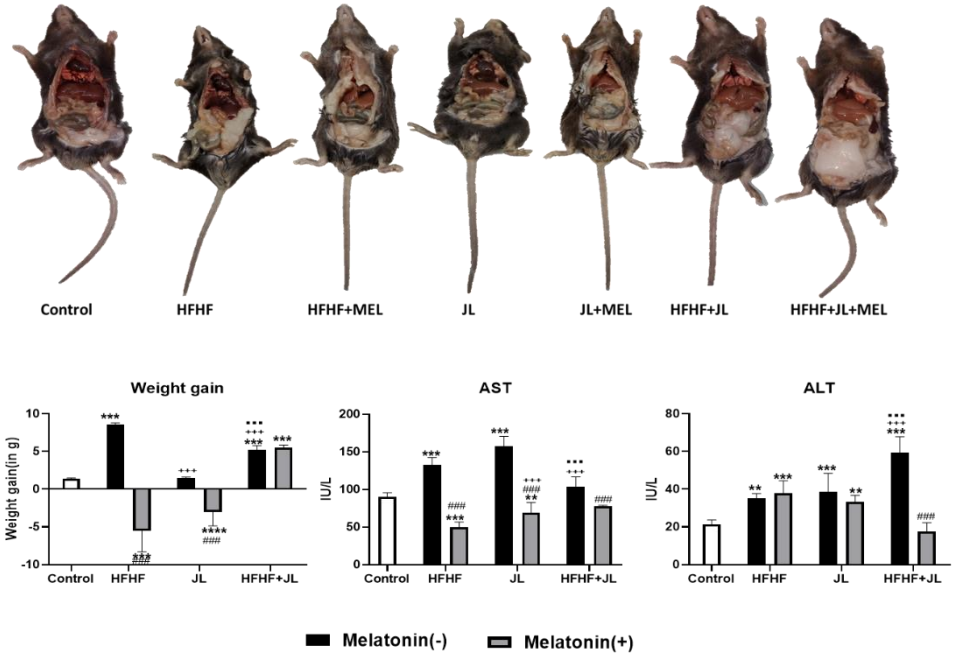


Fig 5: Effect of melatonin on mice subjected to HFHF and/or JL. Data represented as mean±SD *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF,JL and HFHF+JL, +++P<0.001 vs HFHF and …P<0.001. n=6

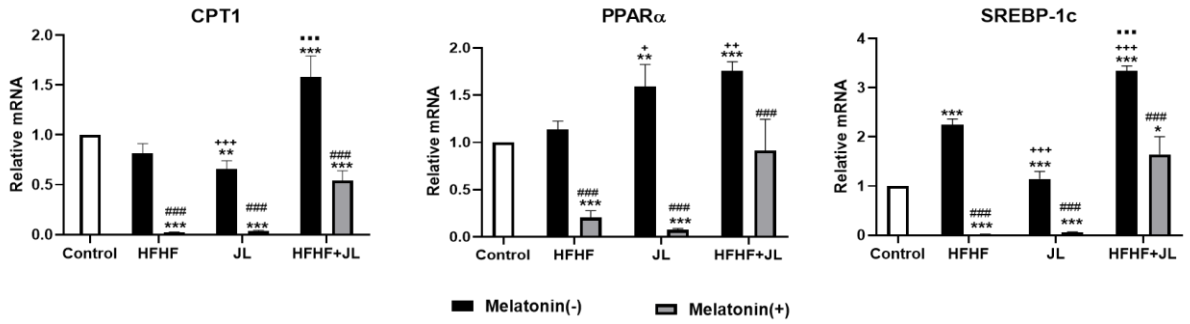
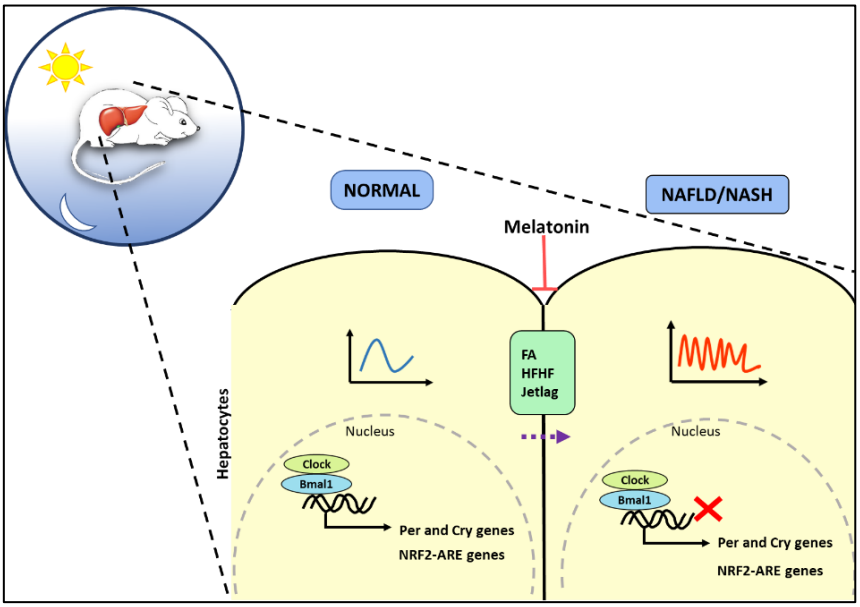


Fig 6: Protective Effect of melatonin on genes governing lipid metabolism in HFHF and/or JL exposed mice liver. Data represented as mean±SD *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF,JL and HFHF+JL, +P<0.05 +++P<0.001 vs HFHF and …P<0.001.

Study 2: Jet lag / HFHF induced chronodisruption in Nonalcoholic fatty liver disease: Role of exogenous melatonin.



Expression of core clock genes (Clock, Bmal1, Per1, Per2 and Cry2) in mice liver subjected to HFHF and/ or JL were examined after the end of the experimental protocol. We observed that HFHF group showed arrhythmic expression of positive regulators (*Clock* and *Bmal1*) but no change observed in negative regulators (*Per and Cry*). Moreover, JL group showed remarkable disruption of all the core clock genes. Melatonin supplementation alleviated the shallow expression of clock transcripts in mice liver induced by dietary and/or photoperiodic manipulation. Further, protein oscillatory expression of Clock-Bmal1 and NRF2-HO-1 was assessed. Circadian clock and NRF2-ARE pathway proteins rhythm showed phase shift and decreased amplitude in HFHF and/or JL group. Melatonin supplementation resulted in restored rhythm of Bmal1 and clock, in contrast moderate changes were observed in NRF2-HO-1proteins. Rhythmicity of clock genes in mice liver by cosinor software is under analysis.

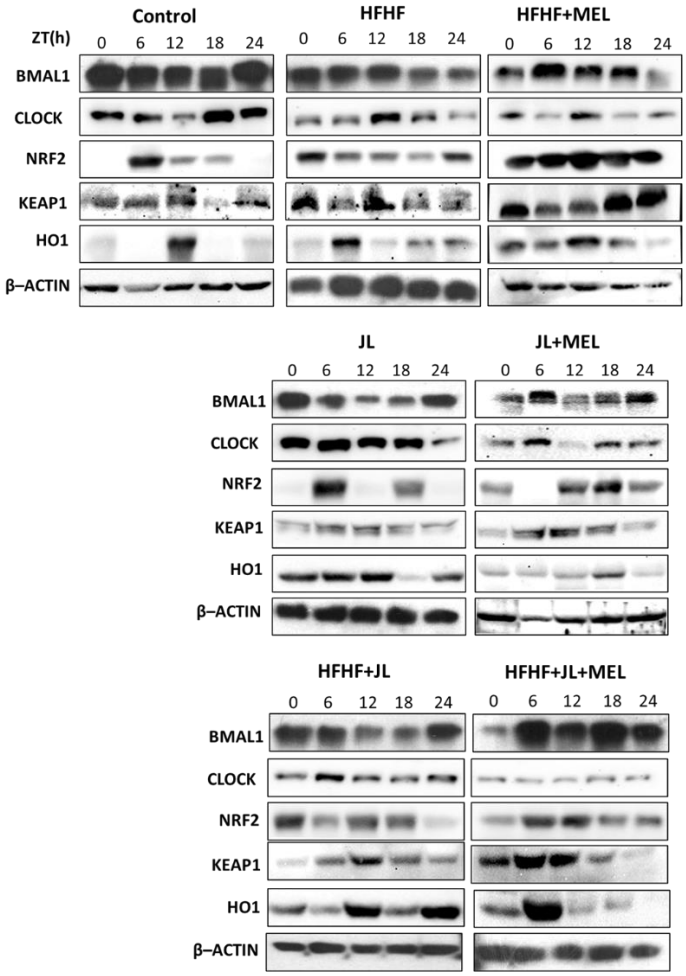


Fig 7: Melatonin modulates protein expression pattern of CLOCK-BMAL1 and NRF2-ARE pathway genes in HFHF and/or JL exposed mice liver. Protein samples from Liver were isolated at an interval of 6h and western blot analysis was carried out. Blots were normalized by β-actin as control.

Key Findings

Exogenous melatonin resulted in lowered OA uptake. Thus, improving cell viability as evidenced by lower indices of lipid accumulation and intracellular oxidative stress. Improved cell viability following melatonin treatment is also attributed to higher mitochondrial membrane potential and possibly healthy functional mitochondria in OA treated HepG2 cells. The key oscillators Bmal1, Clock and Per2 appear to be resynchronized in HepG2 cells that had undergone circadian desynchrony emphasizing the role of exogenous melatonin as a possible therapeutant. However, Cry2, Nrf2 and HO1 are oblivious to these effects. Improved antioxidant status in OA+melatonin treated HepG2 cells and corrective changes in lipid metabolism genes provide evidence on

potential of exogenous melatonin treatment. Improved condition of experimentally induced condition of HFHF and/or JL subjected C57BL6/J mice corroborates findings of invitro study. Serum lipid profile, lipid function and microscopic evaluation imply towards improvement of NASH by exogenous melatonin. It can be surmised that circadian desynchrony is a key player in aggravating HFHF induced NASH and corrective changes induced by exogenous melatonin are partially beneficial.

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