Validation of experimentally induced circadian desynchrony and nonalcoholic steatohepatitis in C57BL/6J mice

Introduction

Nonalcoholic fatty liver diseases (NAFLD) or Nonalcoholic steatohepatitis (NASH) is considered a key challenge because of its occurrence, difficulties in diagnosis, multifaceted pathogenesis and lack of FDA approved drugs. Nonalcoholic steatohepatitis (NASH) is a morbid liver diseases and a major global health problem with limited treatment. NASH is the progressive form of nonalcoholic fatty liver disease (NAFLD) and is one of the most common diseases prevailing in Western countries (Paschos & Paletas, 2009). NASH is characterized by steatosis, liver cell injury, and inflammation (Day & James, 1998; Kleiner et al., 2005). It is a part of the spectrum of NAFLD ranging from simple fatty liver to cirrhosis (Kumar & Malet, 2000). High calorie diet causes an increase in body weight, insulin resistance, high titres of liver enzymes and NASH in patients (Jahn et al., 2019; Schattenberg & Galle, 2010). High calorie diet is the most common experimental dietary method for induction of steatotic liver in mice that mimics western high calorie dietary composition. Various studies emphasize on a major role of high calorie diet, irregular sleep wake cycle and feeding fasting cycle have a significant role in the development of simple steatosis and its progression to NASH (Shetty et al., 2018; Tahara & Shibata, 2016; Tong & Yin, 2013). NASH has also been reported to be frequently associated with metabolic disorders such as insulin resistance, obesity, type 2 diabetes mellitus, cardiac failure, hyperlipidaemia, protein-calorie malnutrition, renal failure and fatty liver (Marchesini & Marzocchi, 2007). Metabolic syndrome is characterized by the presence of abdominal obesity, elevated triglycerides, high levels of hepatic SGOT and SGPT enzymes, reduced HDL-cholesterol and impaired fasting glucose in C57BL/6J mice (Joshi et al., 2021).

The molecular circadian clock system is composed of transcriptional-translational feedback loops. The circadian core loop includes Clock (circadian locomotor output cycles kaput) and Bmal1or Arntl1 (brain and muscle-ARNT-like 1) heterodimerize and generates autonomous circadian rhythm (Gekakis et al., 1998; Takahashi et al., 2008). The heterodimerization of Clock-Bmal1 drives the transcription of clock controlled genes Period (Per1, 2 and 3) and Cryptochrome (Cry1 and 2) genes from their E-box sites (5′-

ACGTG-3′) (Gekakis et al., 1998). In mammals, endogenous circadian clock coordinates majority of physiological processes with environmental conditions such as sleep-wake cycle, feeding/fasting cycle and hormonal secretion (Lamia et al., 2008). Hepatic circadian genes involved in the metabolic functions exhibit circadian rhythmicity in glucose, lipid and protein metabolisms (Jacobi et al., 2015; Joshi et al., 2021; Yin et al., 2018). Desynchrony in the hepatic circadian clock genes exacerbates liver diseases including fatty liver, steato-hepatitis, cholestasis, cirrhosis and liver cancer (Tahara & Shibata, 2016). Bmal1$^{-/-}$ knockout in mice results in reduced sleep, lean body, reduced longevity and irregular energy metabolism as compared to wild type mice suggesting that molecular clocks maintain an overall homeostasis of physiological functions. Disruption or disturbance in the rhythmicity of clock gene function results in impaired lipid absorption, abnormal metabolic phenotype and an altered gut microbiota, thus demonstrating essentiality of circadian system in maintaining normal energy metabolism (Lamia et al., 2009; Pariollaud et al., 2018; Shetty et al., 2018). This study validates the alterations in clock genes and dysregulation in lipid metabolism genes and the role of evening intraperitoneal injections of melatonin in high fat-high fructose and/or chronodisruption subjected C57BL/6J mice. This experimental model mimics a life style disorder since it is a combination of jetlag (photoperiodic manipulation) and high calorie diet (high fat-high fructose diet) that culminates in NASH in C57BL/6J mice.

This study validates the alterations in liver functional markers, lipid profile, lipid regulatory genes, desynchrony in clock genes and endogenous melatonin levels in chronodisruption (CD), high fat-high fructose diet (H) and combination of both i.e. high fat-high fructose diet and chronodisruption (HCD) treated C57BL/6J mice. Also, the merits of timed (evening) melatonin injection in the said groups and subsequent corrective changes in hepatic clock genes, physiology and metabolism are showcased herein.

Materials and methods

Experimental model: C57BL/6J mice male mice aged (6-7 weeks each weighing 20-22 g. Particulars of animal maintenance and ethical statement are provided in Materials and methods section.

Experimental groups:

1. Control
2. Chronodisruption (CD)
3. High fat-high fructose diet (H)
4. High fat-high fructose diet + Chronodisruption (HCD)
5. Chronodisruption + Melatonin (CDM)
6. High fat-high fructose diet + Melatonin (HM)
7. High fat-high fructose diet + Chronodisruption + Melatonin (HCDM)

Food intake, water intake and body weights were recorded thrice every week throughout the period of study. After 18 weeks, mice were euthanized under mild isoflurane anaesthesia and blood, liver and hippocampus were collected as mentioned in materials and methods section.

Parameter tested:

1. Physical parameters (Body weight, Liver, adipose weight and body circumference)
2. Serum liver functional test (ALT, AST and ALP), glucose, lipid profile (Cholesterol, Triglycerides, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, total lipids), protein markers (total protein, albumin and globulin) and rhythmic pattern of melatonin by ELISA
3. Histopathological studies (Hematoxylin eosin (HE) staining)
4. Quantitative RT-PCR: Core clock genes (BMAL1 and CLOCK) and lipid regulatory genes (CPT-1, PPARα, SREBP1C, FAS and CD36)

The experimental protocol for the present study is depicted in Fig. 1.1. Detailed methodology is described in materials and methods section.

Experimental design

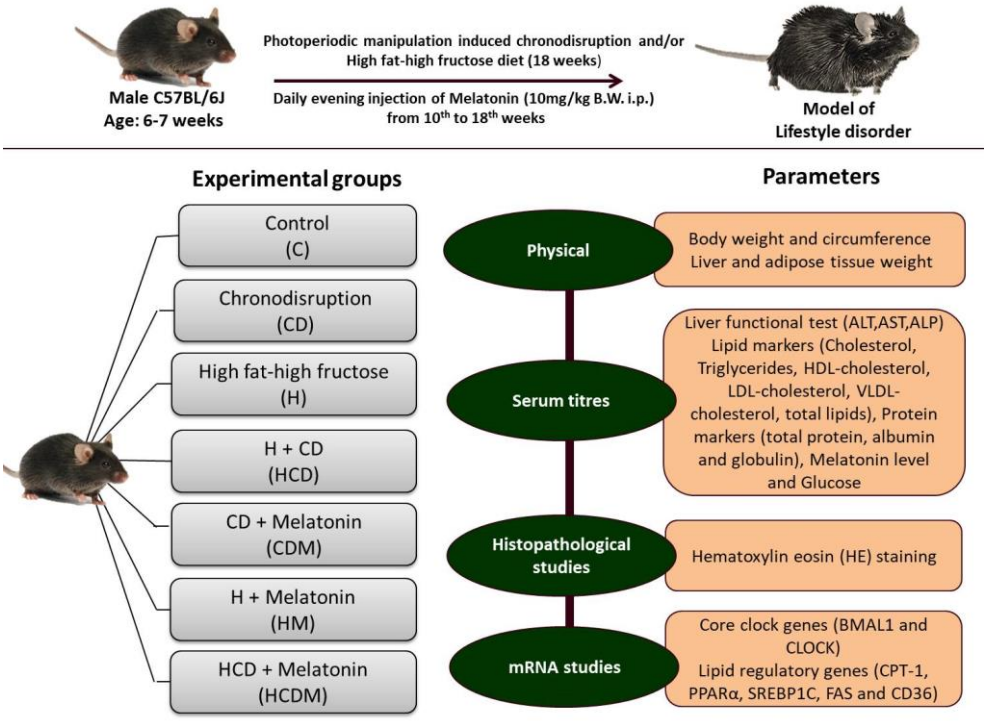

Figure 1.1: Flow chart of experimental protocol followed for validation of experimentally induced Nonalcoholic steatohepatitis by chronodisruption and/or high fat-high fructose diet in C57BL/6J mice.

Results

High fat-high fructose diet (H) and photoperiodic manipulation induced chronodisruption (CD) mediated alterations in body and liver weight in C57BL/6J mice

C57BL/6J mice were maintained on high fat-high fructose diet and/or photoperiodic manipulation induced chronodisruption for the period of 18 weeks for the development of nonalcoholic steatohepatitis. At the end of 9 weeks, mice were treated with daily evening injections of melatonin (10 mg/kg; i.p.) from 10^{th} to 18^{th} week along with high fat-high fructose diet and/or chronodisruption. Body weights of (H) ($p<0.001$) and (HCD) ($p<0.01$) treated group's mice were significantly higher than that of control mice at the end of 18 weeks. Also, high fat-high fructose diet (H) ($p<0.01$) fed mice recorded significant increment in liver to body weight ratio, and HCD mice showed non-significant increment as compared to control (Fig. 1.3a). Timed administration of exogenous melatonin in the said disease control groups recorded no body weight gain and/or increase in liver weight as compared to control groups (Fig. 1.3b). Further, H and HCD groups mice recorded an increment in liver weight as compared to control mice; on the other hand, melatonin administered groups recorded liver weight same as that of control mice (Fig. 1.3b).

Body circumference of H ($p<0.001$) and HCD group showed ~15% increase, but no significant change was observed in the CD group as compared to control (Fig. 1.4b). Melatonin treatment to these groups had recorded a non-significant improvement in these experimental groups. Further, a non-significant increment in the visceral adipose tissue weight was recorded in H and HCD groups; however melatonin treatment to these groups could revert the levels comparable to the control (Fig. 1.4a).

High fat-high fructose diet and/or chronodisruption subjected C57BL/6J mice develop metabolic changes and liver injury characteristic of NASH

The functional status of liver was estimated by assaying the titres of liver marker enzymes (ALT, AST and ALP) in control and treatments groups to corroborate histopathological observation. The liver markers (ALT & AST) were significantly higher in CD, H and HCD groups (Fig. 1.6a & b); whereas non-significant increment in ALP

levels was recorded in H and HCD groups. However, no significant increment in the levels of ALP was recorded in CD group (Fig. 1.6c). Melatonin treatment accounted for a significant decrement in ALT & AST levels in CDM, HM and HCDM groups.

High fat-high fructose diet and/or chronodisruption alters lipid profile in C57BL/6J mice

Experimental group viz. H, CD and HCD showed alterations in lipid profile wherein TG, TC, and LDL, were significantly elevated in H and HCD groups (Fig. 1.7). However, no significant changes were recorded in HDL cholesterol in all the experimental groups. Melatonin treatment accounted for a significant decrement in LDL, total cholesterol and total lipids levels in HM and HCDM groups.

High fat-high fructose diet and/or chronodisruption subjected C57BL/6J mice develops histological features of NASH

Preliminary examination of liver of CD, H and HCD groups recorded a marked difference in colour (pale yellow) as compared to control mice (dark reddish brown). Melatonin administration resulted in restoration of colour along with an improvement in whole liver weight that was comparable to control mice (Fig. 1.8a & b).

H&E staining of Liver

Microscopic evaluation of liver sections (stained with H&E) showed prominent steatotic and inflammatory changes along with microvesicular hepatic steatosis, cellular derangement and mallory hyaline formation in H, CD and HCD groups. Whereas, melatonin treatment accounted for significant decrement in fatty manifestations, lesser cellular derangement with improved histoarchitecture in CDM, HM and HCDM groups.

Glucose, Albumin and globulin protein

Fasting serum glucose was significantly elevated in H and HCD group but non-significant decrement was observed in CD group, but melatonin treatment accounted for a reciprocal change in the said parameter. Also, the serum total protein, albumin and globulin were significantly higher in the H, CD and HCD groups but in melatonin

treatment causes no favourable changes, except HCDM group showed significant decrement in the levels of total protein, albumin and globulin (Fig. 1.9).

High fat-high fructose diet and photoperiodic manipulation induced chronodisruption mediated alterations in hepatic clock genes

Altering the light/dark cycle is known to desynchronize the central circadian clock. We validated the disruption of light/dark cycle due to chronodisruption on the rhythmic expression of the core clock genes (Bmal1 & Clock) in the liver (Fig. 1.10). A time point study was conducted (at ZT0, ZT6, ZT12, ZT18 and ZT24) to validate possible alterations in the core circadian genes in liver of the said experimental groups. In control liver, both Bmal1 and Clock mRNA peaked at ZT6. The (H) group showed a flattened peak of Bmal1 and Clock mRNA. Timed administration of melatonin accounted for an improvement in Bmal1 rhythm at ZT6 whereas the clock mRNA peak recorded a shift from ZT6 to ZT12. Similarly oscillations of Bmal1 and Clock mRNA in liver of CD group recorded flattened peak from ZT0 to ZT6 as compared to control. In HCD group, mRNA of Bmal1 showed non-specific changes, whereas clock mRNA showed complete phase shift from ZT6 to ZT18. In HCDM group, melatonin treatment preserved the diurnal variation in expression of Bmal1, but clock mRNA peaked at ZT12 as comparable to control.

Serum Melatonin ELISA

In C57BL/6J mice subjected to chronodisruption, the serum melatonin levels in CD group was significantly lowered at ZT0, ZT6, ZT12 and ZT24 as compared to control but the peak was recorded at ZT18. In H and HCD groups the melatonin levels were significantly lowered at ZT6 whereas a non-significant decrement was recorded at other time points. Melatonin treatment recorded a reversal of the said patterns in all the groups i.e. CDM, HM and HCDM (Fig. 1.11).

Melatonin regulates mRNA profile of lipid metabolizing genes in liver of CD, H and HCD groups

To determine the effect of high fat-high fructose diet and/or chronodisruption induced nonalcoholic steatohepatitis, we assessed mRNA levels of lipid regulatory genes in liver

samples. A significant increment in mRNA levels of CPT-1, PPARα, and SREBP1C were recorded in CD, H and HCD groups but melatonin treatment accounted for a significant decrement in the mRNA of the said genes in CDM, HM and HCDM groups (Fig. 1.12). Genes regulating lipid uptake (FAS and CD36) were significantly upregulated in all the diseases control (CD, H and HCD) groups. Melatonin treatment accounted for reversal of the mRNA levels of lipid metabolising genes as the same were found comparable to that of control with FAS and CD36 being exceptions in CDM and HCDM group respectively.

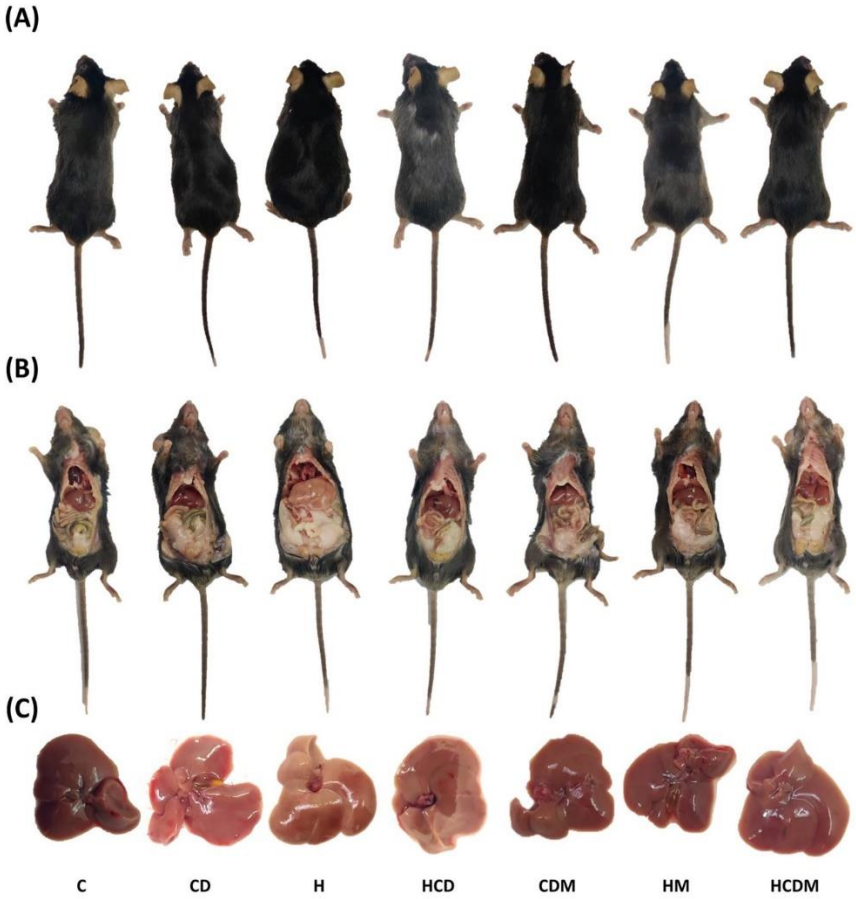

Figure 1.2: C57BL/6J mice subjected to high fat-high fructose diet and/or photoperiodic manipulation induced chronodisruption. (A, B) Mice fed with standard chow diet (C), high fat-high fructose (H) diet, photoperiodic manipulation induced chronodisruption (CD) or a combination (HCD) groups for 18 weeks respectively. Intraperitoneally (i.p. 10mg/kg) melatonin was administered from 10^{th} week to 18^{th} weeks. Pale coloured liver was observed in liver CD, H and HCD groups whereas; melatonin treated groups could revert the same.

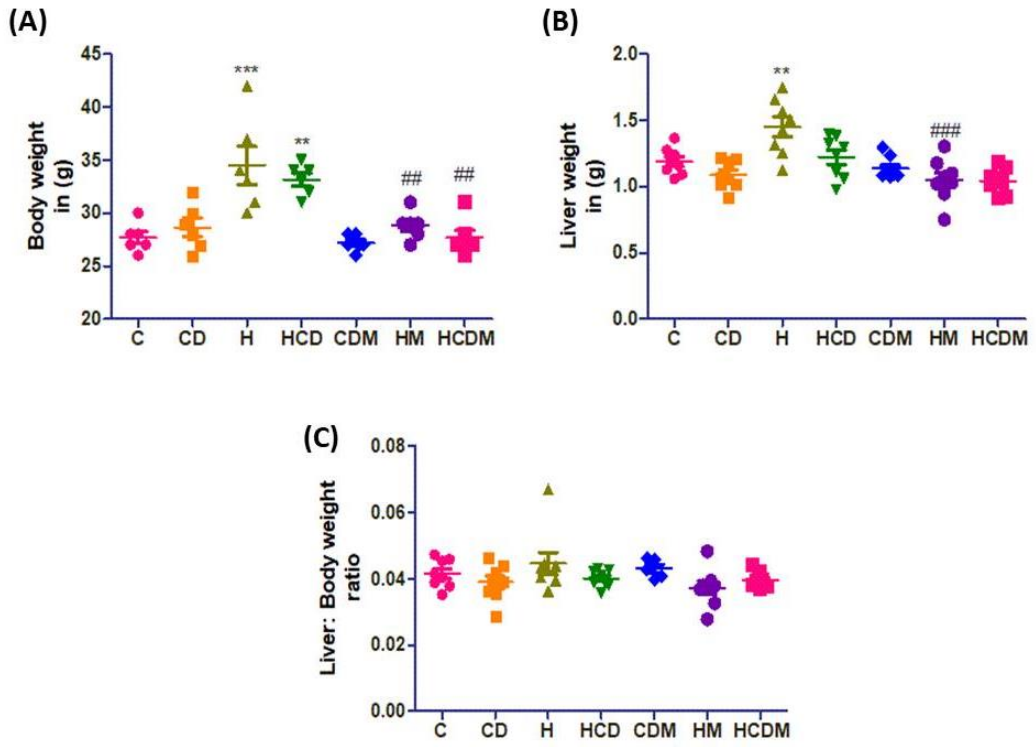

Figure 1.3: Changes in (A) whole body weight, (B) liver weight and (C) liver: body weight ratio of C57BL/6J mice fed with high fat-high fructose diet and/or subjected to chronodisruption and an improvement following melatonin treatment. Results are expressed as mean ± S.E.M. *$p < 0.05$, and ***$p < 0.001$ is when CD, H and HCD compared to Control (C). #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ is when CDM compared with CD, HM with H and HCDM with HCD respectively.

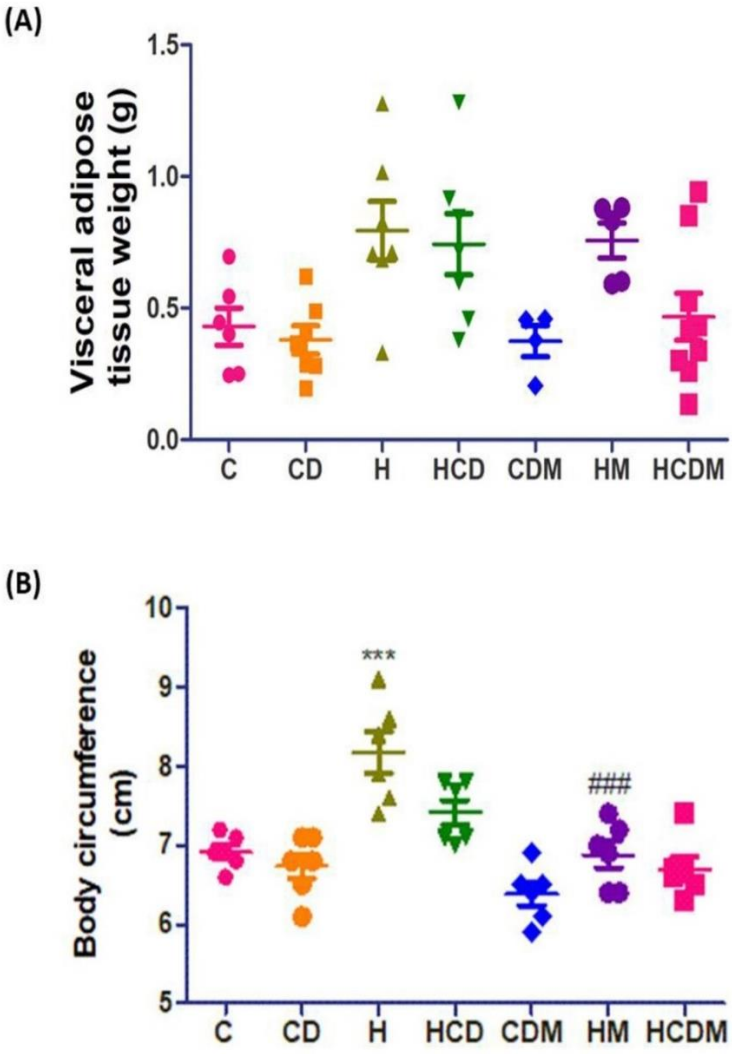

Figure 1.4: (A) visceral adipose tissue weight and (B) body circumference in (H) diet and HCD groups subjected to high fat-high fructose diet and/or chronodisruption subjected C57BL/6J mice for 18 weeks. H and HCD groups gained excess fat deposition and ectopic fat accumulation in the body as compared to control. Melatonin treatment accounted for a reduction in the same. Results are expressed as mean ± S.E.M. *$p < 0.05$, and ***$p < 0.001$ is when CD, H and HCD compared to Control (C). #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ is when CDM compared with CD, HM with H and HCDM with HCD respectively.

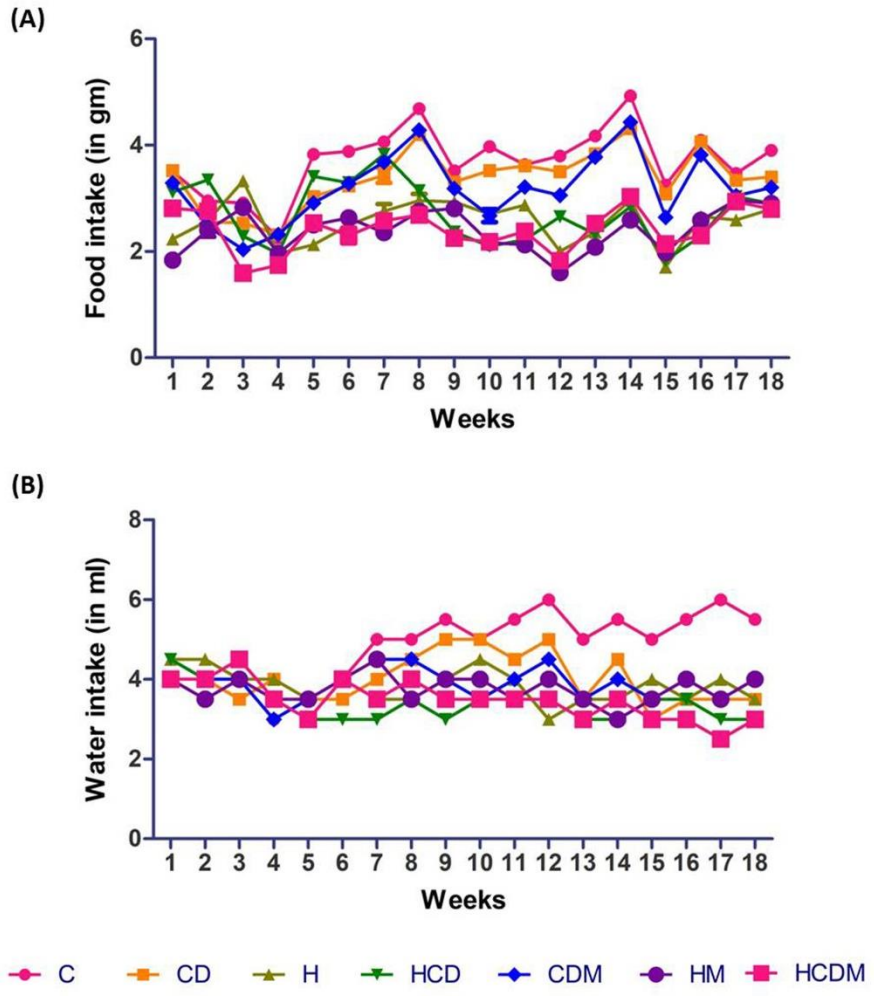

Figure 1.5: (A) Food and (B) water intake in C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption for 18 weeks. No significant difference was observed in control and treated mice.

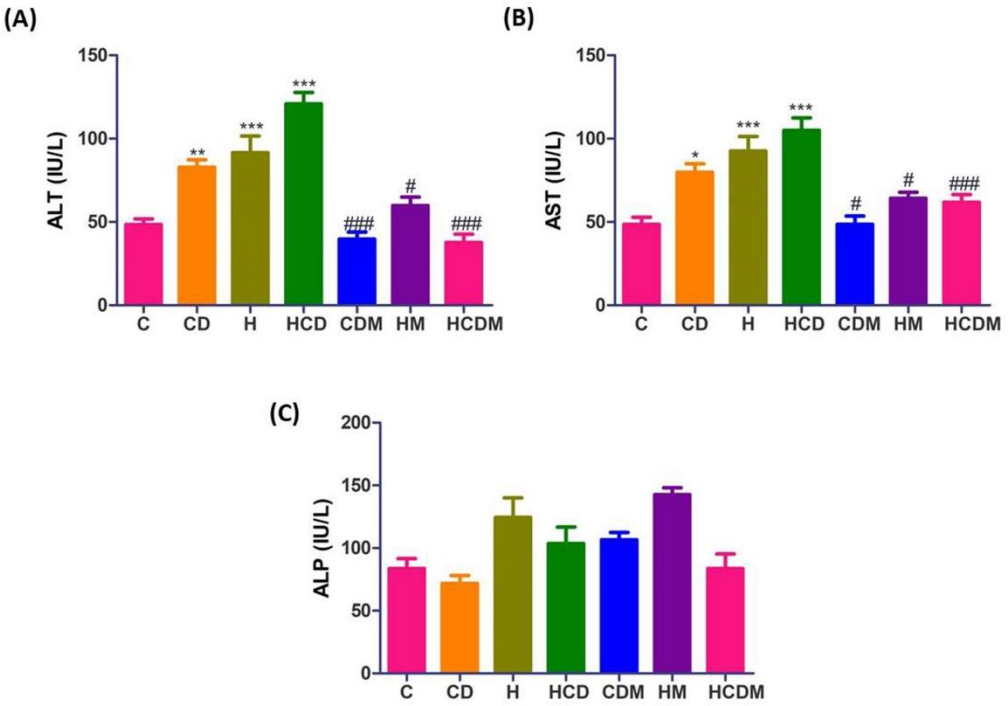

Figure 1.6: High fat-high fructose diet and/or chronodisruption mediated alterations in liver functional markers (A) ALT, (B) AST and (C) ALP in C57BL/6J mice. Results are expressed as mean ± S.E.M. *p < 0.05, and ***p < 0.001 is when CD, H and HCD compared to Control (C). #p < 0.05 and ###p < 0.001 is when CDM compared with CD, HM with H and HCDM with HCD respectively. CD, H and HCD mice showed elevated levels of AST and ALT whereas; melatonin treatment accounted for a reduction in the same.

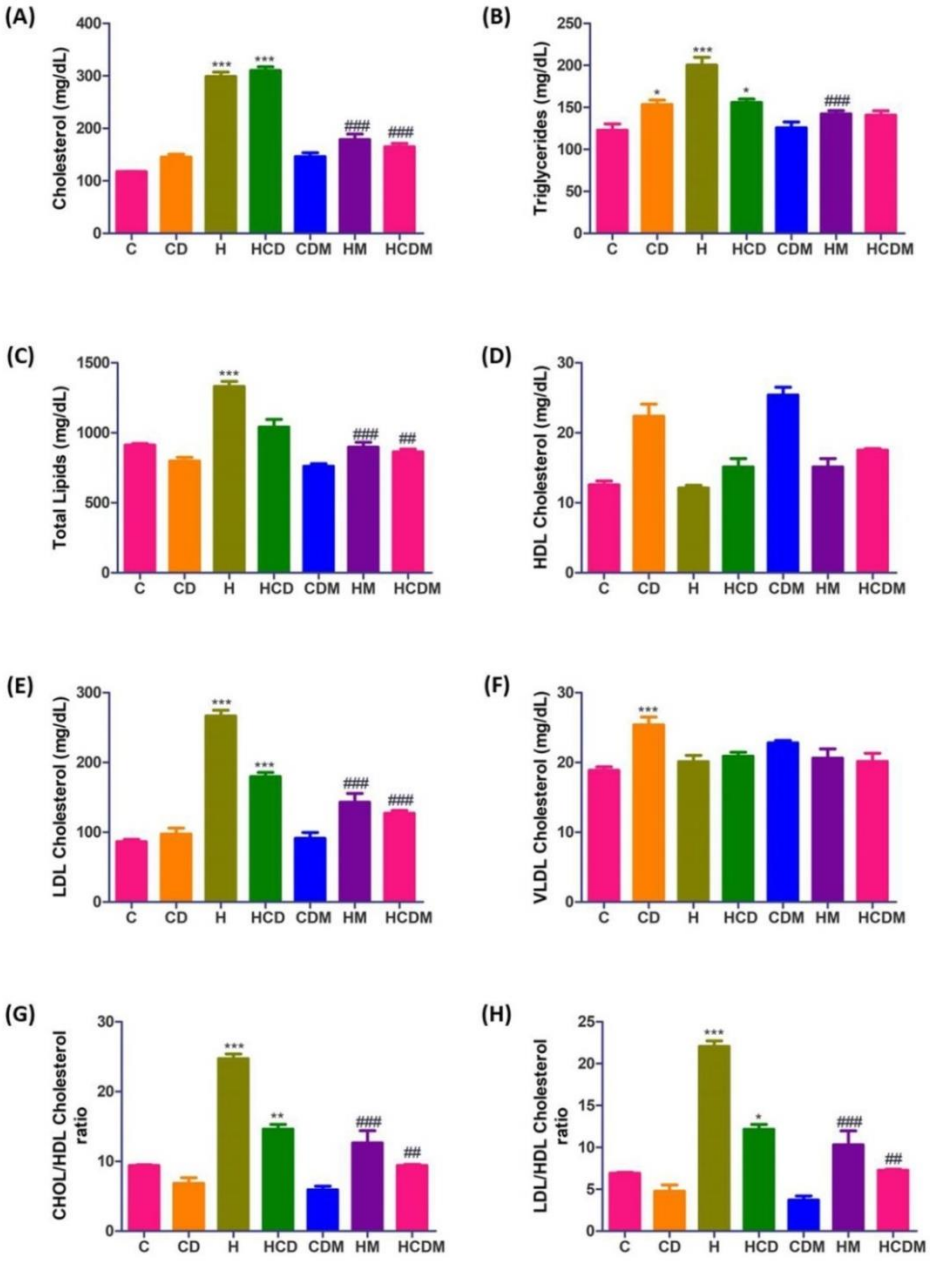

Figure 1.7: Lipid Profile (A) Cholesterol, (B) Triglycerides (C) Total lipids, (D) HDL Cholesterol (E) LDL Cholesterol, (F) VLDL Cholesterol (G) Cholesterol:HDL Cholesterol ratio and (H) LDL cholesterol: HDL Cholesterol. Melatonin treatment maintains a healthier metabolic profile in CDM, HM and HCDM group. Results are expressed as mean ± S.E.M. *p < 0.05, and ***p < 0.001 is when CD, H and HCD compared to Control (C). #p < 0.05, ##p < 0.01, and ###p < 0.001 is when CDM compared with CD, HM with H and HCDM with HCD respectively.

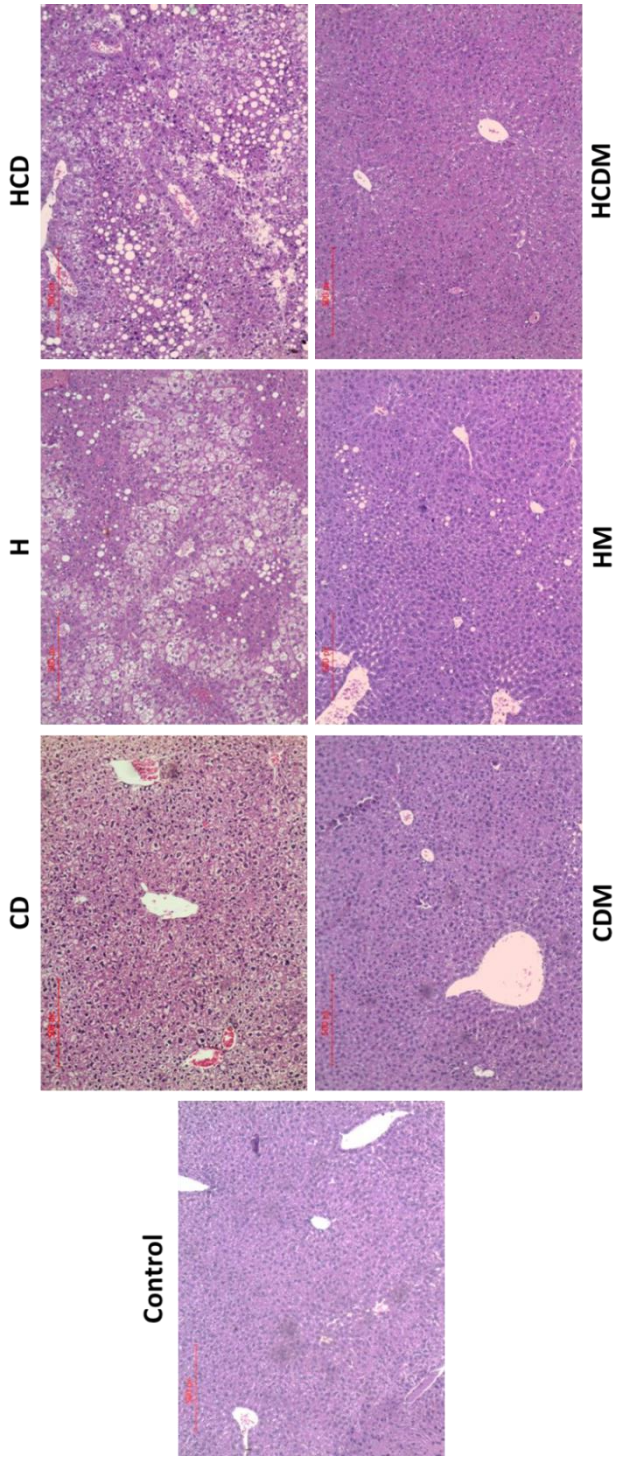

Figure 1.8 a: Microscopic evaluation of the liver (100X). Control (C) group showing normal hepatic cords radiating from the central vein. H and CD groups show moderate distortion of hepatic cords. Melatonin treatment shows an improvement in overall histoarchitecture.

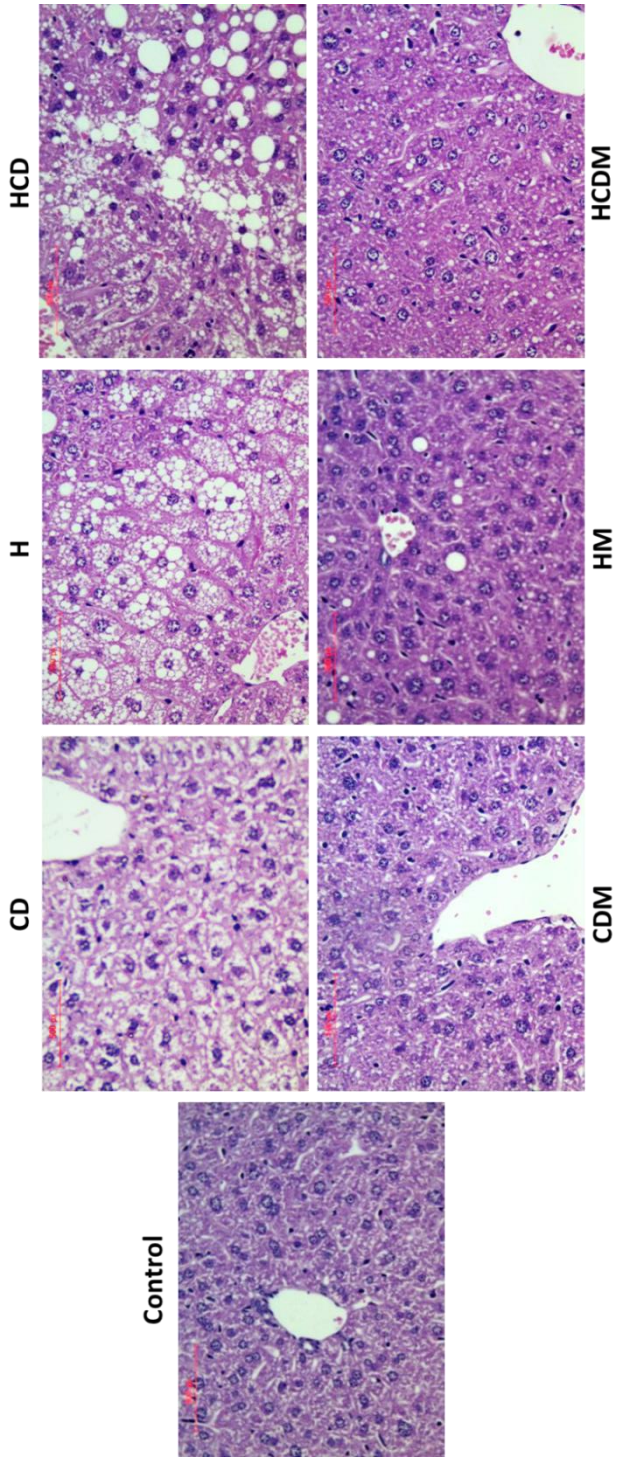

Figure 1.8 b: Microscopic evaluation of the liver (400X). H and CD groups Macrovesicular steatosis, ballooning degeneration and rarefied cytoplasm. Melatonin treated groups showed lesser fatty manifestation.

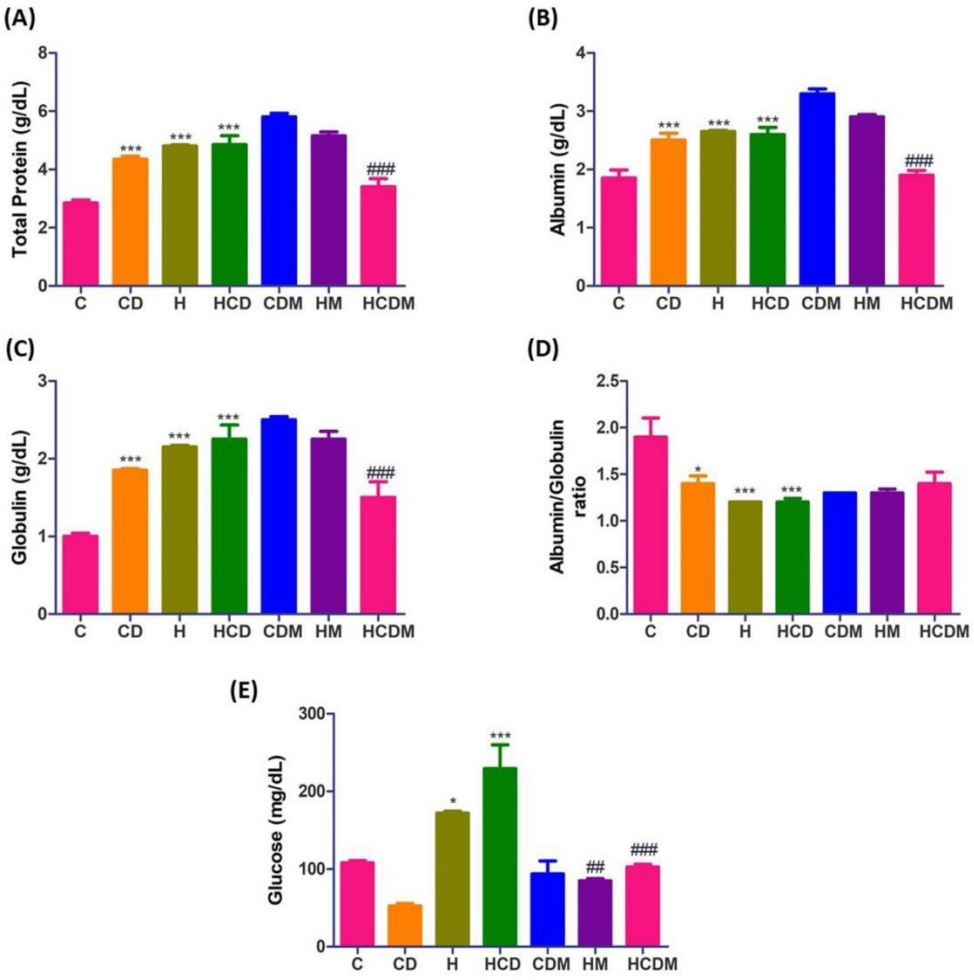

Figure 1.9: Serum (A) total protein, (B) albumin, (C) globulin, (D) albumin: globulin ratio and (E) Glucose in mice fed with high fat-high fructose diet and/or chronodisruption in C57BL/6J mice. Results are expressed as mean ± S.E.M. *p < 0.05, and ***p < 0.001 is when CD, H and HCD compared to Control (C). #p < 0.05, ##p < 0.01, and ###p < 0.001 is when CDM compared with CD, HM with H and HCDM with HCD respectively.

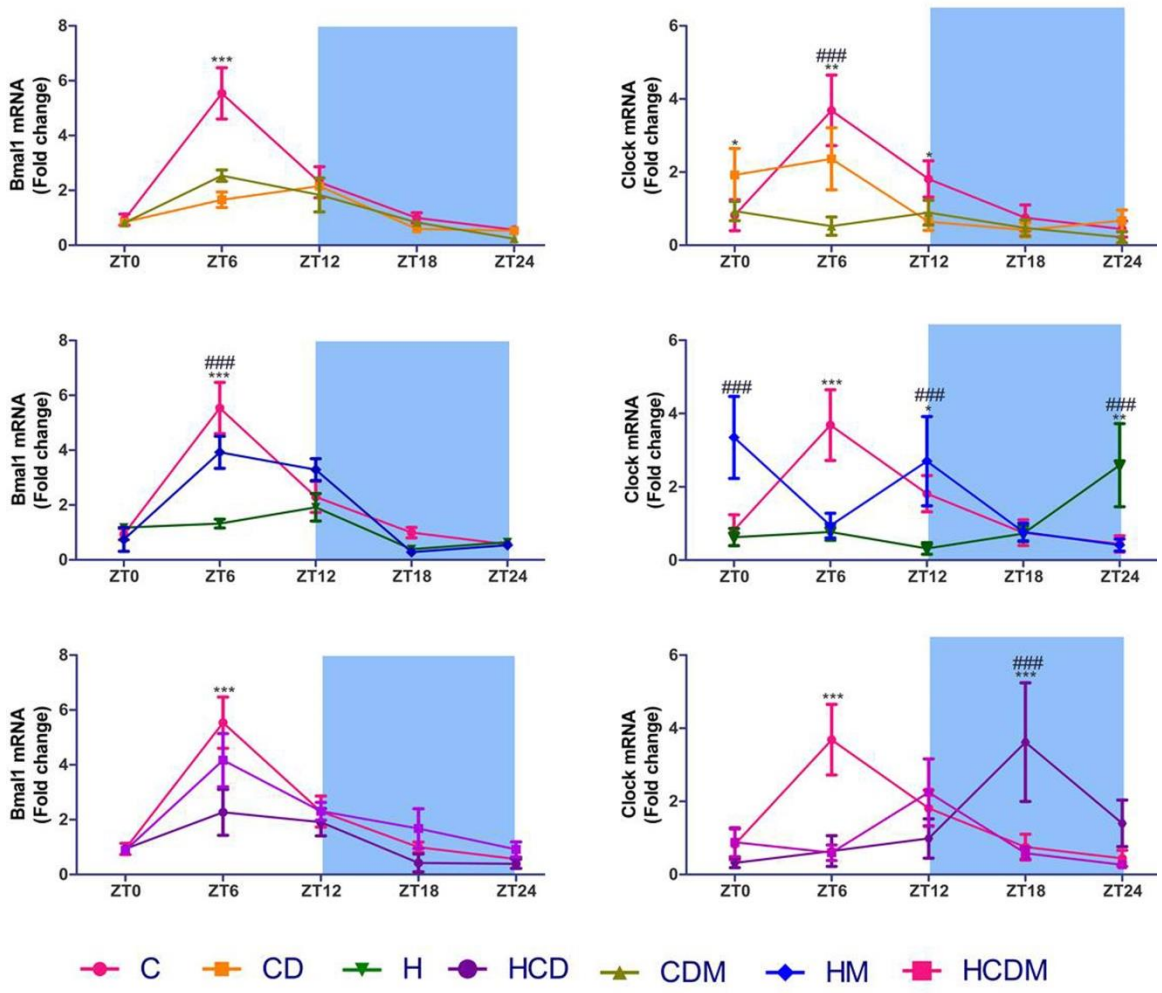

Figure 1.10: Circadian clock gene (Bmal1 and Clock) expression in liver of CD, H and HCD groups as evidenced by their mRNA profiles in ZT0 to ZT12 (no color) and ZT12 to ZT24 (in blue). Results are expressed as mean ± S.E.M. *p < 0.05, and ***p < 0.001 is when CD, H and HCD compared to Control (C). #p < 0.05, ##p < 0.01, and ###p < 0.001 is when CDM compared with CD, HM with H and HCDM with HCD respectively. CD, H and HCD treated groups show flattening/shifts in oscillations of the said genes. Melatonin treatment accounts for moderate corrections in their oscillations.

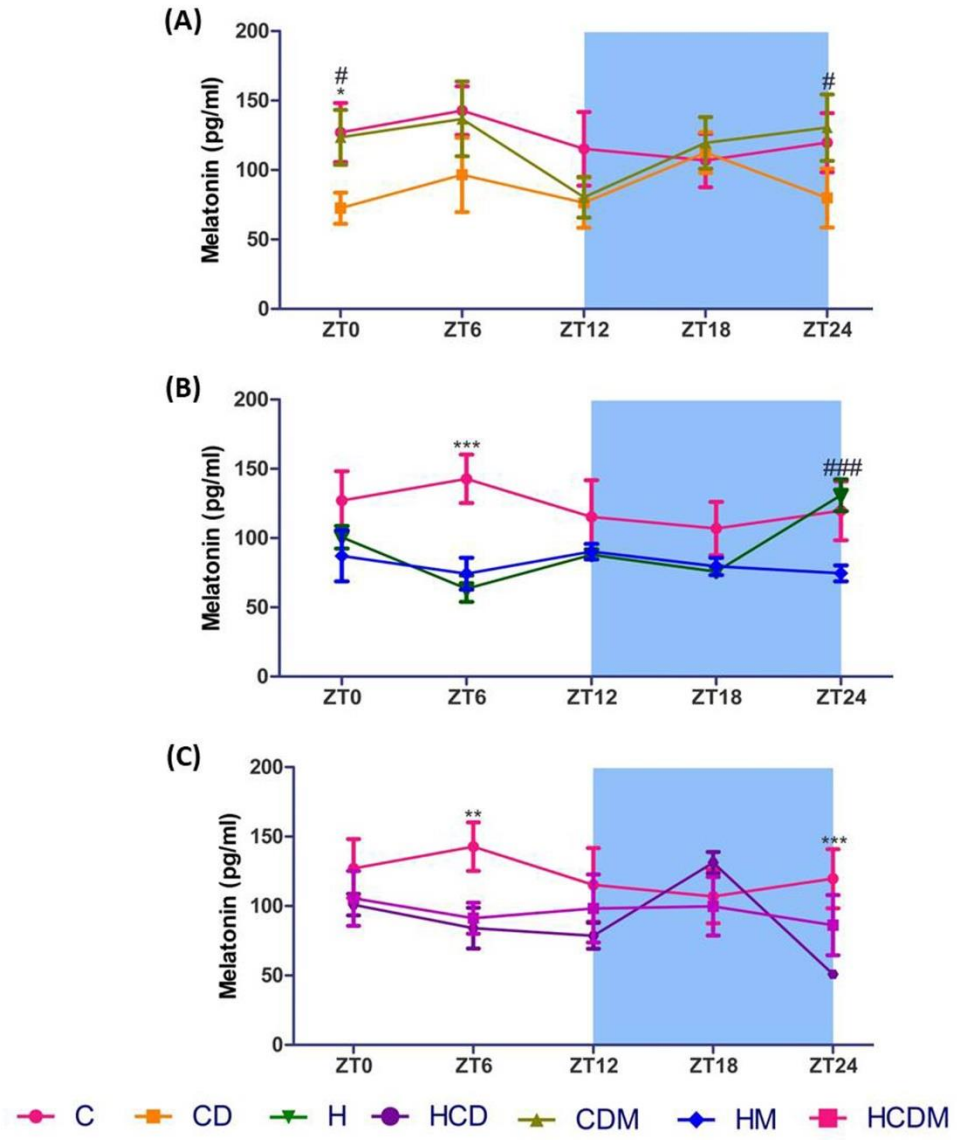

Figure 1.11: Serum melatonin concentrations in mice subjected to high fat-high fructose diet and/or chronodisruption for 18 weeks in ZT0 to ZT12 (no color) and ZT12 to ZT24 (in blue). Results are expressed as mean ± S.E.M. *$p < 0.05$, and ***$p < 0.001$ is when CD, H and HCD compared to Control (C). #$p < 0.05$ and ###$p < 0.001$ is when CDM compared with CD, HM with H and HCDM with HCD respectively. CD, H and HCD treated groups show flattening/shifts in oscillations of the said titres. Melatonin treatment accounts for moderate corrections in their oscillations.

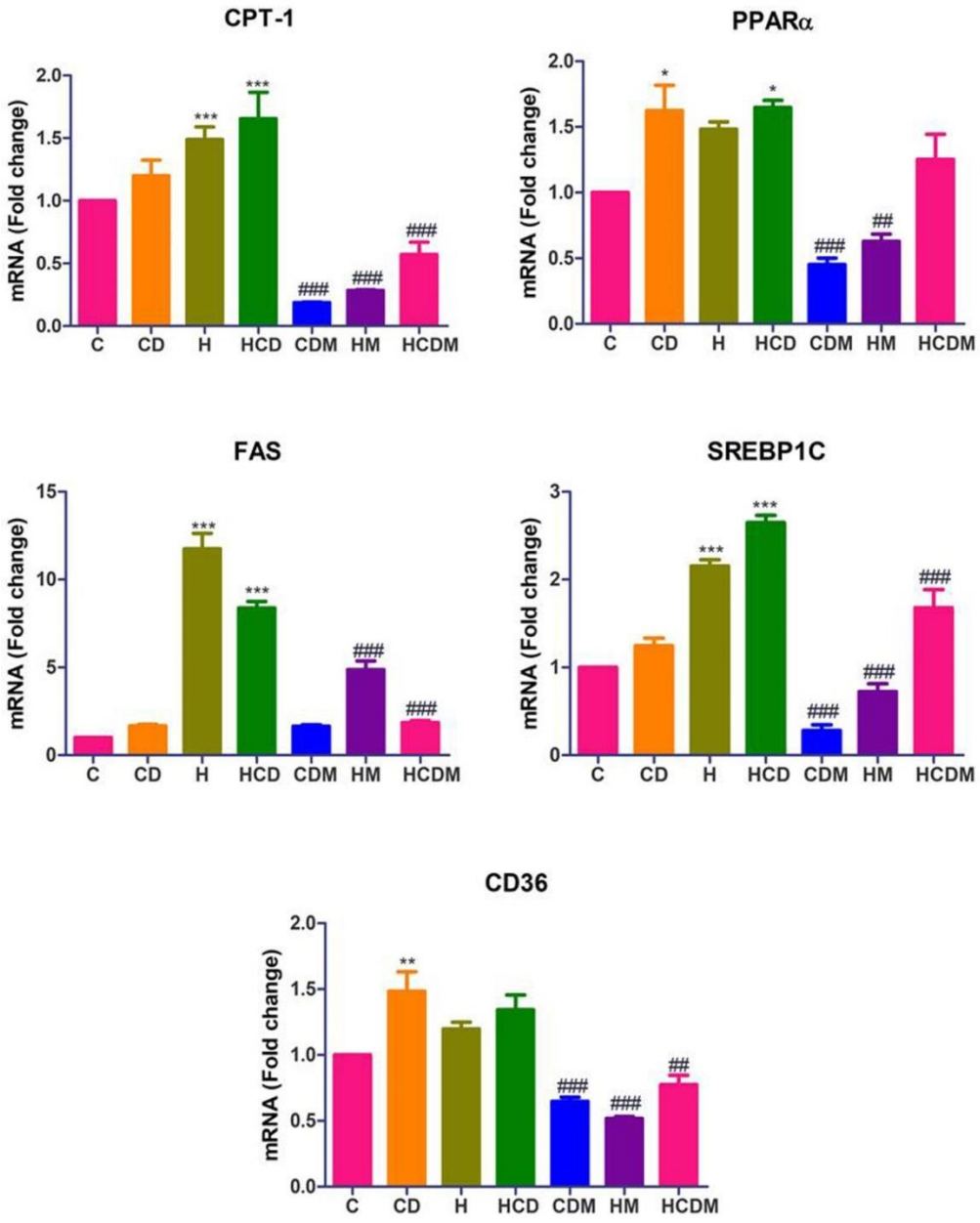

Figure 1.12: Changes in the mRNA expression of genes governing hepatic lipid metabolism in liver mice subjected to high fat-high fructose diet and/or chronodisruption for 18 weeks. Results are expressed as mean ± S.E.M. *p < 0.05, **p < 0.01, and ***p < 0.001 is when CD, H and HCD compared to Control (C). #p < 0.05, ##p < 0.01, and ###p < 0.001 is when CDM compared with CD, HM with H and HCDM with HCD respectively. CD, H and HCD treated groups depict an altered lipid metabolism that appears to be corrected in melatonin treated groups.

Discussion

Nonalcoholic steatohepatitis (NASH) is a lifestyle disorder of the 21st century wherein, obesity, type II diabetes, high blood pressure, and abnormal levels of cholesterol etc. are the major contributors. The chronic impact of untreated conditions of NASH significantly affects vital organs with multifactorial physiological implications. A link between circadian disruption and metabolic disorder is well established using various gene ablation and knockout models (Doi et al., 2010; Kondratov et al., 2006; Shi et al., 2019a; Tahara & Shibata, 2016). Today, artificial light at night (ALAN) is a widespread phenomenon wherein; numerous studies had demonstrated that disturbances in light-dark and feeding-fasting cycles can impose potential health risk, culminating in metabolic dysfunction. Night shifts or rotating shift workers are at risk for sleep disorders, circadian discordance, hormonal imbalance, altered feeding-fasting cycle and development of metabolic syndrome (Kulkarni et al., 2020). An altered photoperiodic regime was linked to several diseases such as obesity, diabetes, fatty liver, stroke, breast and prostate cancer (De Cruz et al., 2012; James et al., 2017; Okuliarova et al., 2020; Yue et al., 2020). Studies in both rodent and human models had shown that chronodisruption was associated with altered food intake patterns, sleep disorders, arrhythmic physical activity, glucose intolerance, dyslipidemia and neurobehavioral perturbations (Ashkenazy et al., 2009; Bravo et al., 2014; Briançon-Marjollet et al., 2015; Chalfant et al., 2020; Krivisky et al., 2011; Pyter et al., 2005, 2006; Skene et al., 1999; Verma et al., 2020). In both mice and humans, chronodisruption or Jetlag has been shown to affect the circadian clocks in the liver, aorta, heart, small intestines and peripheral blood mononuclear cells (Joshi et al., 2021; X. Pan et al., 2019; Rudic et al., 2005; Shi et al., 2019b). Dim light at night (dLAN) led to higher food intake resulting in an unhealthy increment in body mass in rodent model of chronodisruption (Okuliarova et al., 2020).

Melatonin is a neurohormone secreted from the pineal gland in vertebrates that is also known to be a powerful antioxidant and a free radical scavenger. In diseases such as diabetes mellitus, NAFLD, rheumatoid arthritis, Alzheimer's, Parkinson's and other metabolic syndromes, melatonin has been extensively reported for its beneficial effects in alleviating the symptoms and making corrective changes in the metabolism (He et al., 2010; Jahanban-Esfahlan et al., 2018; Joshi et al., 2021; Shieh et al., 2009). Previous

study from our lab had reported that administration of adipose-derived mesenchymal stem cells primed with melatonin improved experimentally induced NAFLD (Vohra et al., 2021). Timed administration of melatonin has been associated with high degree of physiological relevance as melatonin is released during the hours of darkness, regardless of the species (nocturnal or diurnal). Hence, the evening injections of melatonin (10 mg/kg; i.p.) were given in our study (Armstrong, 1989; Baxi et al., 2013; Joshi et al., 2021).

High fat-high fructose diet (H) and a combination of H and chronodisruption (HCD) recorded significant increase in body weight and liver weight gain whereas; melatonin treatment lowered the indices of the said parameters. Ectopic fat accumulation has been reported to occur in mice that are subjected to HCD (Joshi et al., 2021), and the same was observed in our study with a decrement recorded in melatonin treated group. These results are in agreement with the findings of other research groups wherein; mice were fed with a high fat diet (Baek et al., 2015; Joshi et al., 2021; Pfluger et al., 2008). Significantly elevated titres of ALT and AST were observed in H and HCD groups and a non-significant increment was also recorded in CD group indicating liver damage. The same is in agreement with findings of other research groups with H, CD or HCD experimental groups (Ghoneim et al., 2015; Joshi et al., 2021).

The steatotic changes in liver are confirmed using biopsy samples of NASH patients and the same parameter continues to be a gold standard for its validation (Adams & Talwalkar, 2006; Brunt, 2017; Kleiner et al., 2005; Obika & Noguchi, 2011). In our study, pathological score of the liver in CD, H and HCD group mice confirmed fatty manifestation, hepatocyte ballooning and steatotic changes in the liver (H&E staining). The same were reported from our lab (Joshi et al., 2021; Upadhyay et al., 2020; Vohra et al., 2021) with the same dietary composition, by MCD diet (Hwang et al., 2012) and by high fat-high cholesterol diet (Briand et al., 2020; Han et al., 2015). Alterations in photoperiodic regime in rodent models such as continuous lighting, long day photoperiod, or a jetlag protocol has shown histopathological perturbations in the liver that culminate in fatty manifestations (Abulmeaty et al., 2021; Kettner et al., 2016). Hence, the fatty changes observed in the study are comparable to these published reports of altered photoperiodic regime and/or high calorie diet. Administration of melatonin is

known to improve the condition of fatty liver in various experimental models of MCD diet, high fat diet and high fat-high fructose diet fed to the rodent models (Hatzis et al., 2013; Joshi et al., 2021; M. Pan et al., 2006; Stacchiotti et al., 2019; Sun et al., 2016). In our study, melatonin treatment alleviated the histopathological damage in the liver of CDM, HM and HCDM groups.

The suprachiasmatic nucleus (SCN) in the anterior part of the hypothalamus is the central pacemaker of the circadian timing system and regulates most of the peripheral clocks in peripheral tissues (Welsh et al., 2010). Peripheral clocks in the liver maintain the homeostasis by regulating fatty acid, glucose, amino acid metabolism and activity levels of enzymes regulating absorption and xenobiotic metabolism (Bass & Takahashi, 2010; Bugge et al., 2012; Gachon et al., 2006; Lamia et al., 2008; Tahara & Shibata, 2016). In our study, mice fed with either H and/or CD had mediated arrhythmic circadian gene expression in CD, H and HCD groups. In H group, the impairment of hepatic clock was evident in mRNA levels of core clock genes (Bmal1 and Clock) that were lower at ZT6 as compared to the control whereas; in CD group the peak of Bmal1 gene had shifted from ZT6 to ZT12. The said changes in mRNA levels of core clock genes are known to account for chronodisruption (Bravo et al., 2014; De Assis et al., 2018; Froy, 2013; Kolbe & Oster, 2019). Exogenous melatonin caused an improvement in the mRNA titres as evidenced by the levels of core clock genes. These findings agree to the reports on melatonin mediated improvement in clock gene oscillations in conditions of experimentally induced lifestyle disorder (Joshi et al., 2021).

Abnormal lipid accumulation in the liver is a key pathophysiological feature of NAFLD/NASH. Alterations in hepatic lipid and lipoprotein metabolisms in the liver are the central driving factors to the development of NAFLD and its progression to NASH (Okuliarova et al., 2020). In liver, the process of lipid uptake in both physiological and pathological conditions is mediated through lipid regulatory genes such as FAS, FABP1 and CD36 (Inoue et al., 2005). In our study, a significant increment in the mRNA levels of FAS and CD36 were recorded in CD, H and HCD group that had witnessed a reversal following melatonin treatment. In liver, Fatty acid synthase (FAS) and sterol regulatory element-binding protein 1 C (SREBP1C) are critical gene transcription factors that play an important role in the regulation of fatty acid synthesis and lipid metabolism (Chirala et

al., 2003; Horton et al., 2002). In high fat diet induced NASH models, an increased expression of de novo fatty acid synthesis, FA oxidation, FA uptake and transport genes in the steatotic liver were recorded (Han et al., 2015). In our study a similar trend was observed in FA metabolism genes; increase in FAS, PPARα and SREBP1c in all the disease control groups i.e. CD, H and HCD. But CPT1 mRNA expression was only increased in HCD group as compared to control.

Overall, it can be concluded from the present study that, high fat-high fructose diet and/or chronodisruption is able to induce nonalcoholic steatohepatitis in C57BL/6J mice after an exposure of 18 weeks. Exogenous melatonin is able to improve circadian synchrony as evidenced by the oscillations of hepatic core clock genes. Manifestation of fatty changes in liver was validated by cellular derangement and mallory hyaline formation with inflammatory changes hepatic tissue. Further evidence on the same was obtained by the observed desynchrony in mRNA levels of lipid metabolism regulatory genes, complete lipid profile and liver function test.