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Melatonin Ameliorates Intracellular Oxidative Stress and Oleic Acid Uptake in HEPA 1-6 Cells: An *In Vitro* Experimental Model of Nonalcoholic Fatty Liver Disease

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Abstract
Non-Alcoholic fatty liver disease (NAFLD) has emerged as a major health concern, wherein circadian changes have been directly implied as one of the key causative agents. In the present study, HEPA1-6 cells treated with oleic acid (OA) have been used as a model for NAFLD in which cell viability, intracellular oxidative stress and OA uptake are investigated. OA treatment accounted for significant decrement in cell viability at 24 and 48 h treatment. However, presence of melatonin was effective in improving cell viability indices in (OA + Mel) experimental group. Intracellular oxidative stress showed prominent fluorescence in OA treated cells whereas, OA + Mel group recorded relatively weaker fluorescence. The same was confirmed by qualitative and quantitative assessment using DCFDA staining. OA treated cells were stained with Oil red O (ORO) showed visibly higher accumulation of droplets in cytoplasm but melatonin (OA + Mel group) was effective in preventing OA accumulation. These findings provide baseline evidence on melatonin mediated amelioration of OA induced lipotoxicity and NAFLD like changes in HEPA 1-6 cells.

Keywords
HEPA 1-6 cells, Melatonin, Non-Alcoholic fatty liver disease, Oleic acid and Oxidative Stress.

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INTRODUCTION
Non-alcoholic fatty liver disease (NAFLD) is a condition characterized by excessive fat accumulation and triglycerides deposition in the liver in patients with no history of consumption of alcohol. NAFLD has become the most common cause of chronic liver diseases in western countries [1] with growing relevance in developing countries such as India [2]. Pathogenesis of NAFLD is varied and complex having strong association with insulin resistance, obesity, type 2 diabetes and dyslipidemia. Non-alcoholic steatohepatitis (NASH) is an advanced form of NAFLD in which the liver gets scarred due to inflammation and cellular damages. NASH involves hepatocellular steatosis in connection with hepatic inflammation and hepatocyte lesions. Hepatocytes, hepatic macrophages and hepatic stellate cells contribute to the onset and progression of NASH. Hepatic macrophages consisting of resident kupffer cells and recruited bone marrow-derived

macrophages are major producer of inflammatory cytokines such as TNFα, IL-1β, IL-6 that further stimulate stellate cells mediated hepatocyte steatosis and fibrosis [3].

In HepG2 cells, induction of steatosis was reported by incubating cells with oleic acid (OA) wherein, significant increase in PPAR gamma was recorded [4]. Further, OA and Palmitic acid treatments resulted in altered lipid peroxidation, increased triacylglycerol content, decreased insulin mediated glucose uptake and proliferation of HepG2 cells [5, 6, and 7]. Reactive oxygen species (ROS) and caspase-3 activities have also been reported to show significant increment in YAC-1 cells incubated with linoleic acid, OA and stearic acid [8]. Free fatty acids treated HepG2 cells were also reported to upregulate TNF-α mRNA in NAFLD model via cathepsin B (ctsb)-dependent lysosomal destabilization and release of lysosomal protease into the cytosol [5].

Melatonin, a pineal hormone is involved in diverse physiological and biochemical functions such as synchronization of the circadian rhythms including sleep-wake cycle, blood pressure regulation and seasonal reproduction. Further, properties of melatonin such as antioxidant, anti-aging, anti-cancer, anti-atherosclerotic and hepatoprotective are well established [9]. Antioxidant property of melatonin has been attributed for its potential for reactive nitrogen species (RNS) and reactive oxygen species (ROS) scavenging in oxidatively stressed cells [10]. Further, melatonin has been reported to inhibit uptake of monounsaturated, polyunsaturated fatty acids and linoleic acid in hepatoma 7288CTC cells [11, 12]. Exogenous melatonin and altered photoperiod favorably change the hepatic carbohydrate and lipid metabolisms in rat liver [13]. Further, release of neuropeptides (leptin and ghrelin) was reported to be negatively impacted in pinealectomized rats whereas, exogenous supplementation of melatonin could restore the lipid metabolism [14, 15]. Though, leads available with melatonin induced modulation of fatty acid metabolism in liver cells is known, the dose and time dependent efficacy of melatonin in preventing fatty acid uptake and subsequent NAFLD like changes are not known. In the light of available literature and reported therapeutic properties of melatonin in liver diseases, this inventory investigates the efficacy of melatonin in ameliorating NAFLD like changes in OA fed Hepa 1-6 cells.

MATERIALS AND METHODS

Methanol, chloroform, dimethyl sulphoxide (DMSO), and 3-(4, 5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) were purchased from Sisco Research Laboratory Pvt. Ltd. (Mumbai, India). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine Serum (FBS), trypsin phosphate versene glucose (TPVG) and antibiotic-antimycotic solution were purchased from Hi-media Laboratories (Mumbai, India). 2',7'-dichlorfluorescein-diacetate (DCF-DA) was purchased from Sigma Aldrich, USA.

Cell culture protocol

Hepa1-6 (mice liver hepatoma) cells were procured from National Centre for Cell Science (NCCS), Pune, India and maintained in T25 flasks (TPP, Switzerland) at 37°C with 5% CO_2 in DMEM (Hi-media Laboratories, Mumbai, India) containing 10% fetal bovine serum (FBS, Gibco, Invitrogen, USA) and 1% antibiotic-antimycotic solution (Hi-media Laboratories, Mumbai, India). Cells were trypsinized using 1X trypsin phosphate versene glucose (TPVG; Hi-media Laboratories, Mumbai, India) at three-day interval. The experimental groups for this study were: Control (untreated cells), Oleic acid (OA; 50 & 100 µM for 24 h & 48 h) treated, and OA + Mel (co-treated with 100 µM melatonin for 24 h & 48 h) respectively.

Cell viability assay

Hepa1-6 cells were seeded in 96 well plate (1×10^4 cells per well) and allowed to grow overnight. Cells were treated with OA (50 & 100 µM) with or without single dose of melatonin (100µM) for 24 h and 48 h. At the end of treatment, MTT (SRL Pvt. Ltd., Mumbai, India) was added to each well at concentration of 0.5 mg/ml and incubated in dark for 4 h at 37˚C. The resultant purple formazan crystals were dissolved by adding 150 µl/well DMSO (Sisco Research Laboratories Pvt. Ltd., Mumbai, India). The absorbance was measured at 540 nm by using HTX-Multimode Reader (BioTek Instruments Inc., USA) and % cell viability was calculated relative to control.

Intracellular oxidative stress assay

Intracellular oxidative stress caused due to generation of ROS was determined by staining cells with 7.5 µM of 2', 7'-dichlorfluorescein-diacetate (DCF-DA; Sigma Aldrich, USA) for 30 minutes. DCF-DA a non-fluorescent probe gets converted into highly fluorescent 2',7'-dichlorofluorescein (DCF) stain upon oxidation with peroxyl free radical. Hepa1-6 cells were seeded in a 6 well plate for overnight. Cells were treated with 2 doses of OA alone or with melatonin 24 h and 48 h. After incubation media was removed, cells were washed with PBS and stained with DCF-DA in dark. Excess stain was removed by 4-5 washes of PBS and cells were observed and images

were captured using Floid cell imaging station (Invitrogen, USA).

Oleic acid uptake assay

Oil Red O (ORO) solution was made by mixing 2.4 ml of ORO stock solution (0.5% w/v) with 1.6 ml of distilled water then filtered using 0.2 µm filter. After treatment, the cells were fixed with 4% paraformaldehyde for 45 min followed by three washes with double distilled water. A 50 µl of the ORO solution was then added to each well and incubated at room temperature for 15 minutes. After removing the ORO solution from each well, the cells were washed multiple times with double distilled water until the solution became clear and the cells were examined under a light microscope, and the presence of red oil droplets in the cells indicate OA induced accumulation [16]. Images were captured using Nikon Eclipse TS100.

Statistical analysis

The data were expressed as mean ± SEM and analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test using Graph Pad Prism 5.0 (CA, USA). $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Fatty acid laden cells serve as an experimental model for NAFLD that are widely used in generating prima facie evidence on the efficacy of a test therapeutant and the subsequent underlying mechanistic changes. In this study, OA accumulation and lipotoxicity in murine liver hepatoma (Hepa 1-6) cell line and ameliorative changes induced by melatonin were scrutinized at two time points. Intracellular accumulation of toxicants or biomolecules in excess causes cytotoxicity often due to mitochondrial dysfunction [17]. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) is a tetrazolium dye that undergoes reduction by mitochondrial enzymes to form a purple color formazan. The intensity of this color is directly proportional to cell viability [18]. The cytotoxicity of OA in Hepa1-6 cells was evaluated with the MTT assay. Results showed highly significant decrement in cell viability following OA treatment (50 or 100 µM) at 24 and 48 h (Fig. 1). OA treatment accounted for 69% (50 µM) and 59% (100 µM) cell viability at the end of 24 h. Further, a decrement of 14% and 8% respectively were observed in OA treated group at 48 h. Presence of melatonin accounted for significantly higher cell viability of about 80% at 24 and 48 h stages (Fig. 1). These results suggest that melatonin was instrumental in preventing OA induced loss of cell viability. Findings are attributable to the known antioxidant and free radical scavenging potentials of melatonin [9].

Intracellular glutathione is an important non-enzymatic antioxidant that undergoes fast depletion in condition of infection, intracellular ROS or lipotoxicity [19]. The intracellular production of ROS was measured using a fluorescent probe 2', 7'-dichlorfluorescein-diacetate (H2-DCFDA) that produces prominent green fluorescence in Hepa1-6 cells fed with OA. Intracellular oxidative stress generated at 50 and 100 µM doses of OA treatment was seen in form of green fluorescence in cytoplasm of Hepa 1-6 cells (Fig. 2 A&B). Our observations are in agreement with the reported OA mediated heightened levels of intracellular oxidative stress in cultured hepatic cells [20]. In contrast, melatonin supplemented (OA+Mel group) cells showed weak fluorescence as compared to OA treated cells. Further, the quantification of fluorescence intensity provided supportive evidence to the said observations suggesting that melatonin treatment accounted for significant decrement in cellular oxidative stress in OA treated cells (Fig. 2A).

Studies have shown that presence of excessive lipid content activates intracellular enzymes and facilitates intracellular uptake leading to changes in cell morphology [21]. The spindle shaped Hepa 1-6 cells underwent morphological changes and appeared to slightly round at the end of 48 h. Further, accumulation of droplets surrounding the nuclei could be observed in cytoplasm of these cells. It is interesting to note that the spindle shape of the Hepa1-6 cells was restored in presence of melatonin in both 50 and 100 µM (OA+Mel treated) oleic acid treated groups. Morphological alterations resulting due to OA uptake observed in our study are in agreement in published reports [22]. Further confirmation in this regard, was obtained with Oil Red O (ORO) staining that provided important visual evidence on intracellular OA accumulation in form of ORO droplets both at 24 and 48 h. These results are in agreement with published reports on 0.1-2.0 mmol/L OA exposure for 24 h to HepG2 cells [23, 24]. ORO stained OA droplets were visibly higher in 100 µM dose as compared to 50 µM OA dose (Fig. 3). However, OA+Mel (50 µM OA) accounted for visibly less intracellular accumulation of OA both at 24 and 48 h stages but a higher dose of OA (100 µM in OA+Mel group) was found to be less effective (Fig. 3). These observations suggest OA treatment results in lipotoxic changes and NAFLD like condition in Hepa

1-6 cells that is ameliorated significantly by exogenous melatonin.

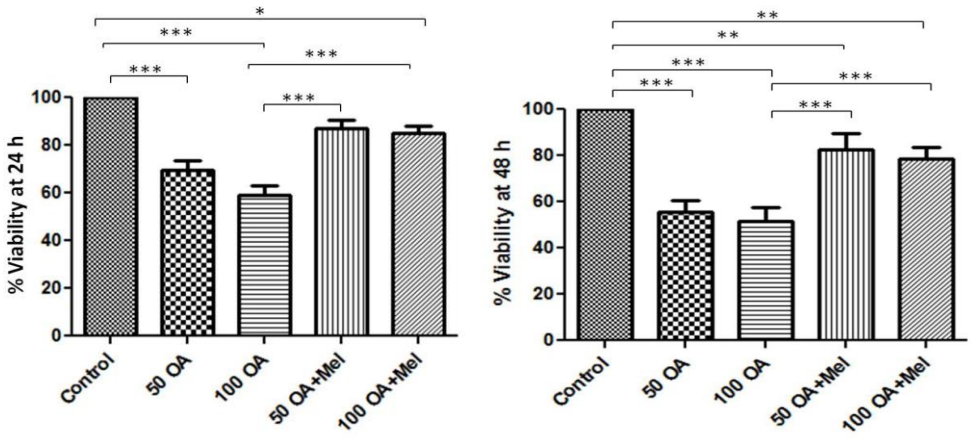
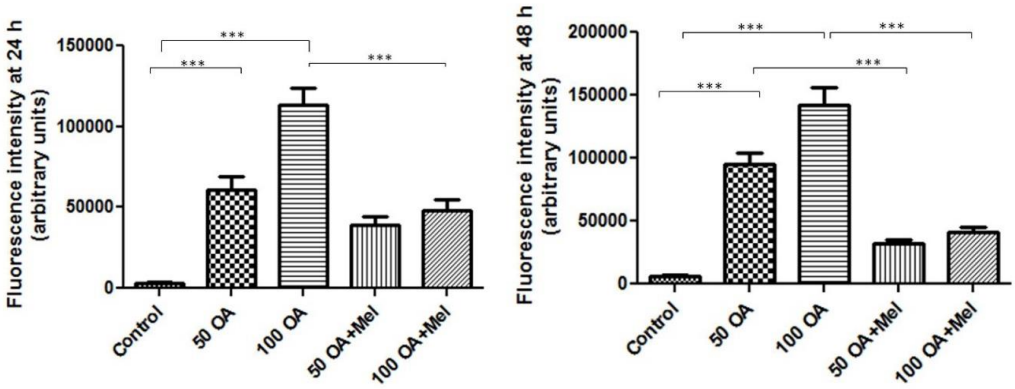
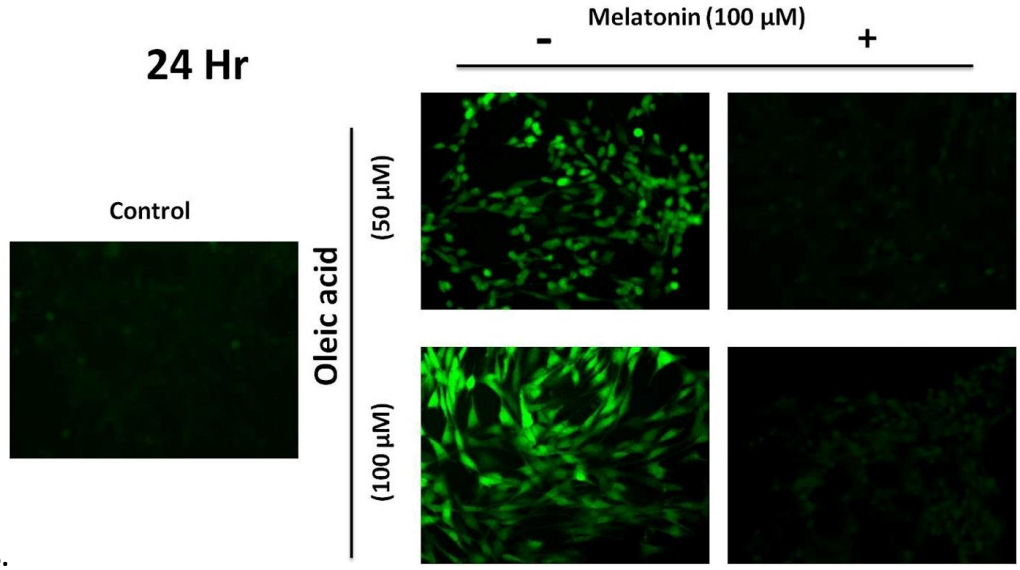


Fig. 1: Effect of melatonin on oleic acid induced cytotoxicity in Hepa1-6 cells at 24 h and 48 h. Data expressed as Mean ± S.E.M. for n=3. *p<0.05, **p<0.01 and ***p<0.001.

A.





B.

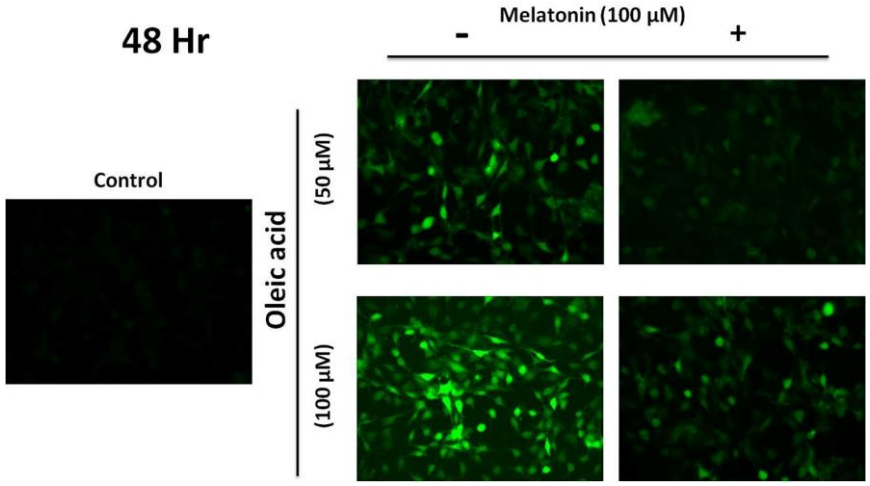


Fig. 2: Effect of melatonin on oleic acid induced intracellular oxidative stress (DCFDA staining). (A) Fluorescent intensity of DCFDA quantified at 24 h and 48 h. Data expressed as Mean ± S.E.M. for n=3. *p<0.05, **p<0.01 and ***p<0.001. (B) Representative images of stained cells at 24 h and 48 h are shown. Magnification= 200X.

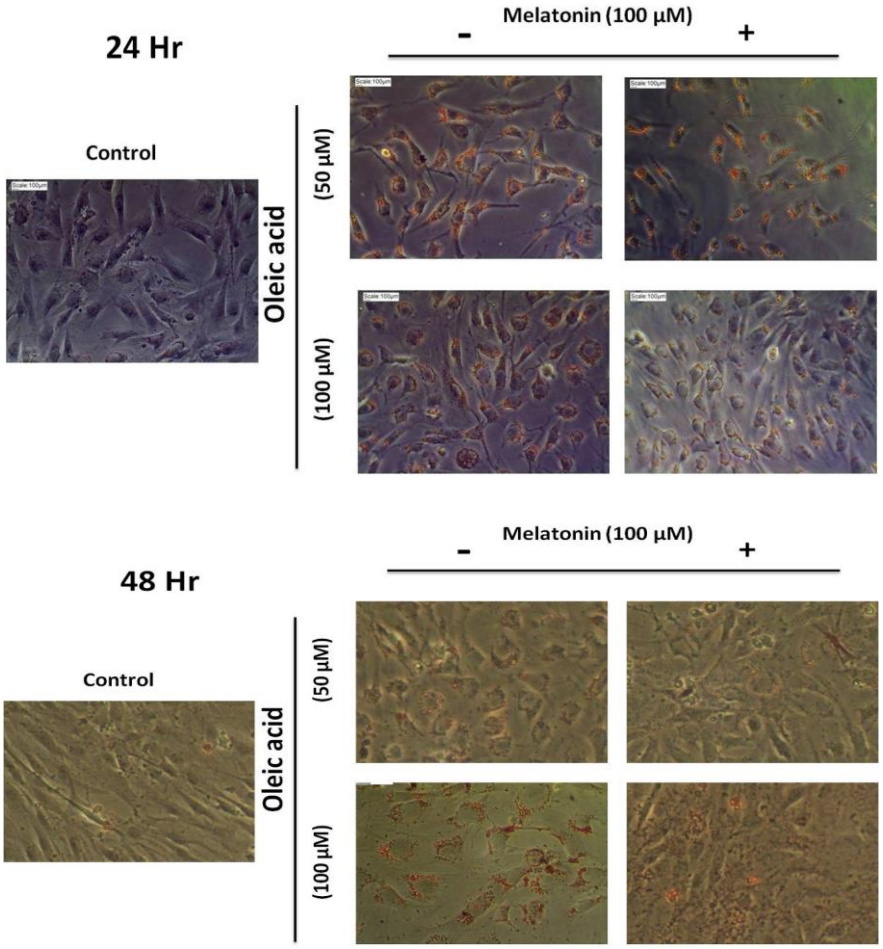


Fig. 3: Effect of melatonin on oleic acid uptake (ORO staining) at 24 h and 48 h. Representative images of the ORO stained images are shown. Magnification= 200X.

CONCLUSION

It can be concluded from the present study that oleic acid treatment manifests NAFLD like events in Hepa 1-6 cells that are characterized by oxidative stress and cell death due to intracellular oleic acid accumulation. Melatonin co-supplementation improves cell viability and lowers intracellular oxidative stress by reducing oleic acid uptake. These findings provide baseline evidence on anti-NAFLD potential of melatonin by preventing the severity of lipotoxic manifestations that needs further scrutiny.

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