Introduction

Circadian rhythms are a 24h biological system that regulates various physiological activities in mammals. Circadian rhythms play an important role in regulating lipid, glucose, and cholesterol metabolism(Ferrell & Chiang, 2015). Strong correlation exists between circadian clock and onset of lifestyle disorders in humans. The suprachiasmatic nucleus (SCN) is the central regulator of the biological clock, whereas the peripheral clocks in various organs are operated by an autoregulatory expression of clock genes. The molecular network comprises of circadian locomotor output cycles kaput (Clock) and brain and muscle ARNT-like 1 (Bmal1) as activators, and period circadian protein homolog 1 (Per1), Per2, cryptochrome circadian regulator 1 (Cry1), and Cry2 as repressors, that work in a transcriptional-translational feedback loop(Buhr & Takahashi, 2013).

Desynchrony of circadian clock due to factors such as shift work, transcontinental travelling (Jetlag), sleep deprivation, artificial light at night exposure can trigger various metabolic disorders like obesity and NAFLD(Hong et al., 2020; Iwamoto et al., 2014; Kettner et al., 2016; Yue et al., 2020). On the other hand, restoration of circadian rhythms often has been reported to improve the status of these lifestyle disorders by reducing morbidity and disease complexity. Various studies with rodent models had shown correlation of circadian disruption with liver metabolism and energy homeostasis(Ferrell & Chiang, 2015; Turek et al., 2005; Xu et al., 2011). Bmal1$^{-/-}$ knock-out mice develop glucose intolerance, hypoinsulinemia, reduced fat storage, increased levels of circulating fatty acids, higher ectopic fat formation in liver and muscle, and hepatic steatosis even in regular chow-fed mice(McDearmon et al., 2006). Clock gene mutant animals display an impaired glucose and lipid metabolism and are susceptible to diet-induced obesity and metabolic dysfunction(Pérez-Mendoza et al.,

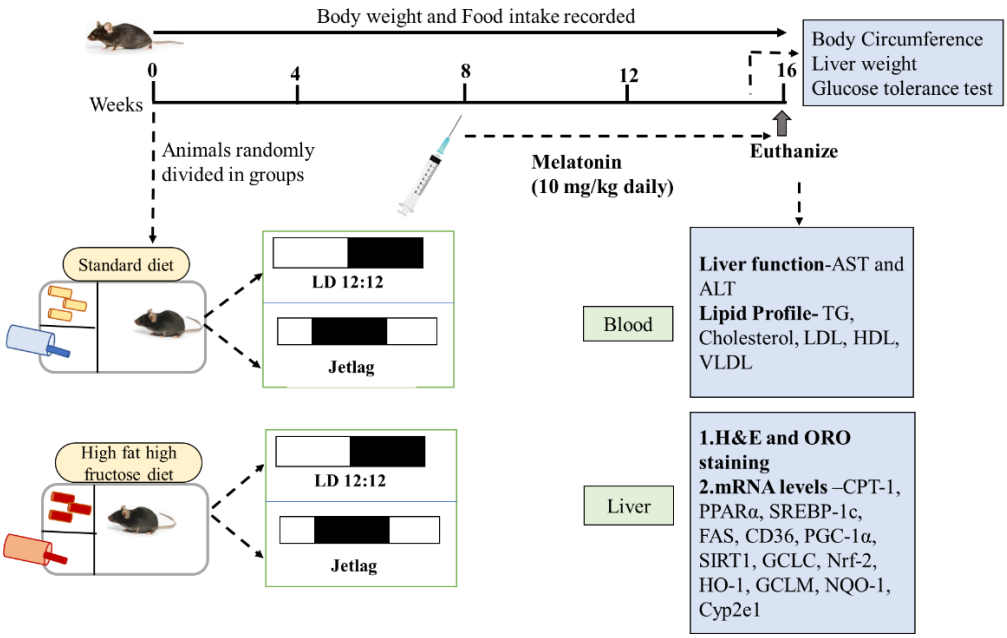
2014). Per2 was shown to have protective effect in the carbon tetrachloride-induced acute liver injury and fibrosis model, $Per2^{-/-}$ knockout mice progressed to a more severe form of hepatic fibrosis with hepatic stellate cell activation(Grimaldi et al., 2010). Chronic jetlag has also been reported to aggravate steatohepatitis and induce hepatocellular carcinoma in $Fxr^{-/-}$ mice(Kettner et al., 2016). These compelling evidence points towards a strong connection between the circadian clock and metabolic homeostasis.

Various coactivators such as PGC-1α and β, other NRs like PPARs and SIRT1 are known to show diurnal rhythms and are key players in the cross talk between circadian clock and metabolic pathways(Mazzoccoli et al., 2012). The circadian expression of PPARα mRNA was abolished in the liver of homozygous Clock mutant mice(Oishi et al., 2005). These results suggested that CLOCK plays an important role in lipid homoeostasis by regulating the transcription of a key protein, PPARα. REV-ERBα controls the timing of the cyclic accumulation of SREBP in the nucleus, which, in turn, regulates the temporal expression of Hmgcr and hence regulates lipogenesis(Shi et al., 2019). Sirt1 is required for the transcriptional activation of core clock genes. SIRT1 binds to CLOCK-BMAL1 in a circadian manner and promotes deacetylation and degradation of PER2. Further, SIRT1 links cellular metabolism to the circadian core clockwork circuitry(Zhou et al., 2014).

Increasing body of evidence on circadian rhythm studies have provided important insights that correlate with expression of the circadian clock gene and metabolism in experimental model of clock gene ablation induced NAFLD. This study investigates role of clock genes and exogenous melatonin in metabolic rewiring under conditions of HFHF and/or JL induced NAFLD. The experimental model comprises of induction of Jet lag (Photoperiodic manipulation) and/or HFHF induces NAFLD in C57BL/6J mice.

The fatty changes in liver, alterations in lipid profile, hepatic antioxidant genes and mitochondrial function has been studied in detail. Merits of exogenous melatonin on the said pathophysiological condition and the corrective changes in physiology and metabolism are showcased herein.

Experimental design



Results

Melatonin alleviates weight gain in HFHF and/or JL subjected C57BL6/J mice.

C57BL/6J mice were subjected to HFHF, JL or combination of both. A significant increase in body weight was noted in HFHF and HFHF+JL group ($P<0.001$), while JL showed no change as compared to control. Further, melatonin treatment accounted for decrement in body weight of HFHF and JL group (Fig 3.2). Body circumference of HFHF and HFHF+JL group showed ~20% increase, while no significant change was observed in JL group as compared to control (Fig. 3.4). Melatonin treatment to these group recorded no significant improvement in these three experimental groups (Fig. 3.4). Further, no significant change was observed in liver: body weight ratio (Fig. 3.4). HFHF, JL and HFHF+JL groups showed a significant increase in blood glucose levels 30 mins after glucose challenge($P<0.001$), wherein HFHF+JL group showed maximum levels ~ 280 mg/dl. However, higher levels of blood glucose persisted even after 60 mins of glucose challenge, suggesting glucose resistance. HFHF alone or in combination with JL accounted for significant increment in blood glucose and AUC (Area under curve) ($P<0.001$). Melatonin treatment to these three experimental groups did not account for a decrement in AUC as the values were significantly higher than control group (Fig. 3.5)

Melatonin improved liver function enzymes and serum lipid profile in HFHF and/or JL induced NAFLD.

The markers of liver function (AST and ALT) were significantly elevated in HFHF, JL and HFHF+JL groups ($P<0.001$). Melatonin treatment accounted for significant decrement ($P<0.001$) in serum AST levels in these experimental groups whereas decrement in ALT levels were recorded only in HFHF and JL groups (Fig3.6 A and B). Experimental groups viz. HFHF, JL and HFHF+JL showed alterations in lipid profile

wherein TG, TC, LDL and VLDL were found to be significantly elevated (P<0.01) and HDL significantly lowered (P<0.001). Melatonin treatment to these experimental groups accounted for a significant (P<0.01) improvement in TG and TC but LDL, VLDL and HDL did not record the said favorable changes. However, relative significant decrement (P<0.001) in LDL and VLDL and an improvement in HDL were observed following melatonin treatment (Fig.3.9).

Melatonin alleviates histopathology in HFHF and/or JL induced NAFLD.

Microscopic evaluations of liver samples showed hepatocyte ballooning, cellular derangement, and Mallory hyaline in HFHF, JL and HFHF+JL groups (fig 3.7). Random scoring of the liver tissue sections revealed that HFHF+JL group accounted for maximum indices of fatty manifestations in liver. Melatonin treatment accounted for significant decrement in indices of fatty manifestations and intracellular fat accumulation with reparative changes observed in HFHF+JL+Mel group (Fig3.7). HFHF and HFHF+JL groups recorded significant increment in fatty changes in hepatocytes as evidenced by ORO staining, but melatonin treatment showed beneficial effect in decreasing the lipid content in the liver (Fig3.8)

Melatonin improves mRNA profiles of lipid regulatory genes, antioxidant genes and inflammatory genes in HFHF and/or JL induced steatotic liver.

To determine the protective role of melatonin in HFHF and/or JL induced NAFLD, we assessed mRNA levels of lipid regulatory genes in liver samples. mRNA levels of *CPT-1, PPARα and SREBP-1c* were significantly elevated in HFHF, JL and HFHF+JL groups (P<0.01). Melatonin treatment accounted for a significant decrement (P<0.001) in the mRNA of the said genes (Fig 3.10). A significant increment of FAS mRNA levels was recorded in HFHF and HFHF+JL group, but melatonin treatment accounted for significantly lowered mRNA expression. By contrast, mRNA levels of CD36 recorded

a decrease in HFHF and/or JL groups whereas moderate corrective changes were observed after melatonin treatment (Fig. 3.11). The mRNA profiles of PGC-1α and SIRT1 recorded moderate decrement in HFHF and/or JL groups but melatonin treatment resulted in significant increment of the same (Fig. 3.12). The mRNA profile of Nrf2, HO-1 and NQO-1 recorded an increment in HFHF and/or JL group, whereas melatonin treatment caused decrement in the expression in said genes. Further, HFHF and/or JL subjected mice accounted for a reduction in the mRNA levels of GCLC and GCLM as compared to control mice. However, melatonin treatment did not record any significant improvement in the said parameters. JL and HFHF+JL group accounted for an increase in levels of Cyp2e1 mRNA levels, whereas melatonin treatment significantly lowered levels of the said genes (Fig. 3.13). HFHF and/ or JL groups accounted for an increment in mRNA levels of IL-6, TNFα and NFκB. However, melatonin treatment resulted in decreased expression of said inflammatory markers.

Figure 3.1: C57BL6/J mice viscera showing adipose build-up.

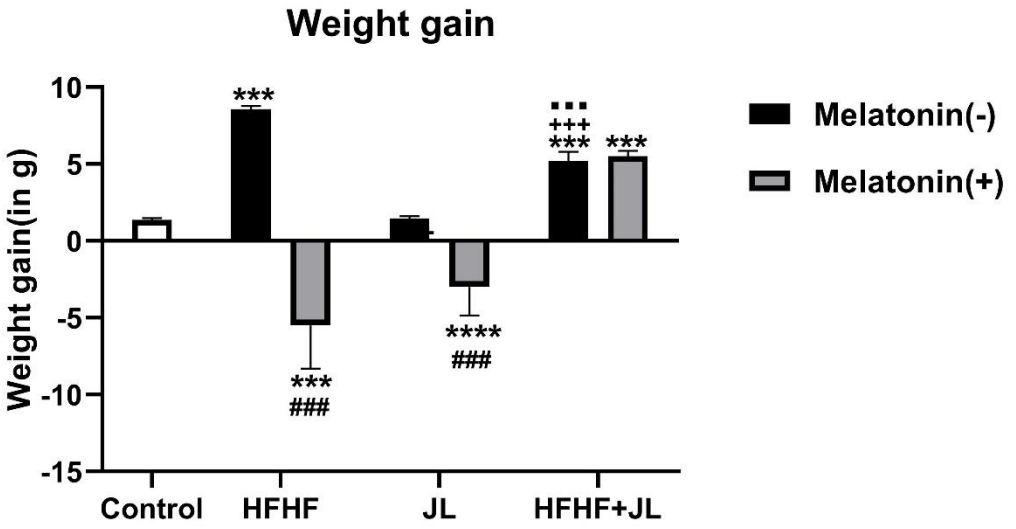


Figure 3.2: Effect of melatonin on weight gain in HFHF and/or JL subjected mice. Data represented as mean±SD ***P<0.001 vs control, ###P<0.001 vs HFHF, JL and HFHF+JL, +++P<0.001 vs HFHF and …P<0.001.

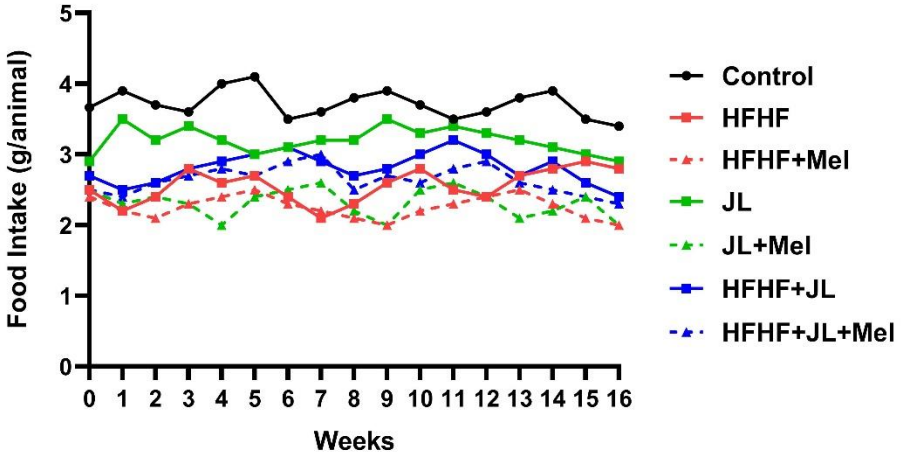


Figure 3.3: Food intake in mice subjected to HFHF and/or JL for 16 weeks.

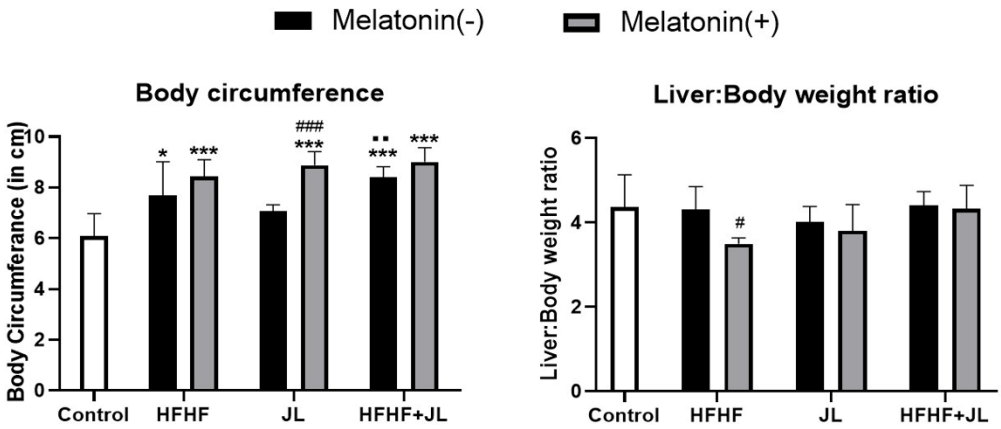


Figure 3.4: Effect of melatonin on Liver: body weight ratio and Body circumference in HFHF and/or JL subjected mice. Data represented as mean±SD ***P<0.001 vs control, ###P<0.001 vs HFHF, JL and HFHF+JL.

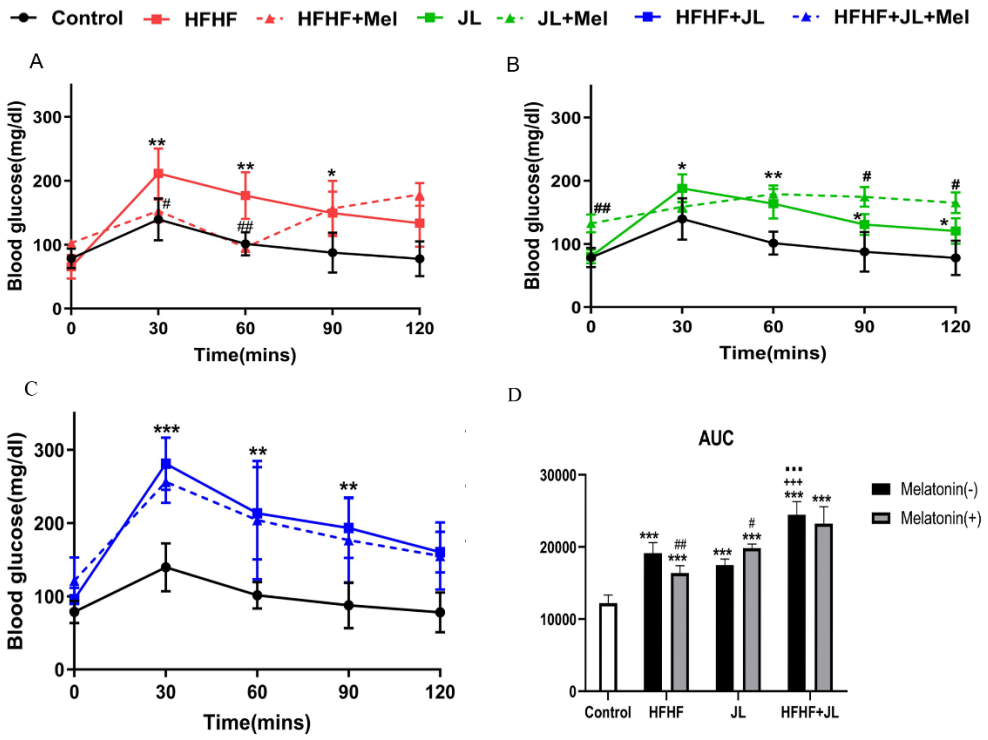


Figure 3.5: Effect of melatonin on blood glucose levels in HFHF and/or JL subjected mice. (A) Blood glucose levels at different time intervals (B) area under curve (AUC). Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL, +++P<0.001 vs HFHF and …P<0.001. n=6

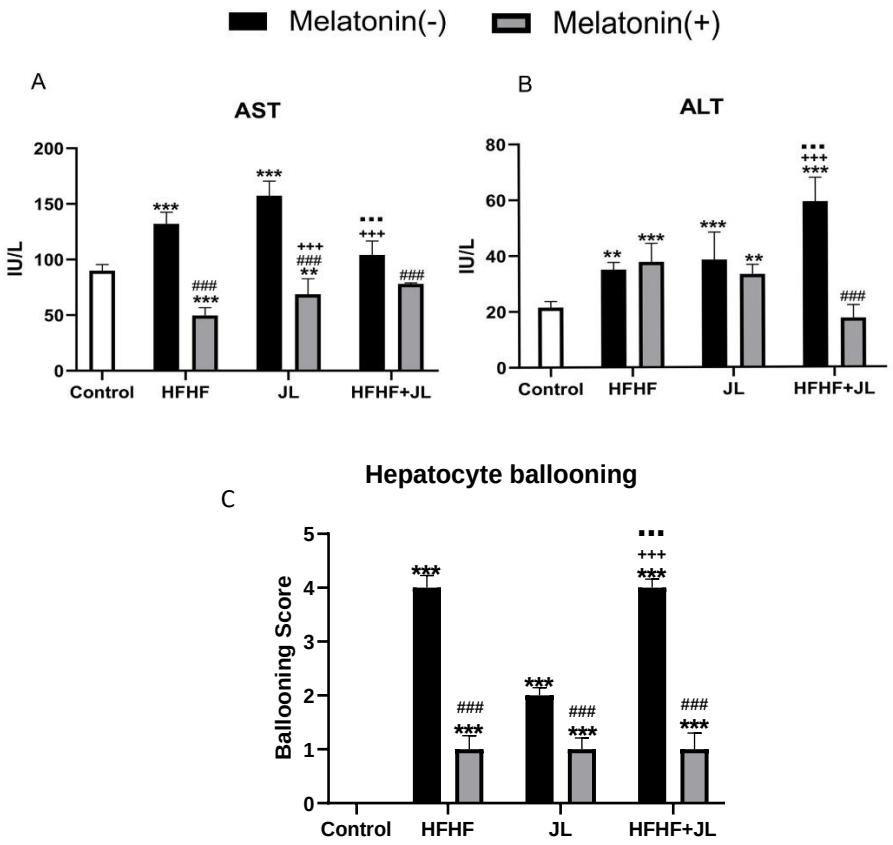


Figure 3.6: Melatonin treatment improves liver function in HFHF and/or JL exposed mice. Graph represent (A) AST and (B) ALT levels in serum (C) Hepatocyte Ballooning Score. Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL, +++P<0.001 vs HFHF and …P<0.001. n=6

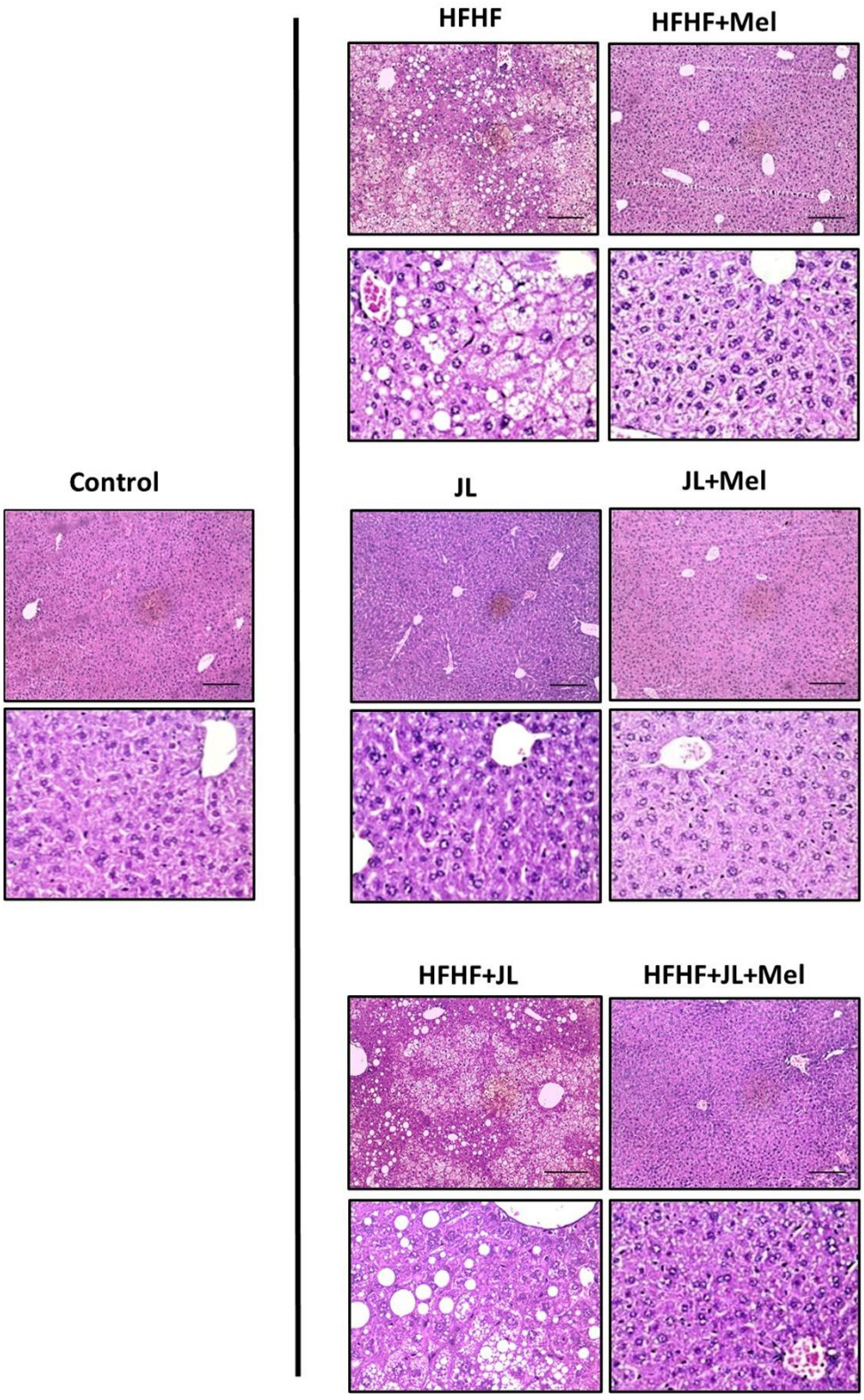


Figure 3.7: Histological analysis of liver done with HE staining of HFHF and/or JL exposed group showing improvement in melatonin administered C57BL6/J mice. Data is depicted Scale bar=100µm. Magnification=100X and 400X.

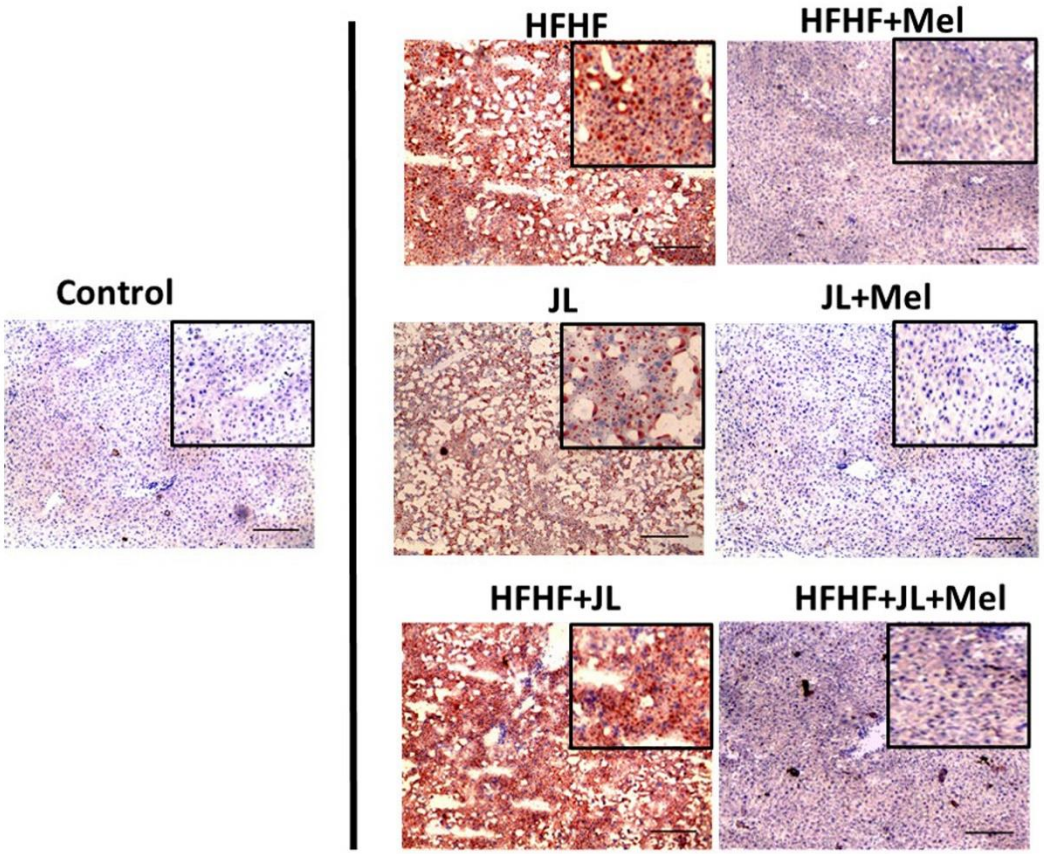


Figure 3.8: ORO staining in fresh frozen section of livers from different groups. Slides depict melatonin treatment improves fatty changes in HFHF and/or JL exposed mice. Scale bar=100µm. Magnification=100X and 400X.

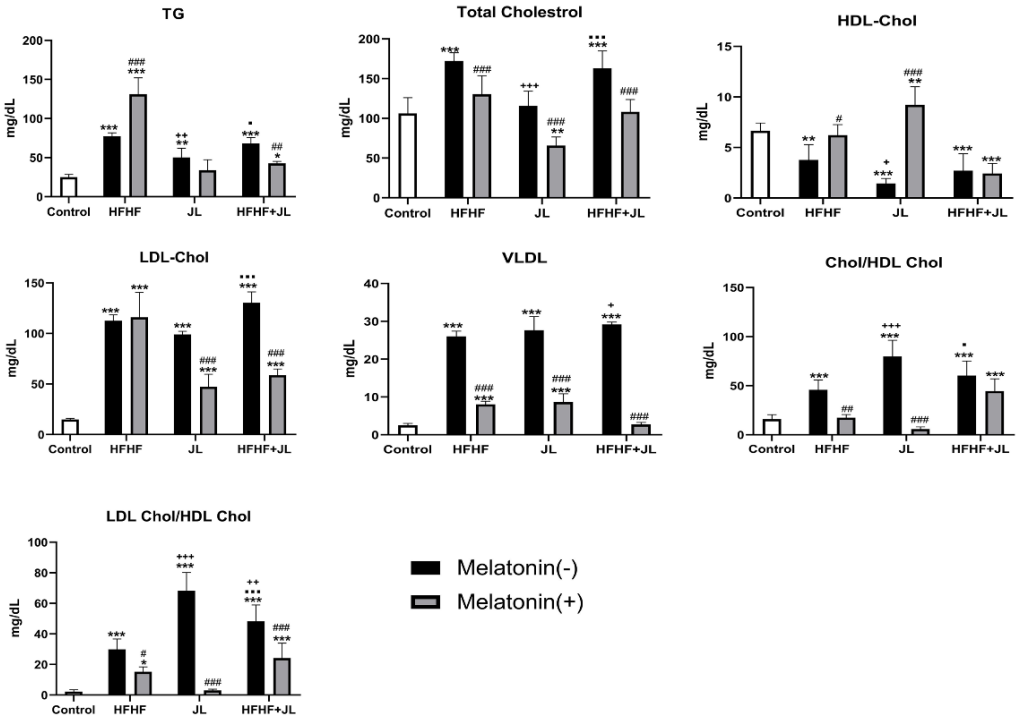


Figure 3.9: Melatonin improves serum lipid profile of mice fed with HFHF and/or exposed to JL. Data represented as mean±SD *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL, +++P<0.001 vs HFHF and …P<0.001. n=6

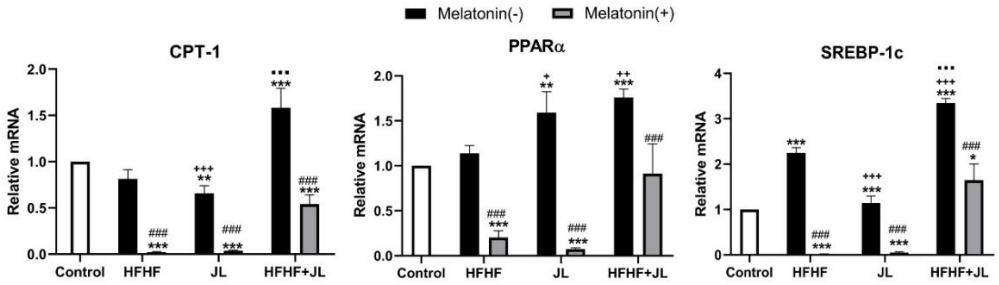


Figure 3.10: Protective effect of melatonin on genes regulating lipid metabolism in HFHF and/or JL exposed mice liver. Graphs represents relative mRNA expression as analyzed by RT-qPCR. Values were normalized with GAPDH. Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL, +P<0.05 +++P<0.001 vs HFHF and …P<0.001.

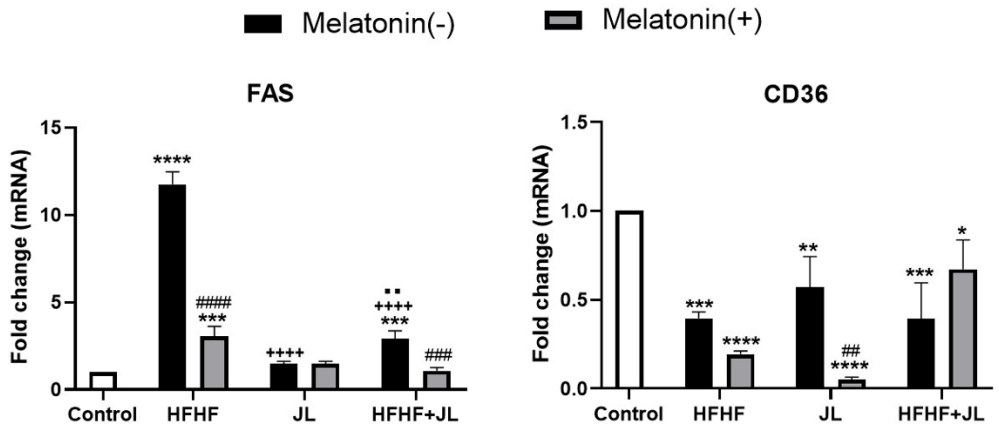


Figure 3.11: Effect of melatonin on genes regulating lipid uptake in HFHF and/or JL exposed mice liver. Graphs represents relative mRNA expression as analyzed by RT-qPCR. Values were normalized with GAPDH. Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL, +P<0.05 +++P<0.001 vs HFHF and …P<0.001.

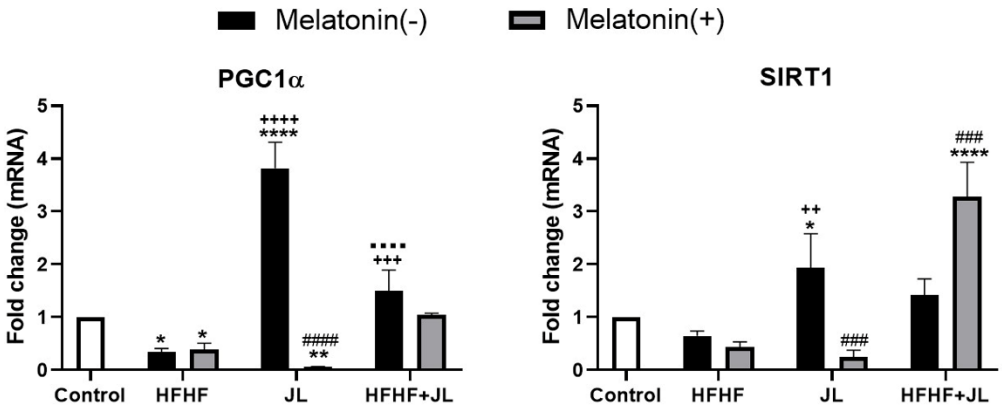


Figure 3.12: Effect of melatonin on genes regulating mitochondrial biogenesis in HFHF and/or JL exposed mice liver. Graphs represents relative mRNA expression as analyzed by RT-qPCR. Values were normalized with GAPDH. Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL, +P<0.05 +++P<0.001 vs HFHF and …P<0.001.

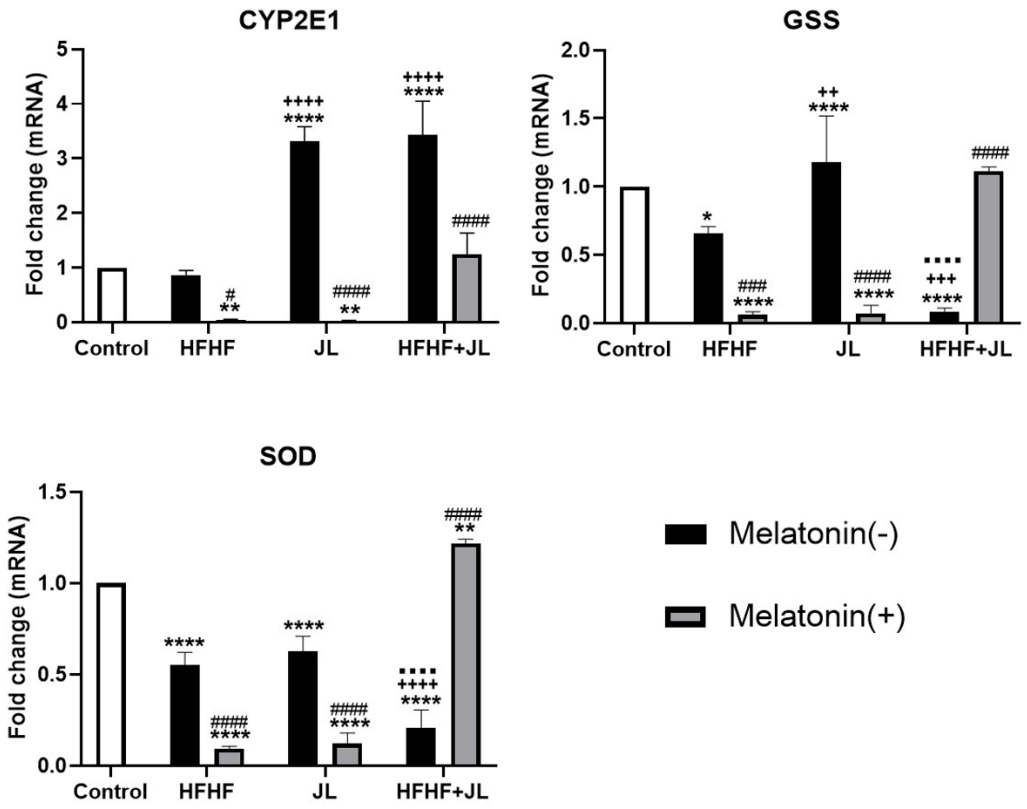


Figure 3.13: Effect of melatonin on genes regulating oxidative stress in HFHF and/or JL exposed mice liver. Graphs represents relative mRNA expression as analyzed by RT-qPCR. Values were normalized with GAPDH. Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL, +P<0.05 +++P<0.001 vs HFHF and …P<0.001.

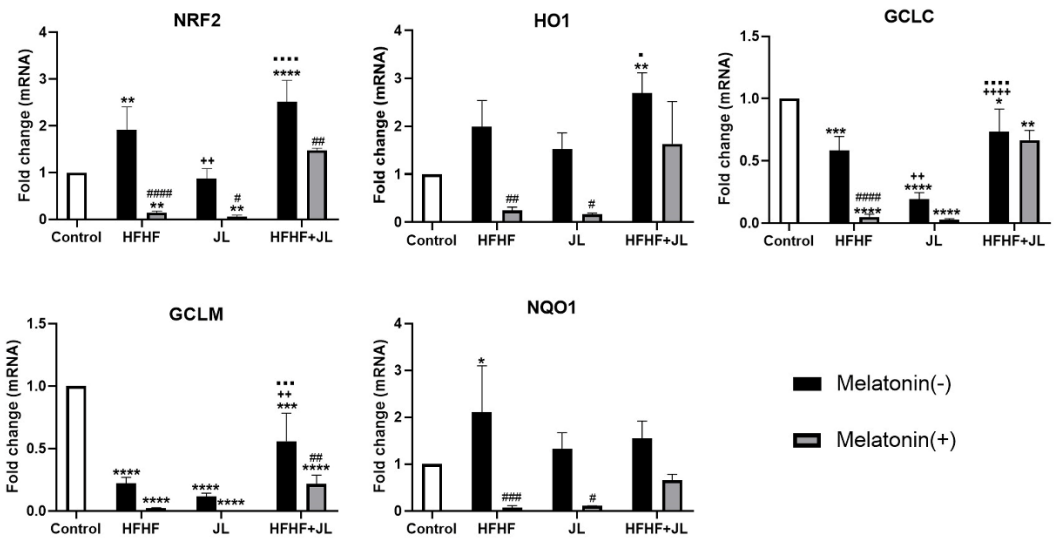


Figure3.14: Effect of melatonin on genes regulating Nrf2-ARE pathway genes in HFHF and/or JL exposed mice liver. Graphs represents relative mRNA expression as analyzed by RT-qPCR. Values were normalized with GAPDH. Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, Graphs represents relative mRNA expression as analyzed by RT-qPCR. ###P<0.001 vs HFHF, JL and HFHF+JL, +P<0.05 +++P<0.001 vs HFHF and …P<0.001.

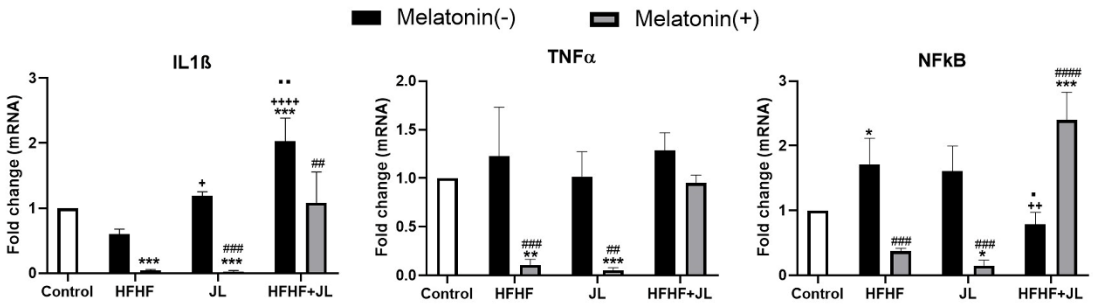


Figure 3.15: Effect of melatonin on genes regulating inflammation in HFHF and/or JL exposed mice liver. Graphs represents relative mRNA expression as analyzed by RT-qPCR. Values were normalized with GAPDH. Data represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs HFHF, JL and HFHF+JL, +P<0.05 +++P<0.001 vs HFHF and …P<0.001.

Discussion

Modern societies have undergone changes in lifestyle, which has increased the risk of metabolic syndrome. NAFLD is the most common chronic liver disease, which may progress to NASH, cirrhosis, or hepatocellular carcinoma(Mulhall et al., 2002). Epidemiological studies have shown an increased risk of obsesity and metabolic disorders among shift workers(Gangwisch, 2009). There are various reports that demonstrate a link between circadian rhythm and metabolic disorders using gene ablation models(Grimaldi et al., 2010; Jacobi et al., 2015; Oishi et al., 2005). However, these models do not closely relate to chronodisruption induced in shift workers and transcontinental travellers. In our study, high fat high fructose diet was fed to mice and photoperiodic regime of 6h phase advance and phase delay was performed to mimic fructose and fat rich diet consumed by humans along with jetlag. Melatonin is extensively reported for its multifaceted physiological role and also in improving hepatic pathophysiology in liver diseases including NAFLD(Mi et al., 2018; Tahan et al., 2009; Tiao et al., 2014; J.-J. Zhang et al., 2017). Evening injections of melatonin has been associated with high degree of physiological relevance in mice and hence, the same was used in our study(Baxi et al., 2013). This study investigates the impact of high fat high fructose diet in combination with jetlag on possible liver damage in C57Bl6/J mice and the role of melatonin in improving the said pathophysiology associated with NAFLD.

HFHF and HFHF+JL group had recorded weight gain, whereas melatonin treatement showed lowered indices of the said parameter. A comparision of data on food intake had revealed that HFHF, JL and HFHF+JL groups had lowered food intake and the same was not improved following melatonin treatment. These results are in accordance

with other studies on high fat diet(Lin et al., 2000), jetlag in CBA/N mice wherein it is reported that time-defined feeding prevents obsesity without affecting the caloric intake (Iwamoto et al., 2014). In our study, significantly elevated titers of AST and ALT were observed in HFHF and/or JL mice indicating liver damage. These results are in agreement to other reports indicating increase in said parameters in HFD and jetlagged mice (Kettner et al., 2016; Upadhyay et al., 2020; Xie et al., 2017), however there are no reports on HFHF+JL groups. Also, melatonin is known to attenuate liver damage by lowering AST and ALT levels in rats fed with HFD(Hatzis et al., 2013).

Dysregulation of lipid metabolism in the liver, especially hepatic de novo fatty acid synthesis—could be attributed to the development of NAFLD. Metabolic perturbations due to increased fat accumulation is a key factor towards initiation of NAFLD. Our results had revealed an improvement in key lipid metabolism genes (CPT-1, PPARα, SREBP-1c, FAS and CD36) following melatonin treatment. These results are in agreement with other studies wherein melatonin mediated improved status of lipid metabolism genes is reported in hamsters and mice fed with high fat diet (de Farias et al., 2019; Ou et al., 2019). Also, Melatonin is reported to alleviate insulin resistance and obesity caused by persistent artificial light exposure in guinea pigs, via activation of the AMPK*α*/PPAR*α* signaling pathway (Liu et al., 2020). Our findings are attributable to a similar set of changes in melatonin treated groups wherein, CPT-1, PPARα, SREBP-1c and FAS showed a decrement in mRNA levels. CD36 levels were found to be oblivious to the treatment schedule and the underlying reason for discrepancies in the result obtained could not be deciphered in our study. Microscopic evaluations (H&E and ORO stainings) had shown distorted fatty accumulation, hepatocyte ballooning and steatotic changes in the livers of HFHF, JL and HFHF+JL

groups. The fatty manifestations in liver have been reported from our lab (Upadhyay et al., 2020) with same dietary composition or by other research groups using MCD diet (Kim et al., 2016), HFD(Lee et al., 2014) etc. Photoperiodic manipulations such as continuous lighting (Hong et al., 2020), or a Jetlag protocol (Kettner et al., 2016) has shown histopathological perturbations in liver that culminate in fatty manifestations over a period of time. Hence, the observed fatty changes are comparable to these published reports. A combination of altered photoperiodic regime and high calorie diet is the best mimic of physiological changes that occur during lifestyle disorders and hence a combination of HFHF+Jl was tried out to understand a possible synergism in experimentally induced NAFLD. Herein, we hypothesize that a combination of HFHF+JL may possibly amplify the pathophysiological changes of NAFLD. The fatty accumulation and steatotic changes were more prominent in HFHF+JL group compared to HFHF and JL groups with JL alone recording minimal steatotic changes. Exogenous melatonin is known to improve the condition of NAFLD in a variety of experiment model such as HFD or MCD fed rodent models (Amin et al., 2017; Hatzis et al., 2013; Pan et al., 2006). In HFHF diet too, melatonin was found to be effective in allievating histopathological damage in the liver. On the other hand, continuous light exposure induced liver damage has been shown to be improved by 50 mg/kg melatonin orally (Hong et al., 2020). This study is the first to report corrective changes in liver histoarchitecture and function following melatonin treatment.

NAFLD is mainly characterised by excess lipid accumulation in liver that leads to altered β-oxidation of fatty acids, eventually resulting in increased ROS and mitochondrial dysfunction (Takaki et al., 2013). In this study, Sirt1 and PGC1α mRNA levels showed decrement in HFHF group but melatonin treatment recorded moderate

corrections in the said genes. However, melatonin is known to increase sirt-1 levels by inhibiting miR-34a-5p (sirt-1 inhibitor) that culminates in cessation of NAFLD progression(Stacchiotti et al., 2019). In contrast, SIRT1 was shown to function as a HDAC which counteracts the activity of the clock machinery by binding to the CLOCK:BMAL1 complex in a circadian fashion and promoting the deacetylation and subsequent degradation of PER2 (Zhou et al., 2014). Oxidative stress and depletion of cellular antioxidant is the key driving force in progression of NAFLD to NASH. Increased intracellular ROS may result in depletion of antioxidants such as glutathione (GSH) and inhibit the activities of antioxidant enzymes such as superoxide dismutase (SOD) (He et al., 2017). We have observed an increment in the mRNA expression of Cyp2e1, Nrf2, HO-1, NQO-1 genes in HFHF, JL and/or HFHF+JL groups, but exogenous melatonin treatment had accounted for a decrement in expression levels of said genes. The mRNA expression of GSS, SOD, GCLC and GCLM showed a decrement in the HFHF and/or JL groups, but melatonin treatment had accounted for an upregulation of these thus accounting for an improved antioxidant status. These results corroborate with findings of other research groups wherein, melatonin is shown to counteract oxidative stress in HFD induced steatosis in mice by increasing the levels of various antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and glutathione reductase in liver tissue (Jarukamjorn et al., 2016) but the observed results in HFHF+JL group are novel and reported for the first time.

Low grade inflammation is a major contributing factor for the transition from NAFLD to NASH (Gao & Tsukamoto, 2016). NF-κB is a transcriptional factor involved in the inflammatory pathways and its activation results in progression of steatosis to NASH. In this study, mRNA levels of pro-inflammatory cytokines IL-1β and TNFα recorded

an increment in HFHF and/or JL group, whereas melatonin resulted in a decrement in the said inflammatory markers. Melatonin treatment resulted in decrement in the mRNA levels of the inflammatory marker genes. These results are in agreement with other findings on HFD induced NAFLD wherein Melatonin mediated decrease in inflammation was attributed to corrective changes in MAPK-JNK/P38 signaling pathway(Sun et al., 2016). It can be comcluded from this study that HFHF+JL does not result in the expected synergistic effect. Steatotic changes observed in this group were comparable to the HFHF group. Melatonin improved steatosis and NAFLD in all 3 disease control groups (HFHF, JL and HFHF+JL) wherein the recovery was maximum in JL+Mel group. These results are attributable to the anti-inflammatory role of melatonin and its ability to restore the cellular antioxidant levels. Melatonin itself is a proven antioxidant (H. Zhang & Zhang, 2014), and hence its contribution cannot be ruled out in improving experimentally induced NAFLD.