Introduction

Non-alcoholic fatty liver disease is caused by an imbalance in lipid metabolism resulting in fat accumulation in the liver (Malaguarnera et al., 2009). Fatty liver is a consequence of excess lipid uptake and *denovo* lipogenesis exceeding β-oxidation and lipid disposal that further leads to oxidative stress and subsequent hepatocyte damage(Friedman et al., 2018). The mitochondrion plays a vital role in homeostasis of lipid metabolism by maintaining a balance between lipogenesis and lipolysis(Mansouri et al., 2018). Various studies have shown a downregulation of lipolytic genes (ACC and SREBP1c) and an upregulation of lipogenic genes (PPARα and CPT-1) in hepatocytes treated with free fatty acids(Michelotti et al., 2013). Insulin stimulates SREBP-1c which transcribes various enzymes required in the lipogenesis pathway. CPT-1 is responsible for the import of long chain fatty acids to the mitochondria and supply reducing equivalents for the mitochondrial electron transport chain (ETC)(Nita & Grzybowski, 2016; Takaki et al., 2013). PPARα has been shown to decrease plasma triglyceride levels through its ability to increase lipoprotein lipase mediated triglyceride clearance as well as decreasing the availability of triglycerides for VLDL secretion(Abdelmegeed et al., 2011). Furthermore, PPARα has been shown to lower TNF-α levels thereby playing a crucial role in improving the pathophysiology of NAFLD(Polyzos et al., 2010). PGC-1α is a key transcription factor which stimulates the expression of mitochondrial genes as well as the nuclear genes required for mitochondrial biogenesis. It has been shown to reduce hepatic lipogenesis, decrease oxidative stress, and improve steatosis in models of NAFLD through activation of Sirt1 and AMP activated kinase (AMPK)(Lamia et al., 2009; Mi et al., 2018). The pathophysiology of NAFLD is best explained by the multiple hit model, wherein oxidative stress plays a key 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(Buzzetti et al., 2016). An increase in intracellular lipid causes mitochondrial dysfunction and subsequent induction of ROS production.

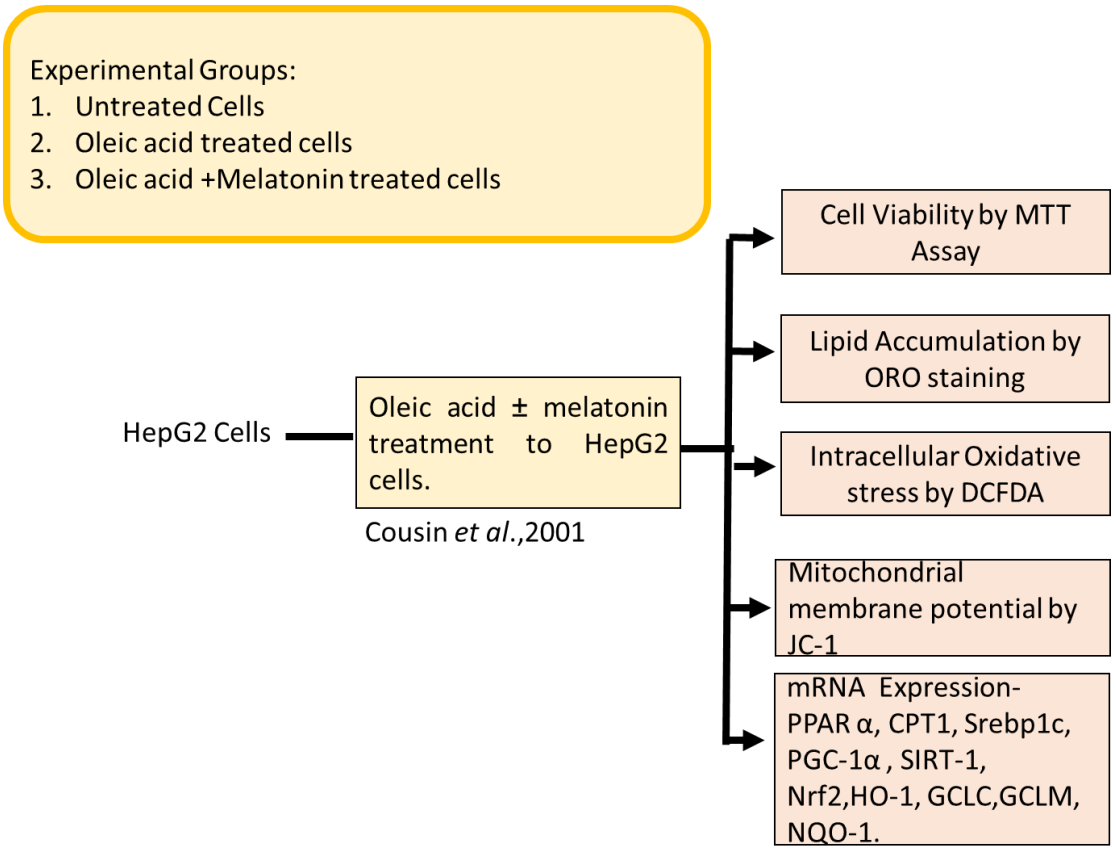
Transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is a positive regulator of a set of genes involved in the protection against oxidative stress. It regulates transcriptional induction of ARE-containing genes that encodes various antioxidant enzymes, electrophile-conjugating enzymes, ubiquitin/proteasomes, chaperone and heat-shock proteins in response to cellular stresses including ROS. In conditions of NASH, Nrf2 has been shown to be downregulated which is accompanied by an increased oxidative stress(Kaspar et al., 2009). 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(Nguyen et al., 2009). 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 the progression of NASH(Chambel et al., 2015).

Melatonin, a neurohormone involved in the regulation of circadian rhythms, has been reported to show potent antioxidant activity. Melatonin exerts protective effect on fatty liver (induced by high-fat diet in rats) possibly through its antioxidant actions(Sun et al., 2015). Studies have shown that melatonin is effective in improving the symptoms of metabolic syndrome(G. Tahan et al., 2011). Recently, Melatonin has been shown to reverse the harmful effects of dietary fructose in animal models by modulating

metabolic pathways like lipogenesis, lipolysis, β-oxidation and gluconeogenesis(Hong et al., 2020; Tiao et al., 2014).

Based on available background information, this study aims to validate the protective role of melatonin in oleic acid (OA) induced lipid accumulation and mitochondrial dysfunction in HepG2 cells. The OA treated HepG2 cells is a suitable experimental model as OA is more steatogenic than other free fatty acids. Herein, the *invitro* protective role of melatonin has been investigated via a series of protocols that scrutinize mitochondrial health, cellular integrity, and antioxidant status.

Experimental design



Results

Melatonin improved cell viability in OA treated HepG2 cells.

Hepatocytes treated with fatty acid (oleic acid, palmitic acid or stearic acid) serves as an ideal *in vitro* model for studying steatosis condition. In this study, HepG2 cells were treated with OA (0.5, 1, 1.5 and 2 mM) alone or in combination of melatonin (5- 1000 µM). Results showed a dose dependent decrease in cell viability following OA treatment, wherein the highest dose (2mM) recorded minimum number of cells (fig. 1.1 A). Melatonin alone did not account for any toxicity upto1000 µM (fig. 1.1 B). Further, melatonin (100 and 500 µM) treatment to OA treated HepG2 cells resulted in significant increase in cell viability (Fig. 1.1 C and D). So, based on the results obtained by MTT assay 0.5 mM OA and 100 µM Melatonin doses were selected for further experiments.

Melatonin attenuated OA induced lipid accumulation, intracellular oxidative stress and mitochondrial membrane potential in HepG2 cells.

After treating the cells with OA in the presence or absence of melatonin lipid uptake was studied by Oil Red O staining. Results indicated that OA treatment accounted for lipid accumulation in HepG2 cells whereas melatonin treatment resulted in a reduced lipid accumulation (fig. 1.2). The same was quantified and was found to be statistically significant ($P<0.001$). The effect of OA on intracellular ROS production was investigated using a fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA)(Eruslanov & Kusmartsev, 2010). Results recorded prominent DCF fluorescence in OA treated cells, while co-supplementation with melatonin significantly ($P<0.01$) lowered the same indicating reduced intracellular ROS (fig. 1.3). Mitochondrial membrane potential was assessed using cationic carbocyanine dye JC-

1. Results revealed that OA treatment significantly (P<0.001) decreased J-aggregate/J-monomer ratio, whereas melatonin co-supplementation showed higher indices of J-aggregate/J-monomer ratio, thus suggesting an improved mitochondrial membrane potential (fig. 1.4).

Melatonin improved mRNA profile in OA treated HepG2 cells.

To investigate the effect of melatonin on the regulation of lipid metabolism and steatosis induced by OA in HepG2 cells, mRNA expression of genes regulating lipid metabolism were studied. OA treatment markedly induced steatosis which was evident by significant (P<0.001) upregulation of lipogenic gene (SREBP1c) and downregulation of lipolytic genes (PPARα and CPT-1). Importantly, Melatonin treatment accounted for a significant (P<0.001) increment in mRNA levels of lipolytic genes (*CPT-1 and PPARα*) and a decrement in the lipogenic genes (*SREBP-1c*) (P<0.001) (Fig. 1.5). Additionally, OA with or without melatonin showed no significant change in the mRNA levels of PGC-1α and SIRT-1; implying towards an unchanged status of mitochondrial biogenesis (fig. 1.6). Further, mRNA profile of Nrf2, HO-1 and GCLM recorded significant (P<0.01) decrement in OA treated HepG2 cells. However, melatonin treatment accounted for a non-significant increment in mRNA levels of the said genes. Also, no significant changes were observed in GCLC and NQO-1 mRNA levels (fig. 1.7).

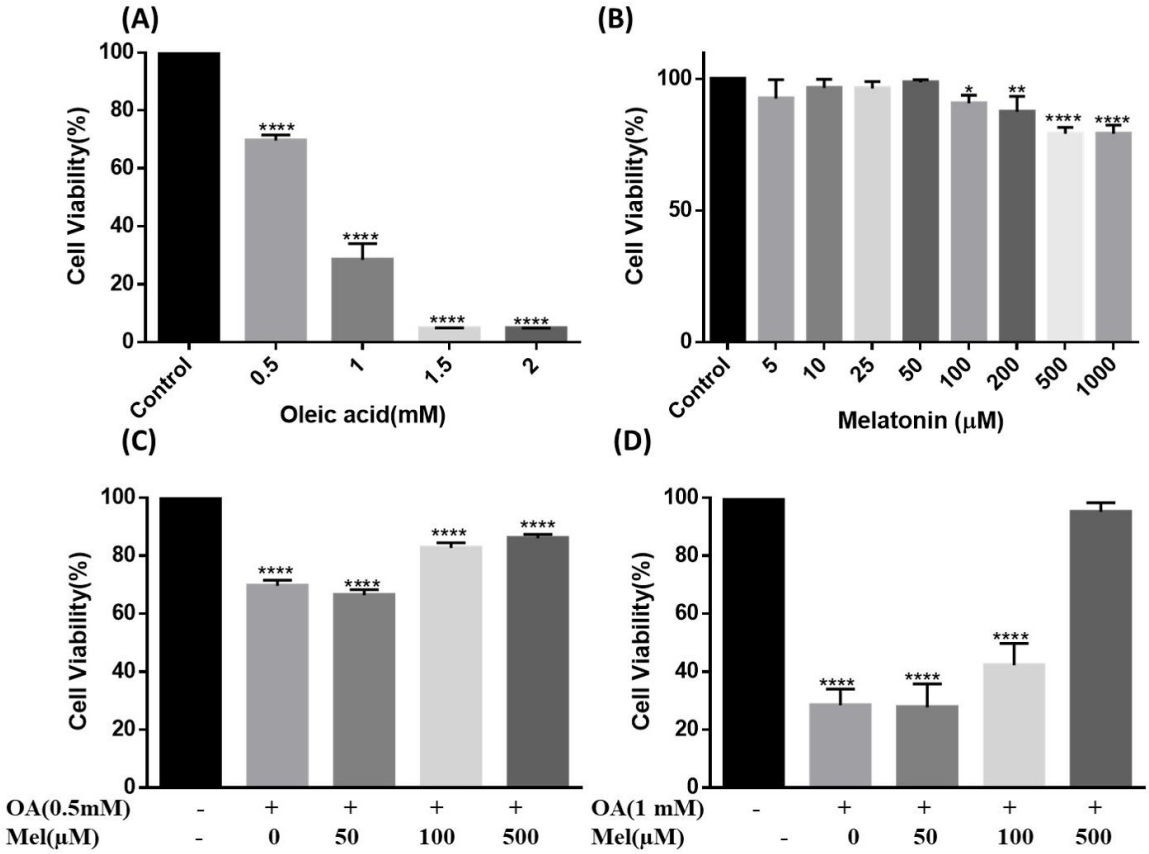


Figure1.1: (A) Cell viability assessment of OA with different concentrations of 0.5, 1, 1.5 and 2 mM dosed to HepG2 cells for 24h. (B) Cell viability assessment of Mel with concentrations of 5 to 1000 µM dosed to HepG2 cells for 24h. (C) and (D) Cytoprotective potential of Mel accessed on OA induced toxicity in HepG2 cells. Data is represented as mean±SD *P<0.05, **P<0.01, ***P<0.001 vs control. n=3

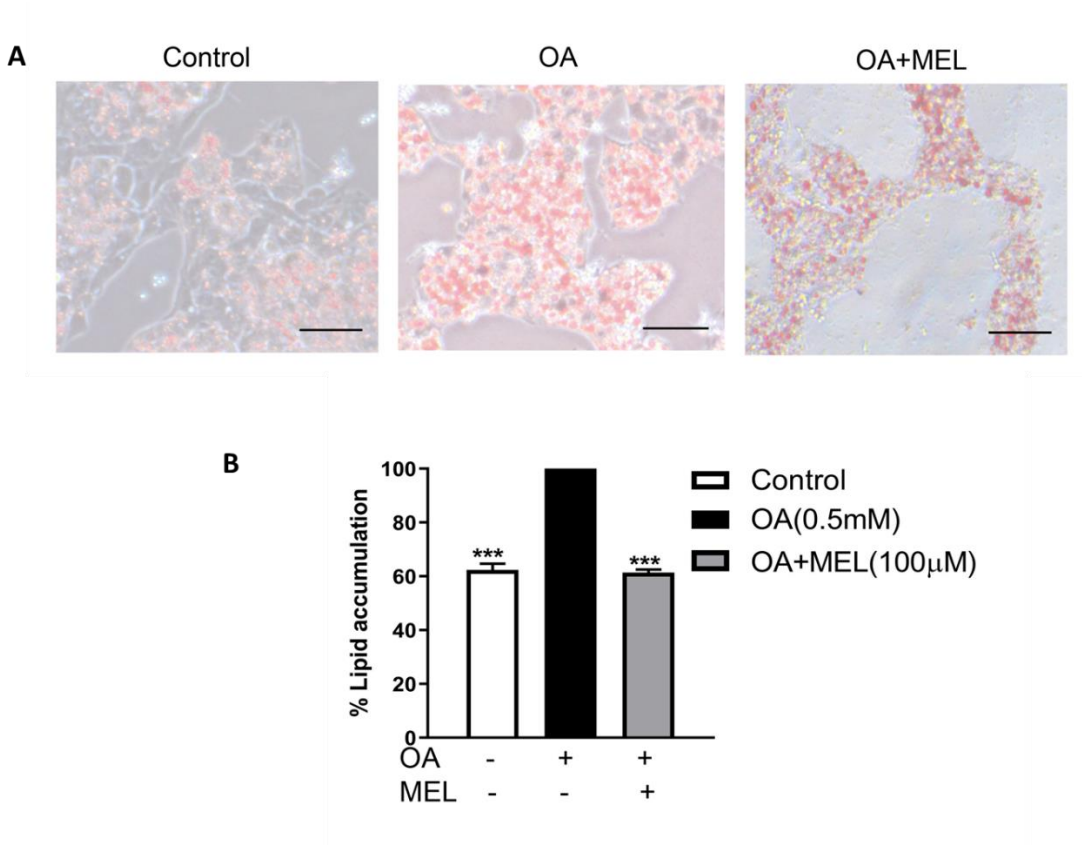


Figure1.2: Lipid accumulation accessed by ORO staining in HepG2 cells treated with OA and OA + Mel for 24h. Data is represented as mean±SD *P<0.05, **P<0.01, ***P<0.001 vs OA. n=3. Scale bar =100 µm

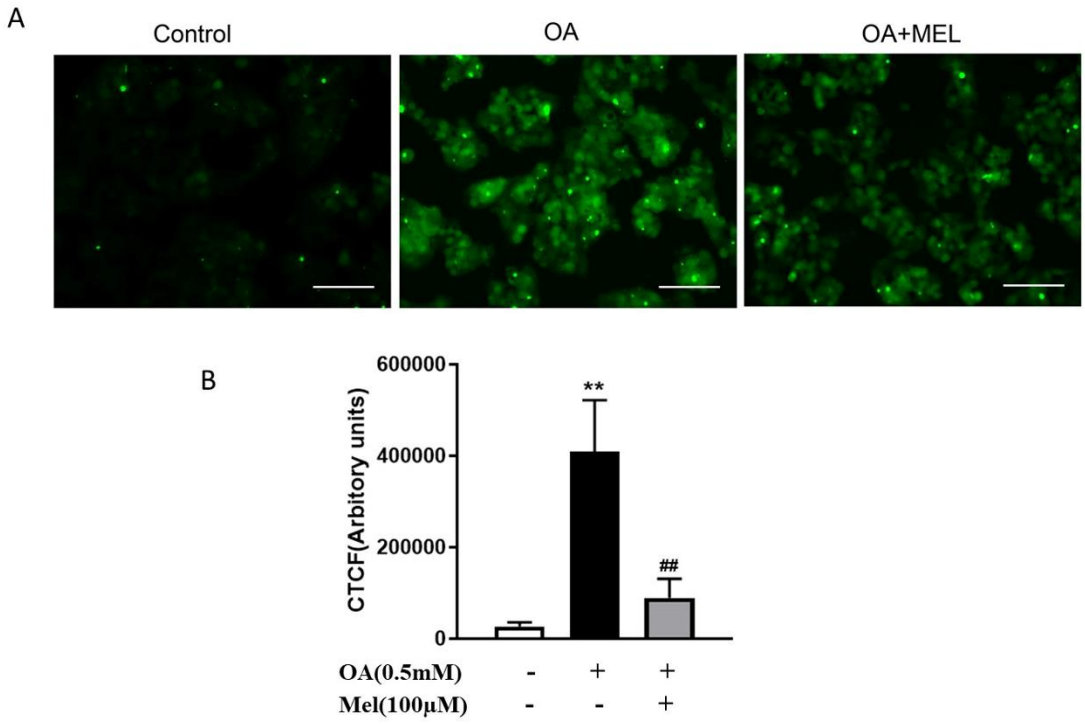


Figure1.3: HepG2 Cells were treated with OA alone and with Mel for 24h. Intracellular ROS was measured by DCFDA staining. Data is represented as mean±SD *P<0.05, **P<0.01, ***P<0.001 vs control. n=3. Scale bar=100 µm

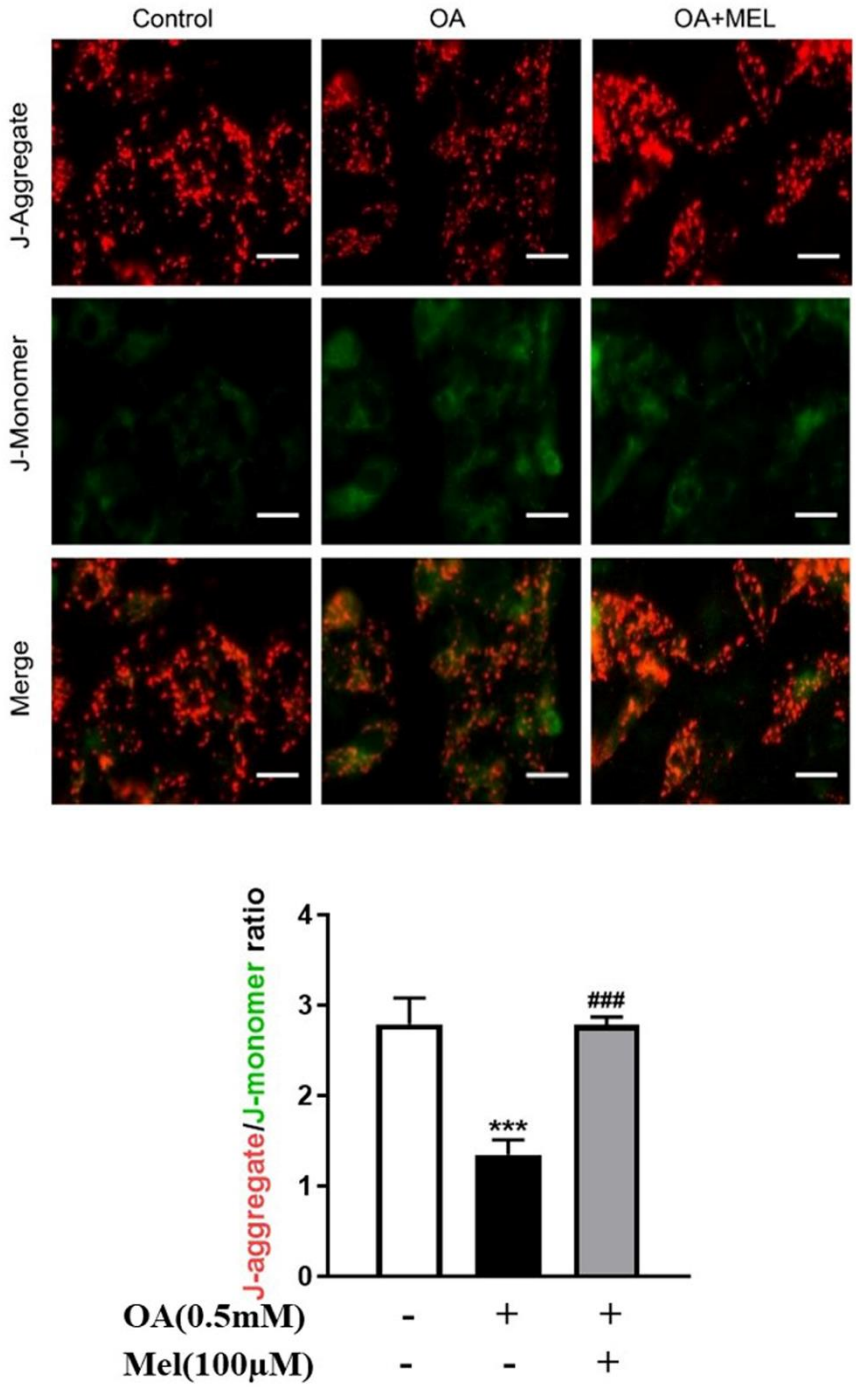


Figure 1.4: Mitochondrial membrane potential is determined by JC-1 staining in OA and OA + Mel treated HepG2 cells for 24h. J-aggregates (red) represents healthy mitochondria and J-monomers (Green) depicts damaged mitochondria. Data is represented as mean ± SD *P<0.05, **P<0.01, ***P<0.001 vs control, ###P<0.001 vs OA group. n=3. Scale bar=20µm

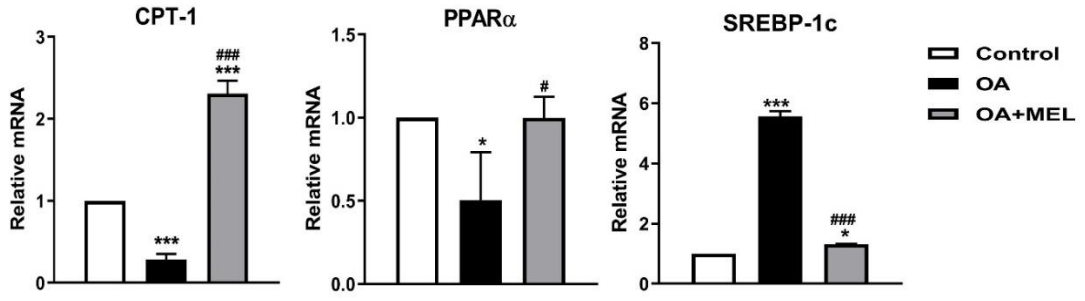


Figure 1.5: HepG2 cells were treated with OA with/without melatonin. mRNA levels of lipid metabolism related genes CPT-1, PPARα and SREBP-1c were assessed by RT-qPCR. Values were normalized with GAPDH. Data is represented as mean±SD. *P<0.05, **P<0.01, ***P<0.001 vs control, #P<0.05, ##P<0.01, ###P<0.001 vs OA group. n=3

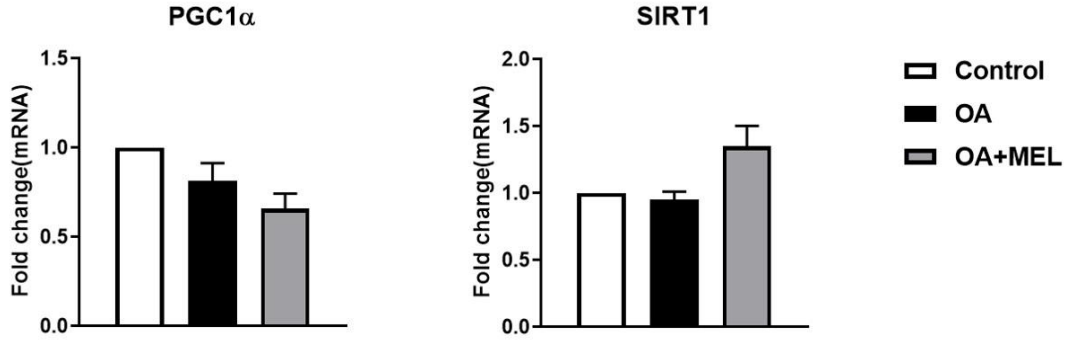


Figure 1.6: HepG2 cells were treated with OA with/without melatonin. mRNA levels of mitochondrial biogenesis genes were assessed. Values were normalized with GAPDH. Data is represented as mean±SD. n=3

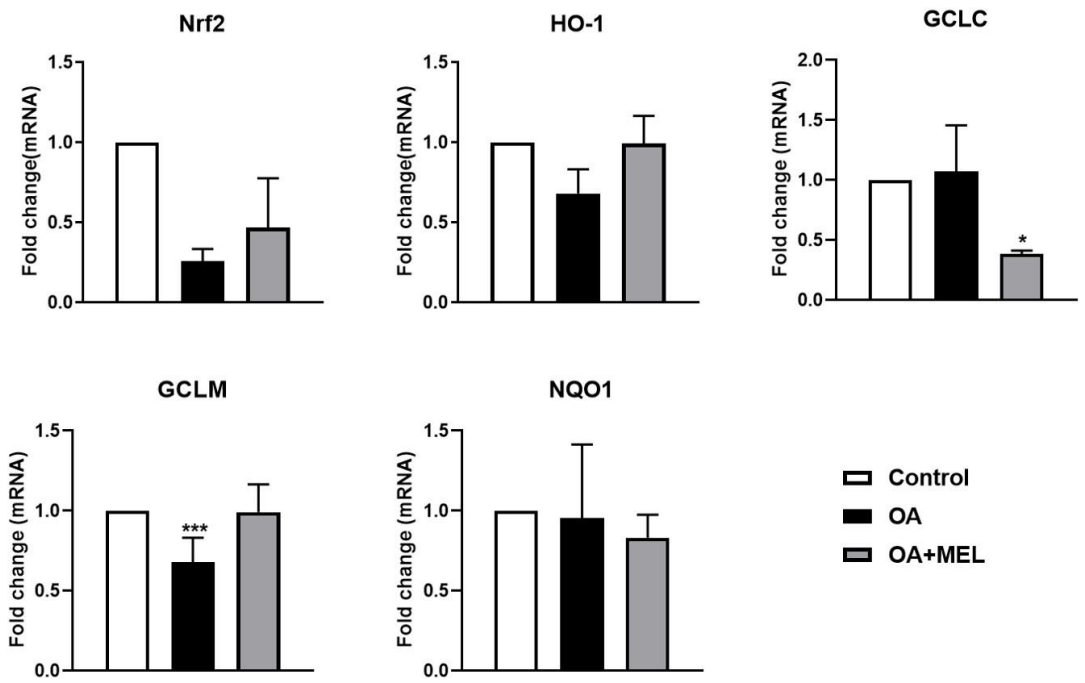


Figure 1.7: HepG2 cells were treated with OA in with/without melatonin. mRNA levels of Nrf2 and ARE related genes was assessed. Values were normalized with GAPDH. Data represented as mean±SD. *P<0.05, ***P<0.001 vs control. n=3

Discussion

Epidemiological studies have shown that 30% of the western population is affected by NAFLD which is associated with dysfunction of lipid and glucose homeostasis. Imbalance in lipid metabolism can result into lipotoxicity, which further leads to induced free radical damages in cells(Nita & Grzybowski, 2016). Oxidative stress is a key player in the pathogenesis and progression of NAFLD. Besides oxidative stress, augmented uptake and intracellular accumulation of TG in hepatocytes is another key event that furthers cellular damage(Friedman et al., 2018). Various research groups have shown use of OA, PA or combination of both as an invitro model of NAFLD, wherein OA is found to be more steatogenic(Ricchi et al., 2009). Based on these previous reports, OA induced steatosis model was used to validate the protective role of melatonin in improving the mitochondrial and antioxidant status in steatotic hepatocytes.

HepG2 cells were treated with a dose range of OA (0.5, 1, 1.5 and 2 mM) and the subsequent effect on the cellular status was assessed by MTT assay. Herein, 0.5 mM dose of OA recorded <30% toxicity along with prominent intracellular fatty manifestations. Hence, 0.5 mM dose was chosen as a suitable dose for subsequent studies. Though, upto 2mM dose of OA has been used for developing HepG2 based steatosis model (N. Li et al., 2020; W. Li et al., 2016; Yao et al., 2011). Dose chosen by us is in agreement with number of other studies on HepG2 cells(Cui et al., 2010; Zhou et al., 2021). Melatonin treatment to HepG2 cells (10-1000 µM) accounted for healthy population of cells and hence, 100 µM dose was chosen for further studies. Reports on decreased PA uptake in 100 µM melatonin supplemented HepG2 cells are in agreement with our study (Jee-In Heo et al., 2018; Jeein Heo, 2017).

HepG2 cells treated with OA recorded significant intracellular lipid accumulation as evidenced by ORO staining. The accumulation indices obtained after quantification of intracellular ORO stain revealed that melatonin supplemented cells accounted for 40% reduced accumulation. These observations are in agreement with other studies wherein 50 % reduction in accumulation has been recorded (Mi et al., 2018).

Balance between genes associated with lipolysis and lipogenesis are critical in maintaining intracellular lipid homeostasis (Saponaro et al., 2015). PPARα is a nuclear receptor that positively regulates fatty acid utilization and catabolism. PPARα activation results in reduced levels of fatty acids required for the synthesis of triglycerides by enhancing the expression of CPT1; which is a rate-limiting enzyme in β-oxidation of fatty acids. SREBPs are transcription factors that translocate into the nucleus and facilitate expression of target genes. SREBP-1c controls de novo hepatic lipogenesis primarily by regulating the expression of genes such as FAS and SCD1(Malaguarnera et al., 2009). In the present study, we investigated the mRNA transcripts of the said genes and found that melatonin upregulated expression of lipolytic genes (PPAR α and CPT1) and downregulated the expression of lipogenic genes (SREBP1c) genes. Our results corroborate with the finding of other research groups wherein PA/OA treatment to HepG2 cells and melatonin supplementation had resulted in an increased expression of PPARα and its target genes and had decreased the expression of SREBP1c whereas melatonin did not change expression of genes regulating cholesterol metabolism, HMGCR and SREBP2(Mi et al., 2018). Anabolic and catabolic genes investigated in our study are the key components of ampk pathway and hence it is hypothesized that the findings are attributable to melatonin induced changes in the AMPK pathway(Rui et al., 2016). Mi et al., (2018) had reported that exogenous melatonin modulates key genes in AMPK pathway in OA treated HepG2

cells and our findings are in agreement with the same. Lipotoxicity is known to cause depletion in Sirt1 mRNA levels, however no significant changes were recorded in OA treated HepG2 cells. In contrast, other studies had reported PA treatment caused a decrement in Sirt1 and PGC1α levels (Tong et al., 2015; Upadhyay et al., 2020) and melatonin treatment had resulted in an improved mitochondrial function by Sirt1 activation (Das et al., 2017).

A majority of past and present studies had attributed the therapeutic potential of melatonin to its antioxidant potential (Reiter et al., 1999; Tan et al., 2015). The same holds true for melatonin induced improvement in functional and pathophysiological condition of experimentally induced liver damage (Jee-In Heo et al., 2018; V. Tahan et al., 2009). Overall, this study provides a prima facie evidence on the protective role of melatonin against OA treatment in HepG2 cells via lowering intracellular oxidative stress and improving mitochondrial membrane potential. Findings of this study forms the basis of detailed investigation envisaged herein.