"Rapid Eye Movement sleep deprivation of rat generates ROS in the hepatocytes and make them more susceptible to oxidative stress"

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Short running title: REM sleep deprivation induces oxidative stress in liver.

Key Words: REM sleep deprivation, ROS in hepatocytes, Nitric Oxide, Nitric oxide synthase, Inflammation in liver.

Abbreviations

REM: rapid eye movement, NREM: non rapid eye movement, REMSD: rapid eye movement sleep deprivation, CC: cage control, LPC: large platform control, EDTA: Ethylene diamine tetra acetic acid, RIPA: Radio immuno precipitation assay, FITC: Fluorescein isothiocyanate, ROS: Reactive oxygen species, NO: Nitric Oxide, DCF-DA, 2,7 Dichlorodihydrofluorescein diacetate, XO: xanthine oxidase X/XO, Xanthine/xanthine oxidase,

Highlights of the study:

- Significant increase in the level of ROS was observed in the hepatocytes of REMSD rats.
- The hepatocytes were found to be more susceptible to the oxidative stress in response to REMSD.
- Nitric oxide levels in the hepatocytes of REMSD rats increased in comparison to control rats which got normalized when sleep deprived rats were allowed to have 5 day of sleep recovery.
- The iNOS gene and protein were also found to be over expressed in response to REMSD as confirmed by real-time PCR and western blot analysis.

Abstract

Background: Rapid Eye Movement sleep deprivation (REMSD) of rats causes inflammation of the liver, apoptotic cell death of the neurons and acute phase response to hepatocytes. Studies suggest that REMSD also facilitates the muscle and cardiac injury while same time causes depression like situation.

Objective and methods: The aim of this research was to determine whether REMSD would create reactive oxygen species (ROS) and oxidative stress in hepatocytes of rats and how stress generated due to REMSD would make the hepatocytes susceptible to oxidative stress? We selectively deprived the rats for REMSD using established flower pot method.

Results: We observed that with increase in the days of deprivation, the levels of ROS in the hepatocytes increased and it became normal when the rats were allowed to have recovery sleep for 5 days. Analysis of gene expression profile by real time PCR at different time of REMSD and the level of proteins by western blot analysis showed that there was increase in the expression of inducible nitric oxide synthase (iNOS) gene and the nitric oxide synthase protein in the hepatocytes. The level of nitric oxide (NO) also increased in response to REMSD compared to control group of rats.

Discussion: We have analyzed here the events that takes place in the hepatocytes of rats after REMSD. Since, REM sleep is important for memory consolidation, synaptic plasticity and survival in most of the animals reported so far. Findings of our study are important as it shows how ensuing loss of REM sleep can affect the hepatocytes in liver and therefore ultimately affecting the body physiology and natural well-being of individuals.

Introduction

Sleep is a natural physiological process which is needed for the survival of living beings studied with few exceptions [1]. In mammals, it is broadly categorized as two types, namely non-rapid eye movement sleep (NREM) and rapid eye movement sleep (REM). Evolutionarily REM sleep is present in mammals and birds with certain essential functions related to physiological and ecological success. In general, it is associated with memory consolidation [2,3], brain maturation, spatial memory acquisition [4] and maintenance of body physiology [5]. Its prolonged loss can alter blood brain barrier functions [6] and can be fatal [7,8]. In terms of energy metabolism, it's as high in wakefulness as in REM sleep, which represents approximately 1/3rd of total sleep [9] Moreover, extended sleep deprivation in animal models is categorized with decreased cerebral glucose utilization compared to expected increased utilization [10]. This theory also justifies the earlier and marked rebound of REM sleep rather than NREM sleep after total sleep deprivation.

Most of studies on REMSD have focused on the biochemical, molecular and anatomical changes taking place in the brain and neurons but very little are known about what happens in other vital organs in parallel. Recent studies have also shown that REMSD can cause apoptosis in the brain [11,12], increased biomarkers of muscle and cardiac injuries [13] and affects newborn's sleep [14]. But, some questions remains unanswered like whether total or partial sleep deprivation can cause damage to only in brain or same time it has certain effects on other body organs or tissue? To answer these questions, we extended our study to liver, which is similar to brain are very important for general physiology and survival. Liver being the metabolic hub contributes significantly to the maintenance of body physiology [15]. After initial screening, we got interested to know how REMSD will affect the oxidative state and well-being of liver. Further, we selected the hepatocytes for detailed analysis to understand

how REMSD affects them as they are known to be involved in metabolism, detoxifying endo- and xenobiotics and synthesis of many proteins like albumin [16–18].

Reactive oxygen species (ROS) is produced as a byproduct of metabolic processes in day-to-day activities for which body has a natural system to maintain the balance using anti-oxidants present in system or generated from dietary sources. The uncommon production of ROS as a byproduct of uncontrolled metabolic processes, diseases or stress leads to a state where maintenance and recovery to normal physiological levels is not possible. This ultimately leads to an inflammation like situation in the system causing disease conditions or cell-death. Several experiments performed for testing the relationship between sleep and oxidative stress has `primarily focused on total sleep deprivation studies. These studies earlier reported that lipid peroxidation levels were increased in the hippocampus of rodents in response to ROS level that have been sleep deprived [19]. Antioxidant capacity was found decreased in peripheral tissues of animals that were deprived of sleep for 5 and 10 days as shown by study [20]; whereas the deleterious parameters were restored after rebound recovery sleep. Overall, the scenario of oxidative stress and sleep deprivation remains inconclusive under the conflicting data collected from past studies. As, few studies reports no evidence of oxidative damage either in brain or peripheral tissues including liver after either short term (8hrs) or long term (3-14 days) of total sleep loss. But at the same time, there were reports of increased superoxide dismutase (SOD) activity in rats which were allowed to recover after total sleep loss (3-14days) in recovery group of rats compared to controls [21]. No increase was reported in another study for antioxidant enzymes like SOD, catalase, glutathione peroxidase or malondialdehyde activity subsequent to sleep deprivation in rats [22]. Furthermore, other reports suggest that sleep deprivation create stress like conditions coupled with oxidative stress with decreased levels of glutathione in whole brain [23] and reduced SOD activity in the hippocampus and brain stem [24]. Fascinatingly enough, studies have also revealed that sleep deprivation itself can cause cellular

stress, a condition that has been associated with the interruption of various physiological processes [25]. Prolonged wakefulness/sleep deprivation activates an adaptive stress pathway termed the unfolded protein response, which momentarily guards against the injurious consequences of ROS [26]. Apparently, recent reports suggest a sharp increase in oxidative and lipid peroxidation in the hepatocytes of sleep deprived rats [27]. To explore further, we inquired here about whether REMSD would generate ROS in hepatocytes of rats and how REM sleep deprived hepatocytes will response to oxidative stress?

Here, we present the data of REMSD affecting the hepatocytes for the increase of ROS and inducible nitric oxide synthase (iNOS) expression in rats and same time REMSD makes them more susceptible to oxidative stress. Our previous study had also shown that REMSD induced acute phase response in the liver, increasing the synthesis of pro-inflammatory cytokines like Interleukin (IL)1β, IL-6 and IL-12 and increasing the levels of liver toxicity marker enzymes, alanine transaminase and aspartic transaminase which circulates in the blood [28]. Our current results provide suggestive evidence that when rats were subjectively deprived of REM sleep, it positively affected the increase of ROS levels in hepatocytes along with increased production and circulation of nitric oxide (NO). The levels of iNOS genes and proteins were also found to be induced in response to REMSD suggesting increased iNOS gene expression and thus same time affecting the nitric oxide production in hepatocytes which in turn getting circulated in system and is responsible for inflammatory conditions and stress being caused.

Materials and Methods

Male wistar rats weighing between 220-260 gm were used in this study. Animals were housed in the institutional animal house facility with a 12:12 hr L: D cycle (lights on at 7.00 am). Food and water were provided *ad libtium.* All experiments were conducted as per the protocol approved by the University's Institutional Animal Ethics Committee.

Methods used for REM sleep deprivation and recovery

Animals were REM sleep deprived by the most widely used flower-pot method [29,30]. In this method, animals were kept on a small raised platform (6.5 cm diameter) surrounded by water compared to large platform control (LPC) rats which were maintained at 12.5 cm diameter pot and cage control (CC) rats, maintained in cages at laboratory condition. Although the animals could sit, crouch and have non-REM sleep on this island, but due to the postural muscle atonia during REM sleep, they were unable to maintain their extended relaxed body posture on the small platform and tended to fall into the surrounding water. As a result they wake up at the onset or prior to the appearance of REM sleep and thus deprived of it. After the days of deprivation the recovery groups of rats were allowed to sleep uninterrupted in individual cages with relentless supply of food and water. The rats were sacrificed on different days (4 & 9 days) of REMSD and after 5 days of REMSD recovery and hepatocytes were prepared for further analysis.

Hepatocytes preparation

Hepatocytes were isolated from the liver of male wistar rats from CC, LPC and REM sleep deprived groups, by method used by Liu [31]. Briefly, the abdomens of the rats were opened through a midline incision and liver was taken out. The portal cannula was then placed and liver was perfused with 0.02% EDTA solution at 37 degree C, at a flow rate of 30 ml per minutes for 15 minutes in a culture dish. Subsequently, the collagenase solution (37degree C) was recirculated through the liver at the same flow rate for 15 minutes. After perfusion the liver capsules were disrupted and the digested liver parenchyma was suspended in the ice-cold Hank's balance salt solution. The resulting cell suspension was washed by centrifuging at 500rpm for 5 min 2-3 times and further centrifuged over 30% percoll at 100g for 5 min to

increase the purity. Viability at the time of labeling as measured by trypan blue exclusion was < 95%.

ROS measurement and treatment dose optimization for creating oxidative stress:

ROS level was quantified in hepatocytes from controls and REMSD group of rats after labeling with 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), which is a cell-permeable dye that becomes fluorescent upon reaction with ROS such as hydroxyl radical, hydrogen peroxide, or peroxynitrite. Briefly, hepatocytes cells from different groups of experiments were loaded with 10 µM DCF-DA for 30 min followed by either ROS measurement or treatment with different doses of H_2O_2 (50-500µM), Xanthine/Xanthine oxidase (X/XO; 10-100mU/ml) for induction and then ROS measurements depending upon experimental design. Whenever, specific inhibitors such as catalase (100-500U/ml) and oxypurinol (10-50mU/ml) used they were incubated firstly with hepatocytes followed by DCF-DA incubation and then induction with H_2O_2 and X/XO for creating the oxidative stress in-vitro following the protocol described for mouse osteoblasts and Human MG63 cells and optimized the suitable dose for our further experiments [32]. The cells were harvested at the indicated time points after incubation, washed three times with phosphate-buffered saline, and then immediately analyzed by flow cytometer with a 488-nm excitation beam. The signals were obtained using a 530-nm band pass filter (FL-1 channel) for DCF. Each determination is based on the mean fluorescence intensity of 5,000 cells.

Assay for hepatocellular NO production:

Hepatocytes were seeded in 24-well, flat-bottom plates (Falcon) at 1 x 10^5 cells/well and incubated at 37°C in humidified 5% CO_2 conditions with and without Lipopolysaccharides. NO was measured indirectly by determination of the concentration of the stable end product, nitrite. Briefly, 100-µl aliquots were removed from hepatocytes cultures and incubated with an

equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylene diamide dihydrochloride in 2.5% phosphoric acid) for 10 min at 37°C. Absolute values were determined using sodium nitrite as standard. Absorbance was read at 550 nm on a microplate reader.

Isolation of RNA and TaqMan Real-time PCR:

RNA was prepared from hepatocytes harvested and stored in RNA later (Sigma, R0901) of CC, LPC and REM sleep deprived groups using RNA purification kit (Qiagen). RNA concentrations and integrity was assessed by Nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). RNA prepared from liver tissue was converted to cDNA using the reverse transcription PCR kit (applied Biosystems). GADPH served as a housekeeping gene while cage control group served as calibrator Probes (Reporter dye FAM labeled on 5' end and quencher VIC labeled on 3' end), PCR master mix, and PCR plate/tubes were obtained from Applied Biosystems and the manufacturer's instruction were followed. The catalogue numbers of gene probes were glyceraldehyde 3 phosphate dehydrogenase *(*GAPDH, Rn01749022_g1), iNOS (Rn02132634_s1) and master mix (Rn99999916-g1).

Western Blot Analysis:

Western blotting was performed as previously described [33]. Primary antibody used were in 1/1000 dilutions (Santacruz Biotechnology, Inc, USA), whereas secondary antibody which is a horseradish peroxidase-conjugated were used at 1/5000 dilutions (Santacruz Biotechnology, Inc, USA). Membranes were developed using enhanced chemiluminescence (ECL) reagent (Promega) and photographed using software (Photo and Imaging 2.0; Hewlett-Packard). The analysis of the images was performed with imaging software (Photoshop 8.0; Adobe).

Statistical Analysis:

The statistical analysis were done using parametric one-way ANOVA test followed by Tukey posthoc test for measures effects across treatment groups. All the graphs were made using Sigma plot (Version 8.02 &12.0), while statistical analyses were carried out using SPSS (Version 2.7.2). The p values over 0.05 were considered significant.

RESULTS:

In-vitro dose optimization results for induction and suppression of ROS

We optimized this in order to have a system of in-vitro ROS induction and suppression which were used further to check the susceptibility of the hepatocytes in response to REMSD from different treatment groups towards created oxidative stresses. Firstly, we treated the normal hepatocytes with different amount of H_2O_2 & X/XO in order to generate superoxide anion. Intracellular ROS level increased in a dose dependent manner with treatments of H_2O_2 (50–500 µM, One way ANOVA, F=354.91, df=4, p<0.001) and X/XO (10–100 mU/ml, One way ANOVA, F=374.83, df=4, p<0.001) for 60 minutes (Fig. 1a &b). We selected the dose of 200µM for H_2O_2 and 20 mU/ml for X/XO for the treatments and induction of oxidative stress based on above experiments (Fig.1a &b). Now, further when we treated the normal hepatocytes using respective concentrations of 200µM-H_2O_2 and 20 mU/ml-X/XO for different time points ranging from 20-120 minutes. We observed that the time point 60 minutes is quite effective and sufficient for induction of superoxide anions (Fig 1c, One way ANOVA, F=410.21, df=4, p<0.001 & Fig.1d, One way ANOVA, F=864.77, df=4, p<0.001). Furthermore, to establish the effective concentrations of the scavengers of the ROS, we firstly treated the normal hepatocytes with respective doses of catalase (100-500U/ml) and oxypurinol (10-50µM) followed by 200µM-H_2O_2 and 20 mU/ml-X/XO treatments. The doses of catalase (500U/ml) and oxypurinol (50µM) were found quite effective (Fig 1e, One way ANOVA,

F=135.71,df=3,p<0.001& Fig.1f, One way ANOVA, F=128.36,df=3,p<0.001) against treatments of H_2O_2 (200µM) and X/XO (20 mU/ml) for 60 minutes and were used for further experiments. These results indicate that we selectively succeeded in inducing the ROS levels after H_2O_2 (200µM) and X/XO (20 mU/ml) treatments and catalase (500U/ml) and oxypurinol (50µM) were able to quench the induced levels of ROS produced due to treatment induction in normal hepatocytes.

REM sleep deprivation increased the ROS production in hepatocytes

ROS production increased significantly with increase in the magnitude of REM sleep loss. We observed 111.15±12.5% increase in production of ROS on day 4 compared to CC group, whereas, on day 9 it became 265.22±10.1%. The LPC group of rats showed comparable levels of ROS against CC group of rats ruling out the procedure related effects. Complete sleep recovery of 5 days showed the decreased levels of ROS (36.36±5.1%) in hepatocytes compare to controls indicating the redressal effects (Fig. 2a, One way ANOVA F=216.92,df=27,p<0.001). Same time, when we exposed the hepatocytes collected from different treatment group of rats with ROS quenchers (500U/ml catalase and 50µM-oxypurinol) which are known and established to reduce the oxidative stresses, in both the treatments we got reduced levels (almost comparable to controls) of ROS in hepatocytes in REM sleep deprived rats on day 4 & 9 (Fig.2a). This further indicates the presence of ROS due to REMSD in hepatocytes which were got cleared due to treatment by catalase and oxypurinol.

Sleep loss increased the susceptibility of hepatocytes for oxidative stress

REM sleep loss not only generated the ROS in the hepatocytes of rats but also made them more susceptible to oxidative challenge We report here the increased production of ROS for REMSD hepatocytes on day 4 (135.50±13.5%) and day 9 (257.1±15.75%) compared to controls, when hepatocytes were exposed to 200µM-H_2O_2 for 60 minutes (Fig.2b, One way ANOVA

F=216.67, df=11, p<0.001 & Fig.2c, One way ANOVA F=442.44, df=15,p<0.001). The levels of ROS became normal when similar hepatocytes were treated with 500U/ml catalase (Fig. 2b &c). Similarly, when hepatocytes from controls and REMSD groups were treated with 20 mU/ml-X/XO and X/XO+Oxypurinol on day 4, 9 and after 5DR. The hepatocytes shown induced ROS levels on day 4 (75.1±6.5%), day 9 (130.5±5.5%) due to X/XO treatments for REMSD cells compared to controls, while, 20 mU/ml-X/XO+50µM-Oxypurinol treatment reduced the ROS levels comparable to controls (Fig.2b&c). The hepatocytes collected from 5DR rats responded normally and where found comparable to controls with both of the treatments (Fig. 2c). The overall results suggest that the REMSD causes hepatocytes to respond differently to oxidative damages thus makes them more susceptible to stress.

Nitic oxide synthase expression and circulation got induced due to REM sleep loss

The iNOS gene expression also got affected against REMSD when measured in hepatocytes of such rats. We report here the log 10 fold change in increased expression of iNOS gene on day 4 (0.6±0.08) compared to CC control, while expression on day 9 further increased 1.5±0.15 fold. After 5days of recovery the expression pattern improved towards normal level but still were found increased (0.71±0.05 fold) compared to control (Fig.3a, One way ANOVA F=357.99, df=6, p<0.001). We also measured for iNOS protein levels using western blot for similar treatments. Our reports suggest that iNOS protein level also got increased 95.1±1.5% on day 4th, which further got increased 162.5±1.25% on day 9th compared to CC group of rats after REMSD (Fig.3 b & c, One way ANOVA F=393.94, df=4, p<0.001). The levels of iNOS protein returned towards normal levels after 5days of recovery sleep (42.5±1.1%), but still found to be circulating higher compared to controls (Fig.3 b & c).

REM sleep loss affected the nitic oxide production in hepatocytes

Further to explore the effects of the iNOS being produced due to REM sleep loss, we collected the hepatocytes from different treatment groups of rats and were measured for their respective NO levels in course of REM sleep deprivation. Our results suggest that, NO levels were significantly increased on day 4 (285±5%) and day 9 (675±11%) compared to cage control. Further, 5 days of sleep recovery shown redressal effects in terms of NO production but overall NO level were still found increased (135±12%) compared to control (Fig. 4, One way ANOVA $F=170.48$, $df=11$, $p<0.001$).

Discussions

We subjected the rats to REMSD using flower-pot method and similarly created the LPC groups and CC groups for effect comparison. We assessed the ROS level measuring out the DCF florescence level using FACS and compared the across groups. We collected the hepatocytes and treated with enzymes known and standardized with us for ROS production induction and suppression. Lastly, we measure the expression levels of iNOS gene & protein as well as NO in hepatocytes in function with REMSD. Our findings suggest that REMSD can induce ROS level in the hepatocytes of rats which ultimately undergo for apoptotic cell death (our unpublished data). Our treatments with H_2O_2 and X/XO selectively induced the ROS levels inside the hepatocytes and same time catalase and oxypurinol treatments succeeded in quenching out the ROS produced due to treatments, which also suggested the presence of super oxides due to treatments or REMSD. This gave us a powerful tool to challenge the hepatocytes from control and REMSD groups and investigate how REMSD will affect their susceptibility to future oxidative stresses? The hepatocytes from REM sleep deprived rats responded poorly to the oxidative stress created indicating the negative effects rendered in hepatocytes due to REMSD. As noted above, the hepatocytes from REMSD group of rats responded more

positively against H_2O_2 and X/XO treatments indicating the REM sleep loss stress induced potentiation of hepatocytes. The 5 days sleep recovery helped the cells to de-stress out its oxidative burden and thus they responded normally to the above treatments. This can be explained as, REMSD might have weakened out the natural cell response by creating more oxidative stress. The stress response got alleviated to certain extent when rats were allowed to have recovery sleep. Other studies also indicate the increased oxidative stress in brain cells of rats after REMSD [21,34], reviewed in for animal models [35]. Conditions like obstructive sleep apnea in humans and rodents were also found to be positively correlated with increased ROS in the system [36,37], while Parkinson disease associated with REM sleep loss were also found to contribute to the colon oxidative stress and mitochondrial function [38]. Sleep deprivation was further found to be associated with anxiety like behavior in rats which can be reduced or prevented by administration of melatonin, possibly by reducing the oxidative stress and maintain the balance between GABAergic and glutamatergic transmission [39]. Administrations of melatonin were found to reduce oxidative stress in rats by blocking the down-regulation of γ-aminobutyric acid A-alpha-2 receptors. These studies suggest that sleep deprivation is somehow directly or indirectly associated with the ROS levels in different tissues studied so far. In our previous study, we had also reported that REMSD can create inflammation in liver by increasing the levels of cytokines such as IL-6, IL-12, IL-1β [13] indicating that cytokines might be involved in induction of iNOS genes. Results published earlier also suggest that cytokines like tumor necrosis factor –α (TNF- α), IL-1β, and Interferon gamma (INF-γ) synergistically activate iNOS gene expression in the liver [35]**.**

Lastly, our results suggest that hepatocytes form REMSD group of rats showed increased NO production which got reduced after sleep recovery. Previous studies claim that NO exerts a protective effect both *in vivo* and *in vitro* by blocking TNF-α induced apoptosis and hepatotoxicity, by inhibiting the caspase-3-like protease activity [40]**.** Thus, our unpublished

data showing that REMSD can cause apoptosis like situation in hepatocytes and increased levels of NO in response to REMSD indicates and suggest that excess of NO were being produced due to sleep loss to counteract the oxidative stress generated due to sleep loss and protect the hepatocytes from apoptosis like processes. Our study is unique in testing the oxidative response as a function of REMSD with optimized enzyme treatment protocols.

This study further justifies and strengthen the evolutionary idea of REM sleep manifesting the role of maintenance and survival.

Conclusions

Our results demonstrate that the REM sleep is very important for individual well-being and its loss can induce the hepatocytes for ROS production and further make them more susceptible to oxidative stress and finally can cause apoptotic cell death (Our unpublished report). Apart from this, REMSD also can induce the iNOS circulation in the system inducing NO production, which might be directly or indirectly is responsible for hepatocytes welfare.

Figures & Figure legends:

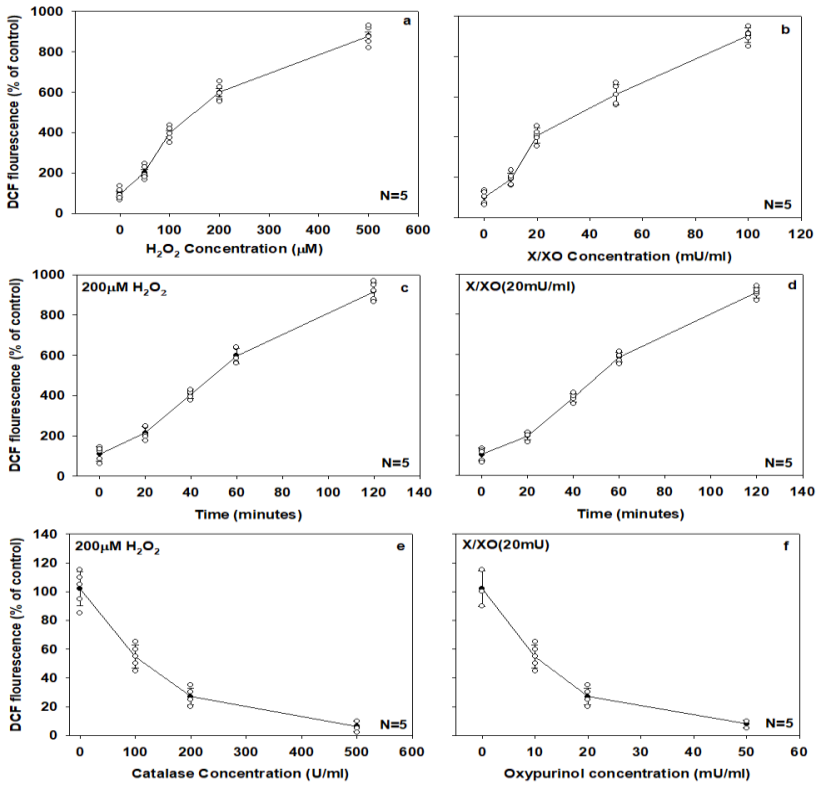

Figure 1: Optimization of ROS level induction and suppression in normal rat hepatocytes by H_2O_2, X/XO, catalase and oxypurinol. Hepatocytes, isolated from rat were loaded with 10 µM 2, 7-DCF-DA for 30 min and then treated with (a), 50–500 µM H_2O_2 for 1 h; (b), 10–100 µM xanthine and 10–100 milliunits/ml XXO for 1 h; (c), 200 µM H_2O_2 for 20–120 min; (d), 50 µM xanthine and 50 milliunits/ml XXO for 20–120 min;

(e), the hepatocytes were treated with different amount of catalase (100-500U/ml) after 200µM H_2O_2 induction for an hour and (f), scavenging of ROS production induced by 20mU X/XO for an hour using oxypurinol (10-50 mU/ml). ROS levels were then determined by FAC calibur as described under "Experimental Procedures." Each point represents the mean ±S.E. of eight determinations from five (N=5) different cell samples. Each determination is the mean DCF fluorescence intensity of 5,000 cells.

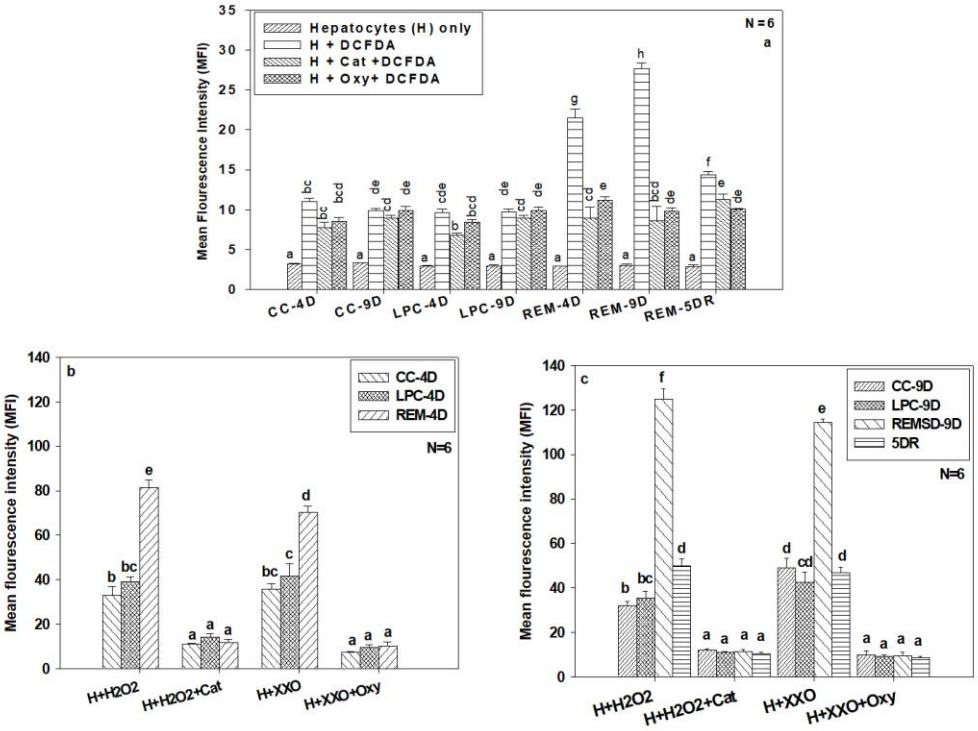

Figure 2: Effect of REM sleep deprivation on ROS production in hepatocytes. Measurement of ROS in hepatocytes from cage control, large platform control, REM sleep deprived group and recovery group of rats. (a), Measurement of ROS in different control groups and REM sleep deprived group of rats treated with scavengers like catalase and oxypurinol after 4 day, 9 days of REM sleep deprivation and 5day of sleep recovery. Differential productions of ROS in progression with REM sleep deprivation compared with control and simultaneously treated with catalase and oxypurinol after 4day of sleep deprivation (b), and 9 day of REMSD and 5day of sleep recovery after 9days of REMSD (c), similarly treated with catalase and oxypurinol. Each vertical bar represents the mean of 6 individual samples with standard error. Treatments that don't share a letter, are statistically different in Tukey post-hoc analysis followed by one way ANOVA across treatment groups.

[CC=Cage control, LPC=Large platform control, REM= Rapid eye movement sleep deprivation. 4D- after 4 days from start of experiment, 9D- after 9 days from start of experiment, 5DR- After 5 days of REM sleep recovery i.e. rats were allowed to sleep in cages for 5 days]

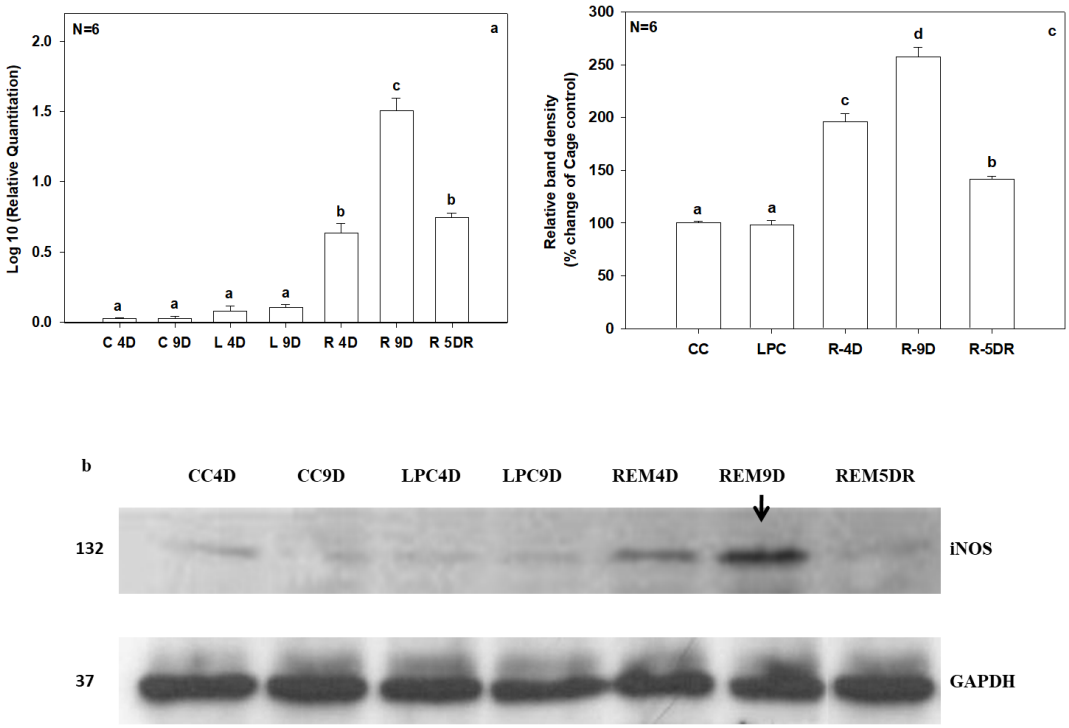

Figure 3: Measurement of iNOS gene and protein levels in response to REMSD in hepatocytes. (a), The graph shows log fold change in expression pattern of iNOS gene, cage control samples were taken as calibrator while GAPDH was endogenous control for respective genes. (b), Analysis of hepatocytes iNOS protein using WB from CC, LPC and REM sleep deprived and recovery group rats. (c), Densitometric analysis of WB samples for different treatment groups using Image-J software, NIH. Data were represented as relative band density reflected as percentage change of control. Refer figure 2 for statistical and legend details as P value < 0.05 were considered as statistically significant.

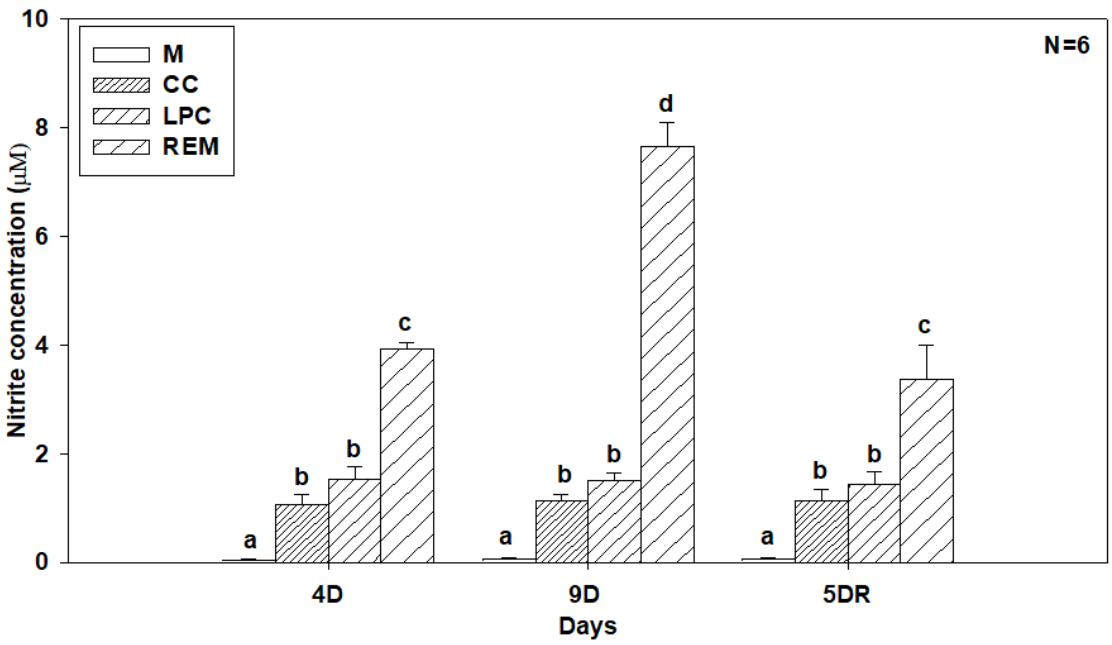

Figure 4: Measurement of NO level in hepatocytes in response to REM sleep loss and sleep recovery. Rats were REM sleep deprived of 4 days and 9 days before recovery of 5 days. The hepatocytes were collected from different time points and cultured for nitrite measurement. The X-axis represents the days of REM sleep deprivation and recovery and Y-axis represents the nitrite concentration (µM) in response to REM sleep deprivation. The Kit (Griess reagent kit, Sigma) was used to measure out the levels of nitrite from cultured hepatocytes. [M=Media only, refer figure 2 for statistical details and other legend abbreviation].

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