Inflammatory changes in liver and brain in experimentally induced Chronodisruption and/or Nonalcoholic steatohepatitis in C57BL/6J mice

Introduction

Liver is a vital organ that is responsible for various fundamental metabolic processes including removal of toxins and regulating systemic immuno-inflammatory build-up. The continuous and exhaustive exposure of endogenous and exogenous pro-inflammatory stressors such as reactive oxygen species (ROS), environmental toxins, drugs etc. contribute to varying degrees of liver inflammation. Mitochondrial dysfunction in liver and subsequent build-up of pro-adipogenic cascade is contributory to fatty liver. Such a scenario resulting in patients without major history of consumption of alcohol leads to Nonalcoholic fatty liver disease (NAFLD) that may progress towards Nonalcoholic steatohepatitis (NASH). Hence, persistent low grade inflammation, hepatocyte injury and cell death are the hallmarks of the NASH (Ludwig et al., 1997).

Pro-inflammatory mediators such as histamines, arachidonic acid derivatives (leukotrienes and prostaglandins), kinins, phospholipid mediators and cytokines are shipped to the site of inflammation wherein; the cytokines are the primary chemical messengers involved in both acute and chronic hepatic inflammation. Chemokines, the largest family in cytokines are known to induce migration and activation of leukocytes to the site of inflammation and activate phagocytosis (Miller & Krangel, 1992; Palomino & Marti, 2015). Additional chemical messengers such as interleukins (IL-1β, IL-4, IL-6, IL-8 and IL-10), tumor necrosis factor-α (TNF- α) and monocyte attraction protein-1 (MCP-1) are also key contributors in this sojourn (Pate et al., 2010; Turner et al., 2014). Interleukins such as IL-1β, IL-6, and TNF-α play an important role in the onset and progression of NAFLD by stimulating insulin resistance, mitochondrial dysfunction, oxidative stress, hepatic inflammation, apoptosis and liver fibrosis (Harmon et al., 2011; Joshi et al., 2021; K. K. Upadhyay et al., 2020). In liver regeneration, production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 play an essential role in repairing the damaged liver (DIEHL & RAI, 1996; Gao, 2012).

The control and co-ordination of humoral, physiological, and behavioral mechanisms is regulated by the circadian rhythms, with the sleep-wakefulness being orchestrated by the

master clock located in the suprachiasmatic nuclei in the hypothalamus (Welsh et al., 2010). Sleep disorders have become a global issue due to shift work and artificial light at night (ALAN) that induces desynchrony of circadian rhythms (via. Jetlag / delayed sleep phase syndrome / inability to sleep at the desired time) which in turn results in depression, chronic illness, insomnia etc. (Koo et al., 2016). The circadian clock genes are also known to regulate physiology, metabolism of nutrients and function of the gastrointestinal tract. Sleep-disturbances have been shown to be a prognostic factor in advanced chronic liver diseases in patients (De Cruz et al., 2012). Further, anomalous circadian rhythm and sleep disorder are ubiquitously diagnosed in patients with hepatitis C, primary biliary cirrhosis, Wilson diseases, liver cirrhosis and hepatic encephalopathy (De Cruz et al., 2012). Circadian abnormalities along with alterations in core circadian clock gene oscillations in liver of experimentally induced NAFLD has been reported from our lab (Joshi et al., 2021). In a cohort of obese patients with NAFLD, obstructive sleep apnea (OSA) was associated with elevated serum ALT as well as histological evidence of progressive liver diseases (Kallwitz et al., 2007). Patients with hypoxic stress of sleep apnea reported development of insulin resistance, hepatic inflammation, hepatocyte ballooning and liver fibrosis (Polotsky et al., 2009). In patients with cirrhosis, the 24 h melatonin rhythm showed reduced sensitivity to light cues (Montagnese et al., 2014). A significant association was found between shift workers and inflammatory markers wherein; high-sensitivity towards CRP levels and leukocyte count was found to be significantly higher in shift workers as compared to daytime workers (Kim et al., 2016). In photoperiodic manipulation induced chronodisruption, significant increment in the mRNA titres of inflammatory markers such as TNF-α, NFκB and IL-1β have been reported (Joshi et al., 2021).

In vertebrates, the pineal hormone melatonin, plays a central role in the regulation of circadian rhythms and acts as a neuroendocrine transducer. The rhythmicity of melatonin secretion is robust and acts as a Zeitgeber or time-giver to the environmental light-dark cycle. The 24 h circadian rhythm of melatonin is responsible for an altered high/low sleep regime in relation to light/dark cues. In circulation, melatonin is released during darkness, irrespective of diurnal/nocturnal activity phase of the target species (Armstrong, 1989). In cirrhotic patients, due to impairment in hepatic melatonin metabolism, a delay in the peak of plasma melatonin was found along with reduced

plasma melatonin secretion that got reversed after a liver transplantation (Montagnese et al., 2010). In high-fat diet fed rats, the amplitude of nocturnal pineal melatonin peak was significantly decreased thus emphasizing on the importance of high calorie intake as a chronomodulator (Cano et al., 2008).

Systemic inflammation signals in the brain are relayed via blood-borne pro-inflammatory cytokines in form of an acute phase response such as activation of hypothalamopituitary-adrenal (HPA) axis, hyperalgesia, behavioral suppression, and sleep disorders (Besedovsky et al., 1991; del Rey et al., 2013). Pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) orchestrate systemic response that often culminates in an acute/chronic inflammation in microglia, astrocytes and infiltrating leukocytes (Maldonado-Ruiz et al., 2017). These events play a critical role in the progression of many neurodegenerative and neuropsychiatric diseases via release of pro- and anti-inflammatory cytokines, chemokines, neurotrophic factors and nitric oxide (Tansey et al., 2007). Activated astrocytes-derived cytokines such as Interleukin 1 beta (IL-1β), tumor necrosis factor-α (TNF- α) are considered to promote neurotoxicity whereas; IL-10 and transforming growth factor beta (TGF-β) are neuroprotective (Deng et al., 2014; Norden et al., 2014). Receptors for IL-1 and IL-6 are located in all parts of the brain, with high congregates in the hippocampus because the same has been implicated as a vital site of learning and memory circuitry (del Rey et al., 2013). Intraventricular injection of IL-1β has been reported to induce spatial memory impairments and cognitive dysfunction in rats with a detrimental effect on hippocampal circuitry (Gemma & Bickford, 2007; Gibertini, 1996). Diets rich in fat and sugars are considered as a major risk factor for NAFLD/NASH with compelling implications on neuro-inflammation and behavioral disorders. In high fat diet fed C57BL/6J and $LDLR^{-/-}$ mice an increased activation of microglia and astrocytes and upregulated mRNA expression of pro-inflammatory (TNF-α, IL-1-β, IL-6 and COX-2) cytokines have been reported in hippocampus resulting in an impaired cognition (Pistell et al., 2010; Thirumangalakudi et al., 2008). Significantly higher levels of brain 3-nitrotyrosine, inducible nitric oxide synthase (i-Nos), IL-6, and glial markers (Iba-1 and GFAP) have been reported in HFD fed Long Evans rats born to HFD dams (White et al., 2009). Obesity-derived neuroinflammation affects major brain areas such as the hippocampus, cortex or amygdala that is also known to control the occurrence of depression, anxiety-like behavior and impaired cognitive functions (Guillemot-Legris &

Muccioli, 2017). In obese patients, waist circumference and circulating inflammatory cytokines have been associated with microglial activation and alterations in proportion of white : grey matters (Maldonado-Ruiz et al., 2017; SATTLER et al., 2015). Thus high levels of cytokines such as IL-1-β and IL-6 can disrupt neurophysiologic mechanism involved in cognition, learning and memory via excessive brain inflammation. Published literature indicates obesity, NAFLD/NASH and metabolic syndrome are important risk factors for the development of neurodegenerative and neuro- psychiatric disorders. Alterations in light/dark cycle regimen affects sleep/wake cycle, cellular redox homeostasis, induces systemic inflammation and neurodegenerative diseases. In old male wistar rats, continuous artificial light at night had revealed significantly higher indices of Reactive oxygen species (ROS), malondialdehyde (MDA), plasma membrane redox system (PMRS), protein carbonyl (PCO), and sialic acid (SA) as compared to the young rats (Verma et al., 2020). Melatonin, a potent anti-inflammatory and free radical scavenging neurohormone, secreted by the pineal gland plays a crucial role in the regulation of circadian rhythms. In aged-ICR mice, oral melatonin administration attenuated alterations of the protein levels of glial fibrillary acidic protein (GFAP), IL-1β, IL-6, TNF-α, phosphor-nuclear factor kappa B (pNFκB) in hippocampus and prefrontal cortex (Permpoonputtana et al., 2018). Melatonin also exerts potent anti-inflammatory and neuroprotective properties in rats subjected to continuous light exposure (CLE) and continuous dark exposure (CDE). In this case, a downregulation of inflammatory cytokines (TNF-α, IL-6), neurodegenerative genes (Neuroglobin (Ngb) and Neuron-specific enolase (NSE) have been reported (Verma et al., 2022). In another study, 10 mg/kg intraperitoneal melatonin had ameliorated low grade systemic inflammation by lowering serum pro-inflammatory cytokines in male Sprague-dawley rats subjected to HFD for 16 weeks (Maher et al., 2020). Oral administration of melatonin lowers the neuro-inflammation in hippocampus by decreasing the pro-inflammatory cytokines IL-1β and TNF-α, improved depression-like behavior in long-term dextran sulphate sodium-(DSS-) treated male SPF SD rats (Lv et al., 2020). Reports on experimental and human studies provide compelling evidences that hippocampal functions are sensitive to systemic inflammatory pathways and hence act as mediators of inflammation. Here, the NAFLD/NASH adversely affects the neuro-psychological functioning of the brain but the mechanism of the same is yet poorly understood.

This study investigates the status of inflammatory markers in hepatic and hippocampal tissues of C57BL/6J mice treated with high fat-high fructose diet and/or chronodisruption so as to assess and correlate with the functional status of liver (hepatic function and histomorphology) and hippocampus (neuro-behavioural studies) envisaged in subsequent chapters in this thesis.

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)

Parameter tested:

1. ELISA of circulating inflammatory cytokines (TNF-α, IL-6, IL-10, IL-12 and IL-17) and rhythmic pattern of corticosterone
2. Quantitative RT-PCR: Pro-inflammatory markers (TNF-α, IL-1β, IL-6, IL-12, IL-17, MCP-1, NF-κB, IBA-1 and CREB) and anti-inflammatory markers (IL-4 and IL-10) in liver and hippocampus

The experimental protocol for the present study is depicted in Fig. 2.1. Detailed methodology is described in materials and methods section.

Experimental design

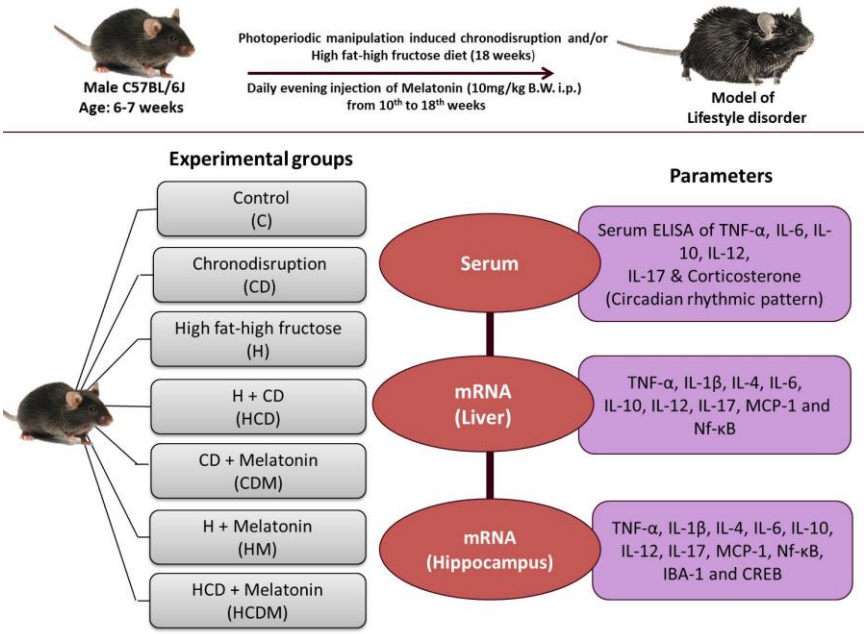

Figure 2.1: Flow chart of experimental protocol followed for inflammatory changes in liver and brain following induction of NASH by chronodisruption and/or high fat-high fructose diet in C57BL/6J mice.

Results

ELISA of cytokines in experimentally induced non-alcoholic steatohepatitis in high fat-high fructose and/or chronodisrupted C57BL/6J mice

In the current study, the array of cytokines such as TNF-α, IL-6, IL-10, IL-12 and IL-17, were checked from the serum sample of all the experimental groups (Fig. 2.2). Pro-inflammatory cytokines TNF-α, IL-6 and IL-17 increased significantly or non-significantly in CD, H and HCD groups; whereas, TNF- α increased significantly in CD ($p<0.01$) and HCD ($p<0.05$) groups and no change was recorded in IL-12. Anti-inflammatory cytokine IL-10 showed significant decrement in all the disease control (CD ($p<0.01$), H ($p<0.001$) and HCD ($p<0.01$)) groups. Exogenous melatonin treatment was able to improve the cytokine levels in serum. It was recorded that TNF-α, and IL-6 showed a significant decrement in melatonin treated groups. However, IL-12 and IL-17 remains unchanged post melatonin treatment as compare to their respective disease control groups, except for HCDM groups with significant decrement was recorded. Anti-inflammatory cytokine (IL-10) recorded a significant increment in HM group, however no change was observed in CDM and HCDM groups (Fig. 2.2e).

Exogenous melatonin administration improves mRNA profiles of pro-inflammatory and anti-inflammatory genes in liver of high fat-high fructose and/or chronodisrupted C57BL/6J mice

Gene expression of pro- and anti-inflammatory markers were assessed in liver of (CD, H and HCD) groups that had accounted for a gross increment in the pro-inflammatory mRNA levels of (TNF-α, IL-1β, IL-6, and MCP-1) (Fig. 2.3) genes including NF-κB ($p<0.001$). Anti-inflammatory genes (IL-10 and IL-4) had recorded a non-significant decrement in CD, H and HCD groups but IL-4 had recorded an increment in all the disease control groups (Fig. 2.4). Timed administration of exogenous melatonin had resulted in significant decrease ($p<0.001$) in the expression of pro-inflammatory markers (TNF-α, IL-1β and NF-κB) in CDM, HM and HCDM groups. But, mRNA levels of IL-6, MCP1, IL-12 and IL-17 showed a non-significant decrement as compared to their respective disease controls. The anti-inflammatory marker (IL-10) showed a significant

increment in CDM (p<0.01) and HCDM (p<0.001) groups whereas; IL-4 in HCDM (p<0.001) group respectively.

Exogenous melatonin administration improves mRNA profiles of pro-inflammatory and anti-inflammatory genes in hippocampus of high fat-high fructose and/or chronodisrupted C57BL/6J mice

To confirm the extent of neuro-inflammation we had assessed mRNA levels of various inflammatory markers in hippocampus of control and treated groups of mice. A non-significant upregulation of the pro-inflammatory cytokines (IL-1β, IL-6, MCP-1, NF-κB, IL-12 and IL-17) was recorded in CD, H and HCD groups (Fig. 2.6 & 2.7). Also, no change was observed in TNF-α mRNA levels in the said experimental groups. Anti-inflammatory markers (IL-4 and IL-10) showed a non-significant decrement in the (CD, H and HCD) groups as compared to control (Fig. 2.8). mRNA levels of melatonin treated (CDM, HM and HCDM) groups showed a non-significant decrement in pro-inflammatory genes (IL-1β, IL-6, MCP-1, NF-κB, IL-12 and IL-17) and a non-significant increment in anti-inflammatory (IL-4 and IL-10) genes of hippocampal tissue.

High fat-high fructose diet and/or chronodisruption subjected in C57BL/6J mice alters the circadian rhythmicity of Corticosterone levels

Corticosterone (CORT) assay in the serum samples of control mice had revealed consistently low levels at all-time points with a single peak recorded at ZT12. CD treated mice had recorded higher serum titres at all the time points with peaks recorded at ZT0 (p<0.01) and ZT18 (p<0.05). H group too had recorded an increase in CORT levels at all the time points with peaks recorded at ZT0 (p<0.05) and ZT18 (p<0.01) respectively. The HCD group did not account for a synergistic effect and the levels with comparable with the other disease control groups (CD and H). Melatonin treatment had accounted for moderate to significant decrement in CORT titres in CDM, HM and HCDM groups at all the time points (Fig. 2.10).

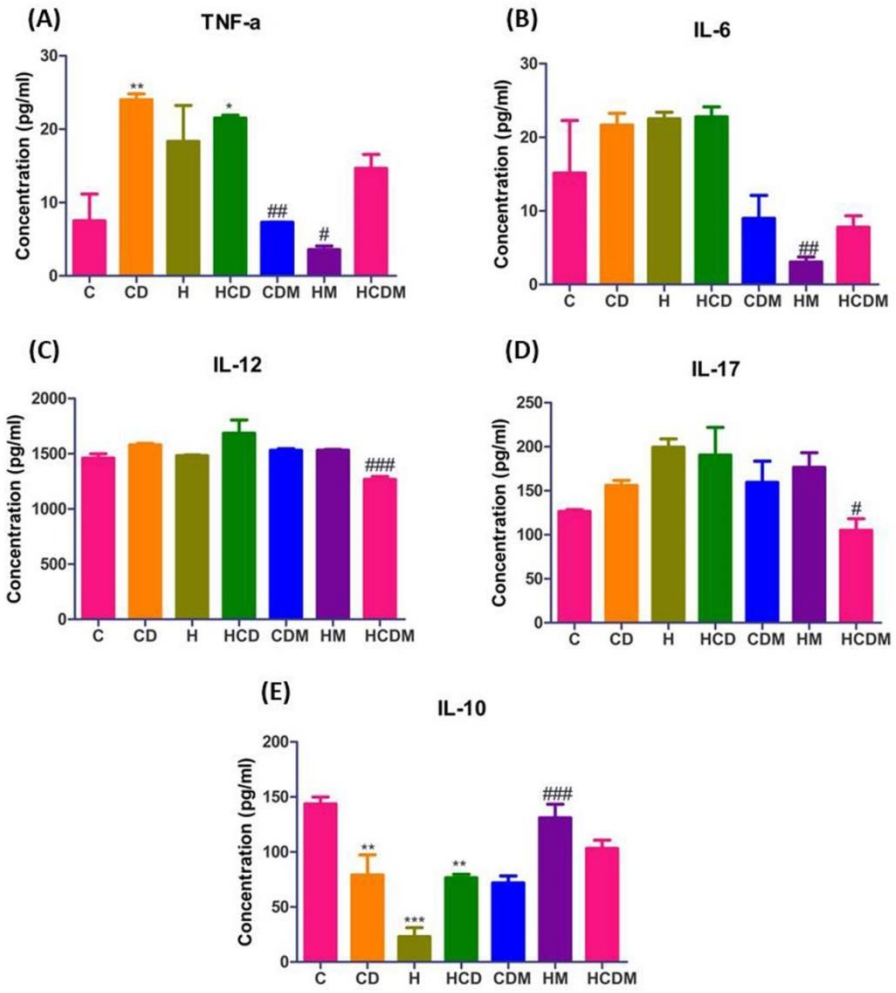

Figure 2.2: Changes in the circulating inflammatory cytokines in serum of mice subjected to mice fed with high fat-high fructose diet and/or subjected to chronodisruption and an improvement following melatonin treatment. Results are expressed as mean ± S.E.M. *p < 0.05, **p < 0.01, and ***p < 0.001 is when CD, H and HCD compared to Control (C). ###p < 0.001 is when CDM is compared with CD, HM with H and HCDM with HCD group respectively.

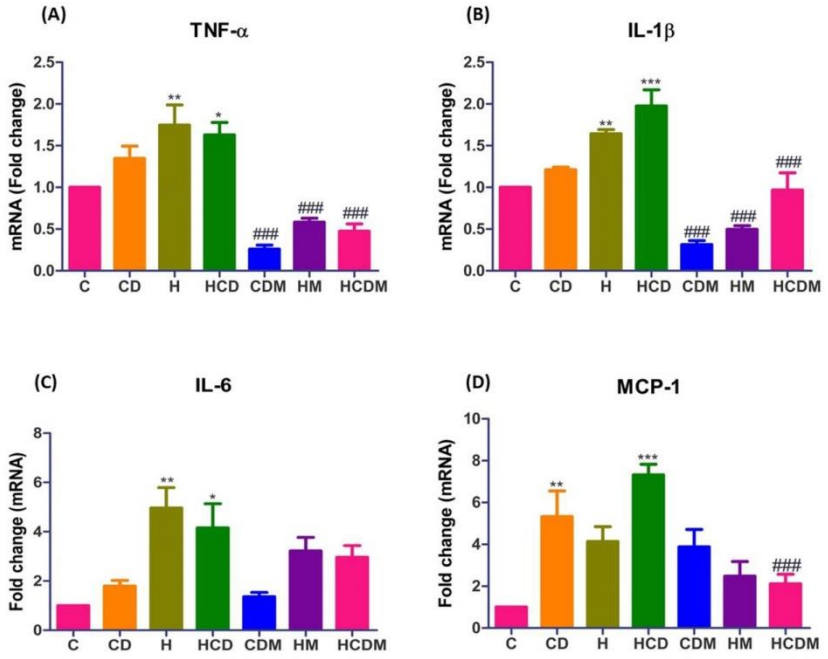

Figure 2.3: Changes in (A) TNF-α (B) IL-1β (C) IL-6 and (D) MCP-1 mRNA expression of pro-inflammatory cytokines in liver of mice fed with high fat-high fructose diet and/or subjected to chronodisruption and an improvement following melatonin treatment. Results are expressed as mean ± S.E.M. *p < 0.05, **p < 0.01, and ***p < 0.001 is when CD, H and HCD compared to Control (C). ###p < 0.001 is when CDM is compared with CD, HM with H and HCDM with HCD group respectively.

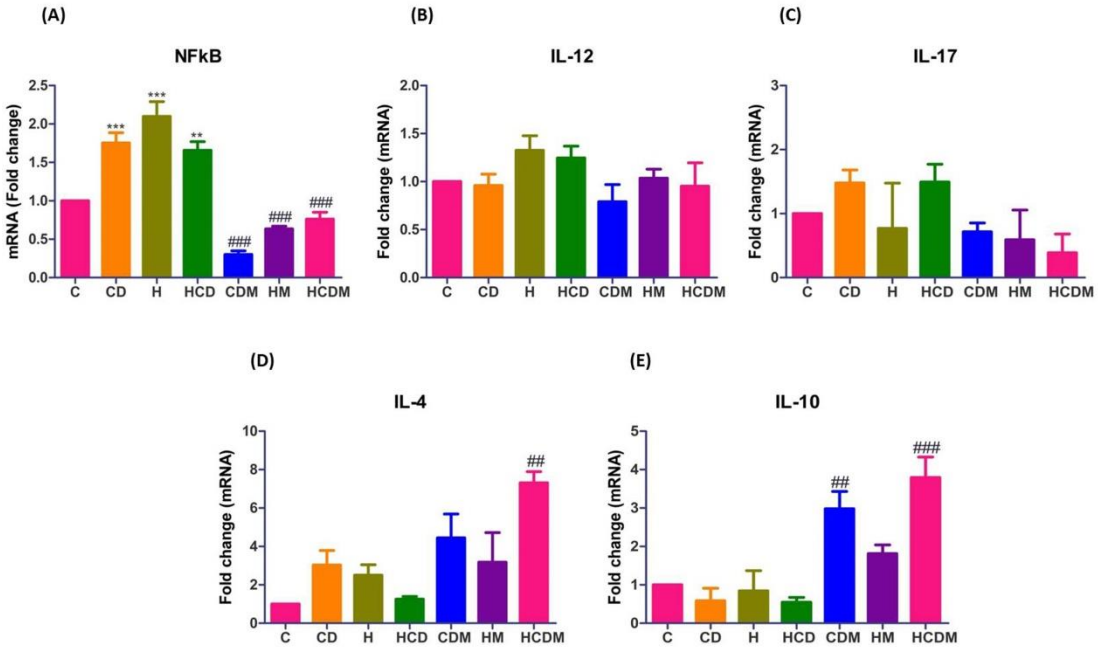

Figure 2.4: Changes in (A) NFκB (B) IL-12 (C) IL-17 (D) IL-4 and (E) IL-10 mRNA expression of pro- and anti- inflammatory genes in liver of mice fed with high fat-high fructose diet and/or subjected to chronodisruption. CD, H and HCD subjected groups depict an altered levels of inflammatory cytokines that appears to be corrected in melatonin treated groups 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 group respectively.

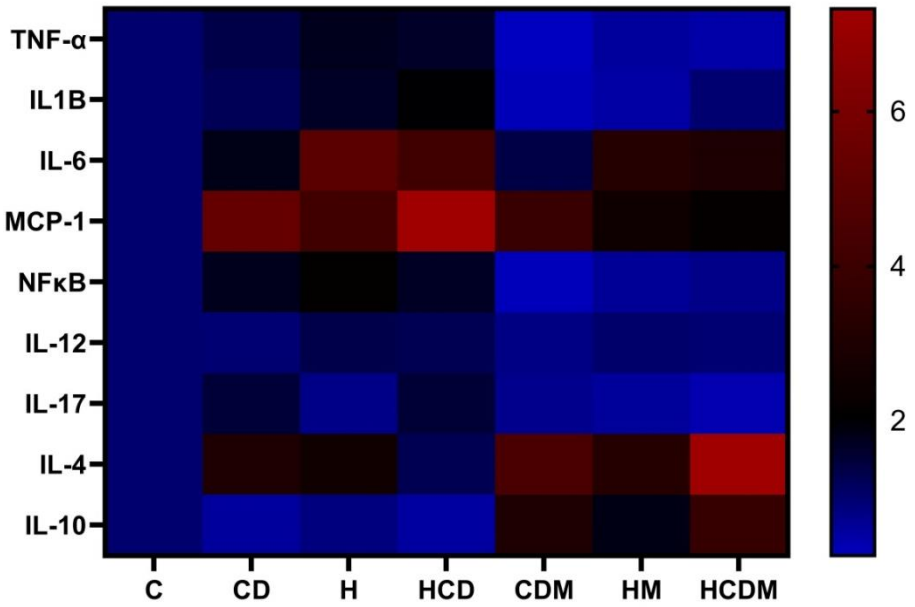

Figure 2.5: Heat map analysis of pro- and anti-inflammatory gene expression in the liver of C57BL/6J mice. The relative fold change in gene expression for genes TNF-α, IL-1β, IL-6, MCP-1, NF-κB, IL-12, IL-17, IL-4 and IL-10 were normalize with internal control GAPDH . Colour from blue to red indicates low to high expression.

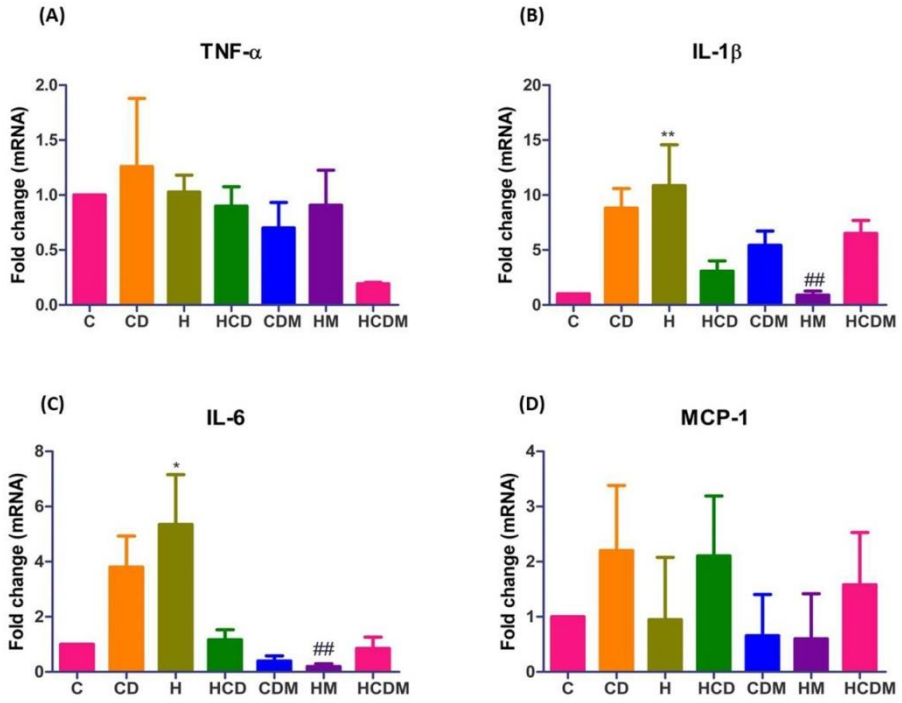

Figure 2.6: Changes in mRNA expression of (A) TNF-α (B) IL-1β (C) IL-6 and (D) MCP-1 pro-inflammatory genes in hippocampus of mice subjected to high fat-high fructose diet and/or chronodisruption. Corrective changes were observed following exogenous melatonin treatment in CDM, HM and HCDM groups. Results 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 group respectively.

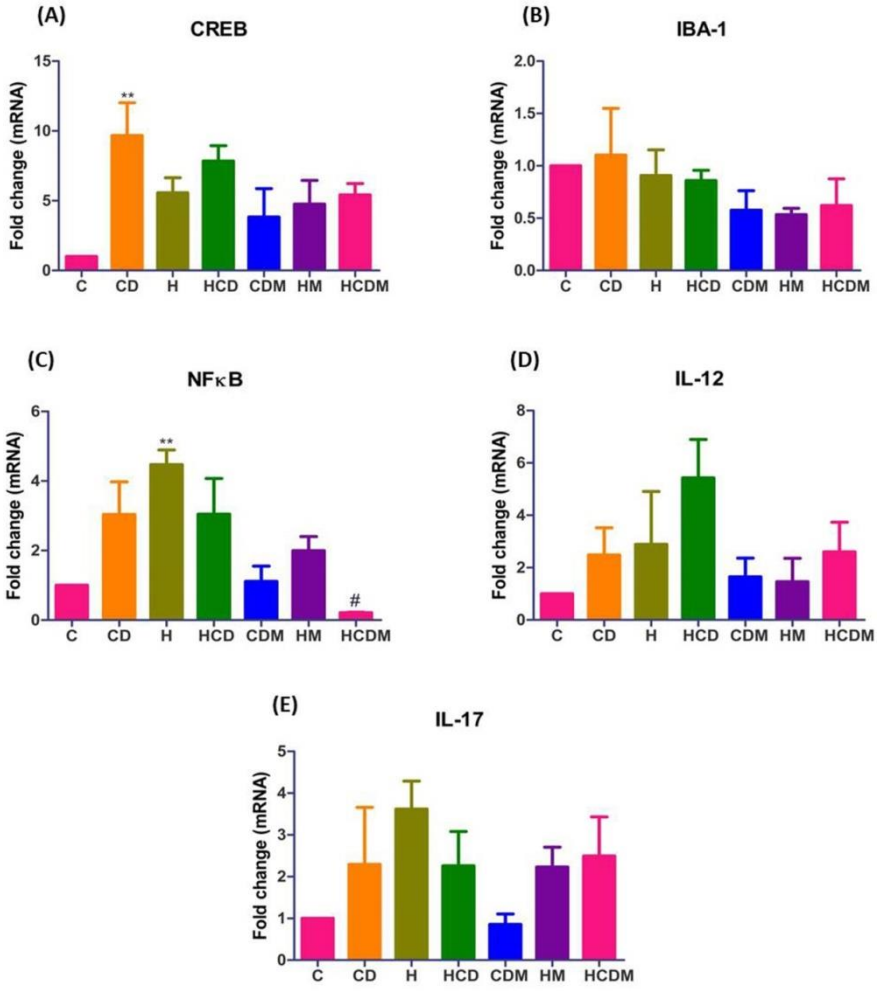

Figure 2.7: Increase in the mRNA expression of pro-inflammatory markers (A) CREB (B) IBA-1 (C) NF-κB (D) IL-12 and (E) IL-17 genes in hippocampus of mice subjected to high fat-high fructose diet and/or chronodisruption. CD, H and HCD treated groups depict increased expression levels that appears to be corrected in melatonin treated (CDM, HM and HCDM) groups. Results are expressed as mean ± S.E.M. **$p < 0.01$, 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 group respectively.

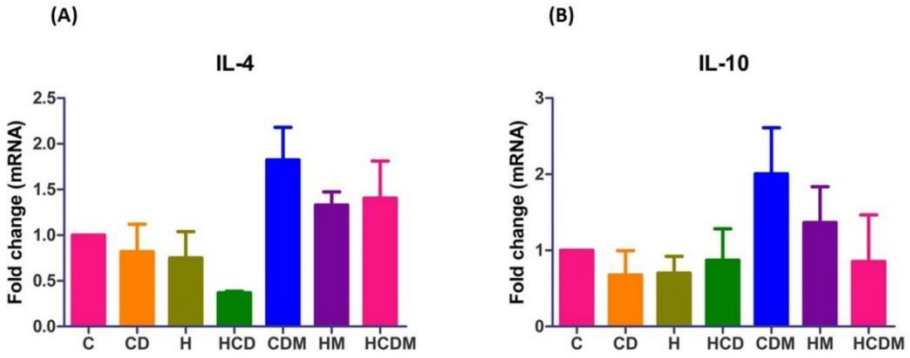

Figure 2.8: Improvement in the anti-inflammatory (IL-4 and IL-10) genes post melatonin treatment in hippocampus of mice subjected to high fat-high fructose diet and/or chronodisruption. Results are expressed as mean ± S.E.M.

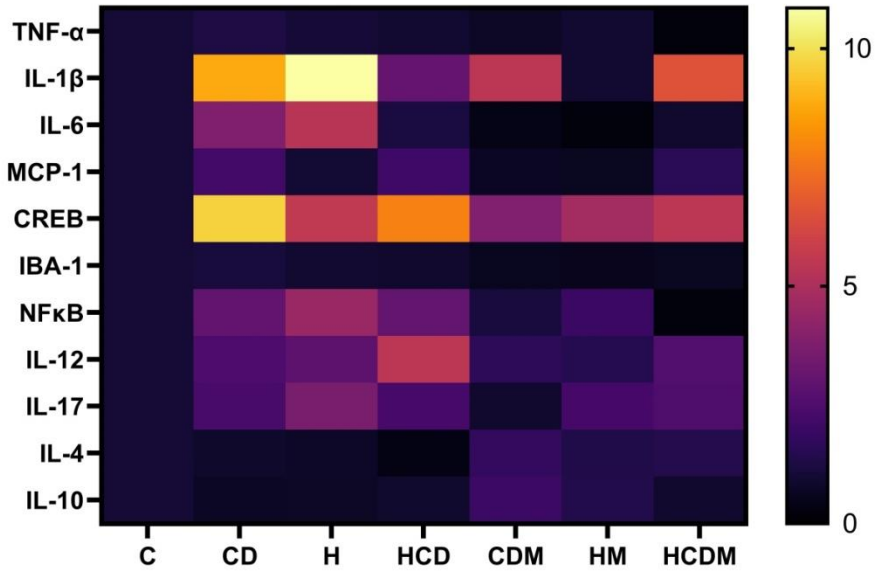

Figure 2.9: Heat map analysis of pro- and anti-inflammatory gene expression in the hippocampus of mice subjected to high fat-high fructose diet and/or chronodisruption C57BL/6J mice. The relative fold change in gene expression for genes TNF-α, IL-1β, IL-6, MCP-1, CREB, IBA-1, NF-κB, IL-12, IL-17, IL-4 and IL-10 were normalize with internal control GAPDH . Colour from black to yellow indicates low to high expression.

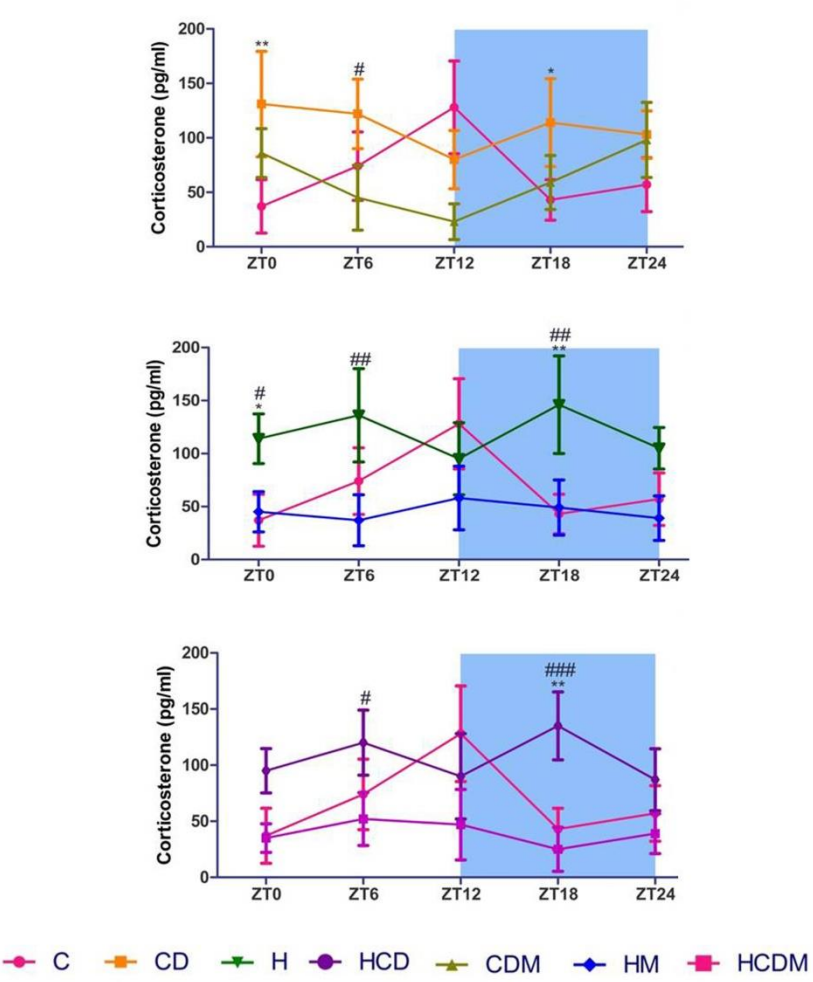

Figure 2.10: Serum corticosterone concentrations in C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption for 18 weeks in ZT0 to ZT12 (no colour) and ZT12 to ZT24 (in blue). Results are expressed as mean ± S.E.M. *p<0.05, **p < 0.01, and ***p < 0.001 is when CD, H and HCD compared 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 group respectively.

Discussion

NASH is considered a progressive form of NAFLD and is characterized by triglyceride accumulation, inflammation, hepatocellular injury and varying degrees of fibrosis (Choi & Diehl, 2005; Harmon et al., 2011; James & Day, 1999). In the pathophysiology of NASH, lipid accumulation is the first step causing liver steatosis and this increases the sensitivity of hepatic injury and inflammation (James & Day, 1999). In NAFLD or NASH, inflammation in the liver has origins inside as well as outside the liver with adipose tissue and gut making contributions to the said effect. Lipotoxicity, mitochondrial dysfunction, endoplasmic reticulum stress and apoptotic cascade in liver are major contributors to the progression of NAFLD to NASH (Joshi et al., 2021; Mansouri et al., 2018; K. Upadhyay et al., 2018; K. K. Upadhyay et al., 2020; Wang et al., 2020). Tissues and cells viz. Spleen, lymph nodes, macrophages and natural killer (NK) cells have been reported to follow a circadian regulation with direct controls on inflammatory pathways (Annamneedi et al., 2021; Gibbs et al., 2012; Hand et al., 2016; Mirsky et al., 2009; Pariollaud et al., 2018). The core circadian gene Bmal1 exhibits anti-inflammatory effects while its heterodimer CLOCK promotes inflammatory responses (Xie et al., 2020; Xu et al., 2021). Heightened levels of inflammation cause circadian rhythm disorders that further aggravate tissue damages including the once caused by fatty manifestations in liver (Joshi et al., 2021; Xu et al., 2021). Circadian clock dysfunction aggravates the development of liver diseases such as fatty liver, hepatitis and cirrhosis (Parent et al., 2012; Xu et al., 2021). Deletion of Bmal1in Ly6Chi monocytes disrupts rhythmicity of monocyte trafficking and develops pathologies associated with acute and chronic inflammation (Nguyen et al., 2013). Chronic sleep deprivation disrupts circadian rhythm in night workers and rotating shift workers with high titres of inflammatory markers (Haack et al., 2007; Kallwitz et al., 2007; Parent et al., 2012; Polotsky et al., 2009). Herein, a combination of high calorie diet and/or photoperiodic manipulation induced chronodisruption was subjected to C57BL/6J mice to mimics a condition of lifestyle disorder.

The pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are crucial in the pathophysiology of NAFLD/NASH (Choi & Diehl, 2005; Zhong & Liu, 2018). The balance between pro- and anti-inflammatory cytokines plays an important role in hepatic

and systemic insulin action and an imbalance has been implicated in the progression of NAFLD to NASH (Tilg, 2010). In this chapter, an increment in the levels of pro-inflammatory (TNF-α, IL-6, IL-12 and IL-17) and a decrement in anti-inflammatory (IL-10) cytokines in serum, of CD, H and HCD groups was recorded. These findings are in agreement with published reports in diet induced NAFLD / NASH in murine models (Barbuio et al., 2007; Headland et al., 2019; Jindal et al., 2015; Neyrinck et al., 2013). Exposure to photoperiod or high calorie diet treatment has been accredited to an increment in stress levels (Desmet et al., 2021; Gomaa et al., 2017; Yang et al., 2021). Hence, serum corticosterone levels were assessed that were found to be significantly elevated in disease groups as compared to the control. Also, a time point based study of serum corticosterone levels had revealed a peak at ZT12 that is comparable to the ZT12 peak reported in C57BL/6J mice or ZT12 and ZT16 in wistar rats (Appiakannan et al., 2019; Cano et al., 2008). The significantly high levels of corticosterone in CD, H and HCD groups at majority of the time points of our study is indeed noteworthy and reiterates heightened stress in the said groups.

Glucocorticoids (cortisol in humans and corticosterone in rodents) are secreted by the adrenal glands in response to stimulation of the hypothalamus-pituitary-adrenal (HPA) axis in response to stressors and induce metabolic and behavioral alterations (Hryhorczuk et al., 2017; Maccari & Morley-Fletcher, 2007). Hyperactivity in the HPA axis has been linked to the development of the metabolic syndrome and neurobehavioral perturbations (Maccari & Morley-Fletcher, 2007; Pasquali et al., 2006). In C57BL/6J$^{-ob/ob}$ and C57BL/6J$^{-ob/+}$ subjected to high fat diet had significant increment in the total plasma corticosterone levels as compared to control (Herberg & Kley, 1975). Photoperiod-sensitive Fischer 344 rats subjected to long- (L:D, 16:8) and short-day (L:D, 8:16) conditions showed significantly lower levels of corticosterone following exposure to long-day condition as compared to short-day condition (Otsuka et al., 2012). Studies in rodents and humans had reported that alterations or elevations in baseline corticosterone or cortisol levels, has been associated with several aspects of blood glucose regulation, fasting hyperglycemia and decreased insulin receptor binding affinity (Andrews et al., 2002; Appiakannan et al., 2019; Briançon-Marjollet et al., 2015). In our study, significantly high levels of glucose was observed in H and HCD groups that support corticosterone upregulation. However, there are no such reports with the combination of

high fat-high fructose diet and chronodisruption and hence our study is the first to investigate the same. Hepatic and hippocampal tissues of CD, H and HCD groups were also studied for inflammatory changes wherein; mRNA levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, MCP-1, NFκB, IL12 and IL17) had shown an increment. Chronodisruption / high fat diet fed inflammatory stress in hepatic and neuronal tissue samples has been reported in various rodent models and a similar trend was observed in our study too (Cavaliere et al., 2019; Froy, 2013; Pistell et al., 2010; Verma et al., 2022; Yanguas-Casás et al., 2021). mRNA expression of anti-inflammatory cytokines (IL-10) showed a decrement in hepatic and hippocampal tissues of CD, H and HCD group.

Melatonin, a powerful antioxidant, is also known for its anti-inflammatory properties. Moderate to significant decrement recorded (CDM, HM and HCDM groups) in levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-12 and IL-17) and improved levels of anti-inflammatory (IL-10) cytokine corroborates this fact. These observations are also supported by a decrement in serum corticosterone levels in melatonin treated experimental groups. In high fat diet fed mice, intragastric administration of melatonin alleviates liver steatosis along with decrement in pro-inflammatory cytokines expressions such as IL-6, TNF-α and activation of mitogen-activated protein kinase (MAPK), JNK and P38 signalling pathways indicating that melatonin inhibits cytokine production, inflammation and apoptosis (Sato et al., 2020). These results corroborate with the findings of other research groups wherein, melatonin is shown to counteract inflammatory stress in liver of high fat diet induced NAFLD/NASH by increasing the levels of anti-inflammatory genes such as (IL-4 and IL-10), and by decreasing the pro-inflammatory marker genes (TNF-α, IL-1β and IL-6) (Headland et al., 2019; Jindal et al., 2015; Joshi et al., 2021; Tahan et al., 2011). Thus, a balance between pro- and anti-inflammatory cytokines in liver appears to play an important role in the management of the nonalcoholic steatohepatitis.

High fat diet induced neuro-inflammation was first recorded in hypothalamus wherein; increased pro-inflammatory cytokine expression (TNF-α, IL-1β & IL-6) and activation of NFκB leading to impaired anorexigenic insulin signalling was reported (De Souza et al., 2005). Levels of pro- and anti-inflammatory cytokines in metabolic syndrome are known to cause cognitive impairments, depression-like behavior, anxiety including memory

deficits (Castanon et al., 2015; Colognesi et al., 2020; Elias et al., 2005; Elwing et al., 2006; Gladding et al., 2018; Moreno-Navarrete et al., 2017). Western diet induced increased body weight and protein levels of IL-6 & MCP-1 in brain along with impaired cognition in C57BL/6J mice (Pistell et al., 2010). In neuronal tissue, cAMP Response Element-Binding Protein (CREB) has been shown to play an important role in immune function, cell proliferation, survival, differentiation, glucose homeostasis, circadian rhythms and synaptic plasticity (Sakamoto & Frank, 2009; Shaywitz & Greenberg, 1999). The expression of CREB is induced by a variety of inflammatory and growth factors wherein; it mediates the transcription of several inflammatory genes such as IL-2, IL-6, IL-10 and TNF-α (Wen et al., 2010). IL-12 and IL-10 are primarily produced by dendritic cells, macrophages, and neutrophils in response to antigenic stimulation and had a major role in the progression of (NAFLD) from simple steatosis to NASH (Darmadi & Ruslie, 2021; Grazia Roncarolo et al., 2006; Mariano et al., 2013; Sousa et al., 1997; Zahran et al., 2013). IBA-1 is a microglia/macrophage-specific marker used for microglial detection and an increased expression of IBA-1 has been reported in activated microglia (Kanazawa et al., 2002; Shi et al., 2021). In mouse brain, upregulated IBA-1 in response to influenza viral infection and play an important role in immunological and pathophysiological function in microglial activation (Mori et al., 2000). In our study, though IBA-1 expression was unchanged, an increment in the mRNA expression of CREB in disease groups (CD, H and HCD groups) provides comprehensive evidence that high fat-high fructose diet and/or chronodisruption promote hippocampal inflammation.

The overall reduction in corticosterone levels in melatonin treated groups along with an improvement in depression and anxiety-like behavior (elaborated in chapter-4) are in agreement with other studies wherein; exogenous melatonin was shown to manifest positive neurobehavioral changes in experimental models (Gomaa et al., 2017; Yang et al., 2021). Our observations can therefore be accredited to the anti-inflammatory potential of melatonin that is successfully able to downregulate serum cytokine levels (TNF-α, IL-6, IL-12 and IL-17), hepatic mRNA levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, MCP-1, and NFκB) and neuroinflammation in hippocampus (IL-1β, IL-6, CREB, NFκB, IL-12 and MCP-1) along with upregulation in the anti-inflammatory markers (IL-4 and IL-10) in hepatic and hippocampal tissue.