"Rapid eye movement sleep deprivation causes apoptotic cell-death of the hepatocytes in rat"

Atul Pandey[1, 2], Devesh Kumar[1, 3], Gopesh Ray[1, 4], Santosh Kar[1, 4*]

[1]School of Biotechnology, Jawaharlal Nehru University, New Delhi-110067, India

Present addresses:

[2] Department of Ecology, Evolution, and Behavior, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, 91904, Israel

[3] University Hospital Brussels, Brussels, Belgium

[4] School of Biotechnology, KIIT University, Bhubaneswar-751024, Odisha, India

***Corresponding Author**: santoshkariis@rediffmail.com, Ph: +91-9937085111

Short running title: REM sleep deprivation causes liver cell death.

Key Words: REM sleep deprivation, Apoptosis, cell-death, Hepatocytes, Sleep recovery, rat.

Abbreviations: REM; rapid eye movement, NREM; non rapid eye movement, REMSD; rapid eye movement sleep deprivation, CC; cage control, LPC; large platform control, TUNEL; Terminal deoxynucleotidyl transferase dUTP nick end labeling, EDTA; Ethylene diamine tetra acetic acid, RIPA; Radio immuno precipitation assay, FITC; Fluorescein isothiocyanate, TDT; Terminal deoxynucleotidyl transferase, FAM; 6-carboxyfluorescein, ROS: Reactive oxygen species, NO: Nitric Oxide.

ABSTRACT

Rapid eye movement sleep deprivation (REMSD) of rats are associated with neuronal cell loss, oxidative damage and neurodegenerative diseases. Assuming the noxious role of REMSD, we examined its effect on liver, which controls the most of the metabolic processes of the body and thus are very much needed for survival. Here, we report REMSD induced apoptotic cell death in hepatocyte, which were got alleviated up to certain extent after relentless sleep recovery of 5 days. Further investigation revealed the involvement of mitochondrial gene/protein cascade in the apoptotic process. Our current study shows that REMSD can causes the apoptosis in hepatocytes of rats involving the well evolutionary conserved mitochondrial pathways. Our results further signifies the functional and preventive role of REM sleep which is unique to mammals and avians with certain exceptions, as its loss can negatively affect the natural well-being and survival of individuals.

INTRODUCTION

Sleep is an important evolutionary physiological and behavioral process required for the survival and well-being of animals studied, although no predominant hypothesis has emerged to explain its functions [1,2]. In mammals, it is broadly categorized as two types, namely non rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. Evolutionary REM sleep is present in mammals and birds with essential functions related to physiological and ecological success. REM sleep, also called as paradoxical sleep in general is associated with memory consolidation, brain maturation, spatial memory acquisition, muscle regeneration and maintenance of body physiology [3–9]. Functional aspect of REM sleep can be seen as it affecting amygdala activity in response to previous emotional experiences, reorganizing the hippocampal

excitability, pruning and maintenance of new synapses in development and learning [10–12]. Its prolonged loss can alter blood brain barrier functions and can be fatal [13–15]. Some recent studies have shown that REMSD can cause apoptosis in rat's brain [16–18]. Our previous study had shown that REMSD induces acute phase response in the liver, increasing the synthesis of pro-inflammatory cytokines like IL1β, IL-6 and IL-12 and increasing the levels of liver toxicity marker enzymes, alanine transaminase (ALT) and aspartic transaminase (AST) which circulates in the blood [19]. Similarly, we have also shown that REMSD can induce the ROS level and NO production in the hepatocytes along with making them more susceptible to oxidative stress (Communicated for publication). Recent, reports also suggest the effect of REMSD on the new born baby's sleep [20]. Liver being the metabolic hub contributes significantly to the maintenance of body physiology and well-being. It synthesizes complement proteins and houses Kupffer cells and natural killer (NK) cells, which are important components of the innate immune system. Based on previous literature, we hypothesized that REMSD will might affect the liver? As it controls the most of the physiological processes in body. Furthermore, we selected the hepatocytes for 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.

Apoptosis, a highly regulated form of cell death with multiple check points and molecular mediators, can be initiated in hepatocytes via the death receptors mediated extrinsic pathway, or by cellular perturbations that constitute the intrinsic pathway [21]. Unlike other cells, in hepatocytes, both the pathways converge on mitochondria. Mitochondrial permeabilization is just sufficient to induce apoptosis of hepatocytes [22,23]. Generally, apoptosis can aggravate tissue injury, inflammation and fibrosis

[24–26]. Apoptosis of hepatocytes were also evident in viral hepatitis, metabolic diseases, alcoholic steatohepatitis, autoimmune hepatitis and drug induced liver injury which links liver injury to death of hepatocytes [27–31].

In current study, we observed that substantial REMSD in rats induced apoptosis of hepatocytes by 4th & 9th day as indicated by DNA laddering, Annexin V labeling and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. Further, real time PCR and western blot analysis revealed the downregulation & upregulation of anti-apoptotic and pro-apoptotic genes like Bcl2, Bax, Caspase 3 and p53 in the hepatocytes of REMSD rats. Furthermore, Sleep recovery affected positively for all parameters studied in REMSD group of rats indicating redressal effects of sleep recovery. Our results demonstrate that the REM sleep is very important for individual well-being and its loss can induce the hepatocytes cell death. This indicates the protective and adaptive evolutionary role of the REM sleep for the maintenance and survival of the organisms it present.

MATERIALS AND METHODS

All the male Wistar rats weight 220-260gms when used for study. During all the studies rats were kept in the institutional animal house facility of the University with a 12:12hrs L:D cycle (lights on at 7.00 am). For all experimental groups and treatments food and water were supplied *ad libtium.* We were got approval from University's Institutional Animal Ethics Committee for all the protocols and experiments conducted.

Methods used for REM sleep deprivation and recovery:

We used classical flower-pot method for depriving the REM sleep in rats [32,33]. For REMSD rats were kept on a small raised platform (6.5cm diameter) surrounded by

water compared to large platform control (LPC) rats, which were maintained on platform of 12.5cm diameter. Similarly, the cage control (CC) rats which remained in cages throughout the experiments. Due to lack of enough space rats only 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. This happens to wake up REMSD group of rats at the onset or prior to the appearance of REM sleep and thus ultimately deprived of it. After the intended days of deprivation the rats were allowed to sleep uninterrupted in cages individually (to reduce aggression behavior) with relentless supply of food and water. The rats were collected on different days (4 & 9 days) of REMSD and after 5 days of REMSD recovery and samples were prepared for further analysis.

Histopathological examination:

Liver sections were prepared from different group and stained with Hematoxylin & eosin (H&E) [34] and further comparatively examined taking into CC sections as reference. Inflammatory cells, necrotic cells and apoptotic cells were marked and labeled appropriately.

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 [35]. 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%.

Annexin V labeling of hepatocytes for detection of Apoptosis:

Annexin V assay was performed using the Flow-TACS kit (4835-01-K) from R&D systems. After labeling sample were analyzed by flow cytometer (BD FACS Calibur) within one hour. Annexin V^+ and PI^- cells were considered as early apoptotic, Annexin $V^+$and PI^+ cells were considered as late apoptotic whereas Annexin V^- & PI^+ cells were considered to be necrotic.

TUNEL labeling of hepatocytes DNA for detection of Apoptosis:

TUNEL assay was performed following instructions from Flow-TACS kit (4817-60-K) from R&D systems. Samples were analyzed by flow cytometer within one hour. $TUNEL^+$ and PI^- cells were considered as early apoptotic, TUNEL $^+$and PI^+ cells were considered as late apoptotic whereas TUNEL $^-$ & PI^+ cells were considered to be necrotic.

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 GAPDH (Rn01749022_g1), Bcl2 (Rn99999125_m1), Bax (Rn02532082_g1), p53 (Rn00755717_m1), Caspase 3 (Rn01488068_g1) and master mix (Rn99999916-g1).

Western Blot Analysis:

Western blotting was performed as previously described [36]. 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 ECL reagent 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 statistical analyses were carried out using Sigma plot (Version 8.02 &12.0) and SPSS (Version 2.7.2), while p values over 0.05 were considered significant.

RESULT & DISCUSSION

There are inconsistent reports about sleep loss (Total and selective) and cellular apoptotic responses. No evidence of brain-cell degeneration following total sleep deprivation in rats were reported [37,38]. However, in few recent studies apoptotic neuronal cell degeneration were recorded against sleep deprivation [16,39–42]. To investigate further the effect of REM sleep loss in liver. We firstly examined the liver sections of hematoxylin/eosin (H&E) staining after REMSD and after 5 days of recovery (5DR) along with control groups. The Normal cage control group rats along with large platform control group rats showed normal histology on 4th day (SF1a & b) as well as on 9th day (SF1d & e). Whereas, REM sleep deprived group rats sections on 4th day (SF1c) showed mild lymphocytic infiltration, dilated central vein and parenchymatous cells injury while sections on *9th day* (SF1f) showed hepatic degeneration, lymphocytic infiltration and mild apoptotic cells along with some necrotic cells. Rats with 5*DR* (SF1g) showed mild lymphocytic infiltration along with normal histology indicates the redressal effect of sleep recovery.

We hypothesized and tested here based on our previous report (In correction, Sleep science) that ROS generated due to stress exerted by REMSD can induced cell death like situation in hepatocytes. Furthermore, Annexin V and TUNEL labeling of hepatocytes were done to estimate the cell death involving apoptosis. Our finding suggest that in contrast to the control groups (CC and LPC), there were significant number of Annexin V positive hepatocytes present in the REMSD group by 4th day as well as by 9th day of sleep deprivation, while recovery of 5 days restored the situation by decreasing the number of Annexin V positive cells (Fig. 1A, One way ANOVA $F=84.13$, $df=6$, $p<0.001$). To further support our findings so far, we screened hepatocytes for TUNEL-positive nuclei after different days of REMSD and 5DR and

observed that there is significant change in labeling by 4th (10±2.75%, Fig 2 c & d) and 9th (20.54±2.02%, Fig 2 c & d) day of experiment in comparison to controls (Fig. 1B, One way ANOVA F=181.72, df=6, p<0.001). There were no significant apoptosis of hepatocytes in the LPC group of rats, which indicated that stress due to confinement is not contributing significantly to apoptosis of hepatocytes (Fig. 1B). TUNEL assay is most often used to identify apoptosis of cells [43]; however, recent reports have indicated that TUNEL can detect DNA fragmentation in necrotic tissues [44]. The non-specific staining in TUNEL assay was analyzed by using hepatocytes from CC and LPC groups of rats. Both the control groups showed small percentage of cells (<2%) being stained in TUNEL assay indicating that there was no major nonspecific staining.

Previous studies also suggest that even moderate levels of continuous stress can induce apoptosis in cultured hepatic cells [45–48]. Superoxide's produced due to oxidative stress can kill the hepatocytes by apoptosis involving the activation of caspases and JNK [49]. The phenomenon of apoptosis at cellular levels were highly correlated with iNOS induction and concomitant massive and sustained circulation of NO, which were strongly correlated with high ROS levels and can be observed by chromatin condensation and DNA laddering. Tumor suppressor p53 precedes DNA fragmentation in cells in response to NO generation [50]**.** Studies suggest that the cytokines like TNF-α, IL-1β, and INF-γ synergistically activate iNOS expression in the liver**.** 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 [51,52]**.**

Cellular susceptibility toward NO varies between different types of cells and tissues [53]. NO has been shown to cause accumulation of the nuclear phospho-protein p53 in RAW 264.7 cells [54]. There is suggestive evidence of the role of p53 in apoptotic pathway in response to DNA damage [50]. In our experimental condition expression

of p53 gene (Fig. 2A, One way ANOVA F=54.31, df=6, p<0.001) was found to be elevated in respect to controls, as analyzed by real time PCR assay. To investigate further when we performed the western blot analysis of hepatocytic proteins. To our expectations the level of p53 protein found increased due to REMSD. As expected further, the expression pattern of mRNA and proteins were observed toward normalcy after 5 days of unhindered sleep recovery (Fig.3A&B, One way ANOVA F=24.92, df=14, p<0.001). Accumulation of the tumor suppressor p53 in response to endogenously generated NO, as an early accompanying event of apoptosis [55]. Exposure of NO, generated from an NO donor or from overexpression of inducible-type NOS, results in p53 protein accumulation [56]. Earlier report suggests that in apoptotic condition, ROS production was observed along with increased synthesis and circulation of NO and p53 genes and further they were correlated with Bcl2 family proteins [57]. To explore further, we measured the expression of apoptotic genes and proteins in the hepatocytes of controls versus REMSD rats, analyzed by RT-PCR and WB. The expression pattern of Bcl2, Bax, Caspase 3 and P53 genes were measured and compared between CC, LPC and REMSD groups. The expression of Bcl2, an anti-apoptotic gene whose protein product binds to Bcl-x family of pro-apoptotic proteins such as Bax, PUMA, Noxa etc, preventing them to permeabilize the mitochondrial membrane, were found to be decreased significantly after 4th and 9th day of REMSD (Fig. 2B, One way ANOVA F=24.80, df=6, p<0.001). P53 gene which was reported to be involved in apoptosis regulation at early stage, which were found in our case over expressed and secreted (Fig.3A) might have induced the secretion of Bax gene (Fig. 2C, One way ANOVA F=23.89, df=6, p<0.001) and caspase 3 gene (Fig.4A, One way ANOVA F=79.52, df=6, p<0.001), which were supposed to be involved during the final stages of apoptosis [58,59]. WB analysis of proteins level

further supported our RT-PCR data for caspase 3 (Fig.4B&C, One way ANOVA $F=47.88$, $df=19$, $p<0.001$). Reports suggest that, increase in the level of p53, which also transcriptionally regulates Bax, also contributes the redistribution of Bax from the cytosol to mitochondria [60]. We observed the decreased levels of Bcl2 while increased levels of Bax proteins (Fig. 4A&B). The decreased Bcl2 and increased Bax used to alter the Bcl2/Bax protein ratio affecting mitochondrial membrane potential. Taken all together these events were reported to facilitate permeabilization of mitochondrial membrane and release of cytochrome C which activate caspases [61–63]. In our hand also, we observed higher circulation of cytochrome C, Cas 3 and Cas 9 (Fig.4B&C). Activated caspases (particularly Cas 3) induced apoptosis of the hepatocytes (Fig.1A&B). In a study reported earlier REMSD of rats was similarly reported to cause apoptosis of neurons involving Bcl2 family proteins and caspases [9; 10], which further supports our current line of observations. The physiological consequence of hepatocytic cell death also signifies and justifies the evolutionary and conserved roles of REM sleep which evolved lately in avians and mammals with certain exceptions.

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FIGURE LEGENDS

Figure 1: Detection of apoptosis in the hepatocytes of rats; (A), Annexin v labeling of hepatocytes from CC, LPC and REM sleep deprived group of rats; (B), TUNEL staining of hepatocytes from CC, LPC and REMSD group of rats. X-axis represents the different days of sleep deprivation for treatment groups and Y-axis represents the percentage of Annexin V and TUNEL positive cells. Treatment groups marked with different small letters are statistically different in a Tukey post-hoc tests followed by ANOVA. The boxes shows the median (continuous line) and mean (discontinuous line), while the upper and lower lines of the box show the interquartile range between the first quartile and the third quartile which represents data range of $25^{th}/75^{th}$ percentile of data. The whiskers are the upper and lower adjacent values representing $5^{th}/95^{th}$ percentile of data, outliers if any are indicated with black circle dot. P value < 0.05 was considered statistically significant.

[CC-4=Cage control-Day 4, LPC-4=Large platform control-Day 4, CC-9= Cage control-Day 9, LPC-9=Large platform control-Day 9, REM-4=REMSD-Day4, REM-9=REMSD-Day 9 and REM-5DR, 5 days of sleep recovery after 9 days of sleep deprivation]

Figure 2: Analysis of apoptotic genes by real time PCR. The graph shows log fold change in expression pattern of p53 genes (a), Bcl2 (b) and Bax (c) respectively. Cage control samples were taken as calibrator while GAPDH was endogenous control for respective genes. X-axis represents the different days of sleep deprivation for treatment groups and Y-axis represents the log fold expression of genes. See figure1 for further statistical and legend specific details.

Figure 3: Analysis of hepatocytes proteins using WB from CC, LPC and REM sleep deprived group rats. (a), Lane 1(CC-4D), lane 2 (CC-9D), Lane 3(LPC-4D) and Lane 4 (LPC-9D) represent samples from the CC and LPC group of rats after 4th

and 9th day of the start of experiment, while Lane 5 (REM-4D), Lane 6 (REM-9D) and Lane 6 (REM-5DR) represent samples from REMSD group rats after 4thday, 9th day and after 5DR of REM sleep deprivation. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as an endogenous loading control. (b), Densitometric analysis of the protein bands expressed in reference to the endogenous loading control GAPDH. X-axis represents the different days of sleep deprivation for treatment groups and Y-axis represents the gel band density. P value < 0.05 were considered as statistically significant, see figure 1 for additional details.

Figure 4: Analysis of Caspases involved in apoptosis from CC, LPC and REM sleep deprived group rats. (A), the graph shows log fold change in expression pattern of caspase 3 gene. Cage control samples were taken as calibrator while GAPDH was endogenous control for respective genes. (B), Lane 1(CC-4D), lane 2 (CC-9D), Lane 3(LPC-4D) and Lane 4 (LPC-9D) represent samples from the CC and LPC group of rats after 4th and 9th day of the start of experiment, while Lane 5 (REM-4D), Lane 6 (REM-9D) and Lane 6 (REM-5DR) represent samples from REMSD group rats after 4thday, 9th day and after 5DR of REM sleep deprivation. (C), Densitometric analysis of the protein bands expressed in reference to the endogenous loading control GAPDH. X-axis represents the different days of sleep deprivation for treatment groups and Y-axis represents the gel band density. Value of P < 0.05 were considered as statistically significant. Other statistical details as reflected in figure 1.

Supplementary figure 1: **Histopathological examination of rat liver tissue from cage control, large platform control and REM sleep deprived group.** H&E-stained sections of the liver in progression with REMSD in rats showing inflammatory cells. (Original magnification, 200x). (a) CC 4th day, (b) LPC 4th day, (c) REM sleep deprived

4th day, (d) CC 9th day, (e) LPC 9th day, (f) REM sleep deprived 9th day and (g) REM sleep deprived 5 day recovery group.

Figures:

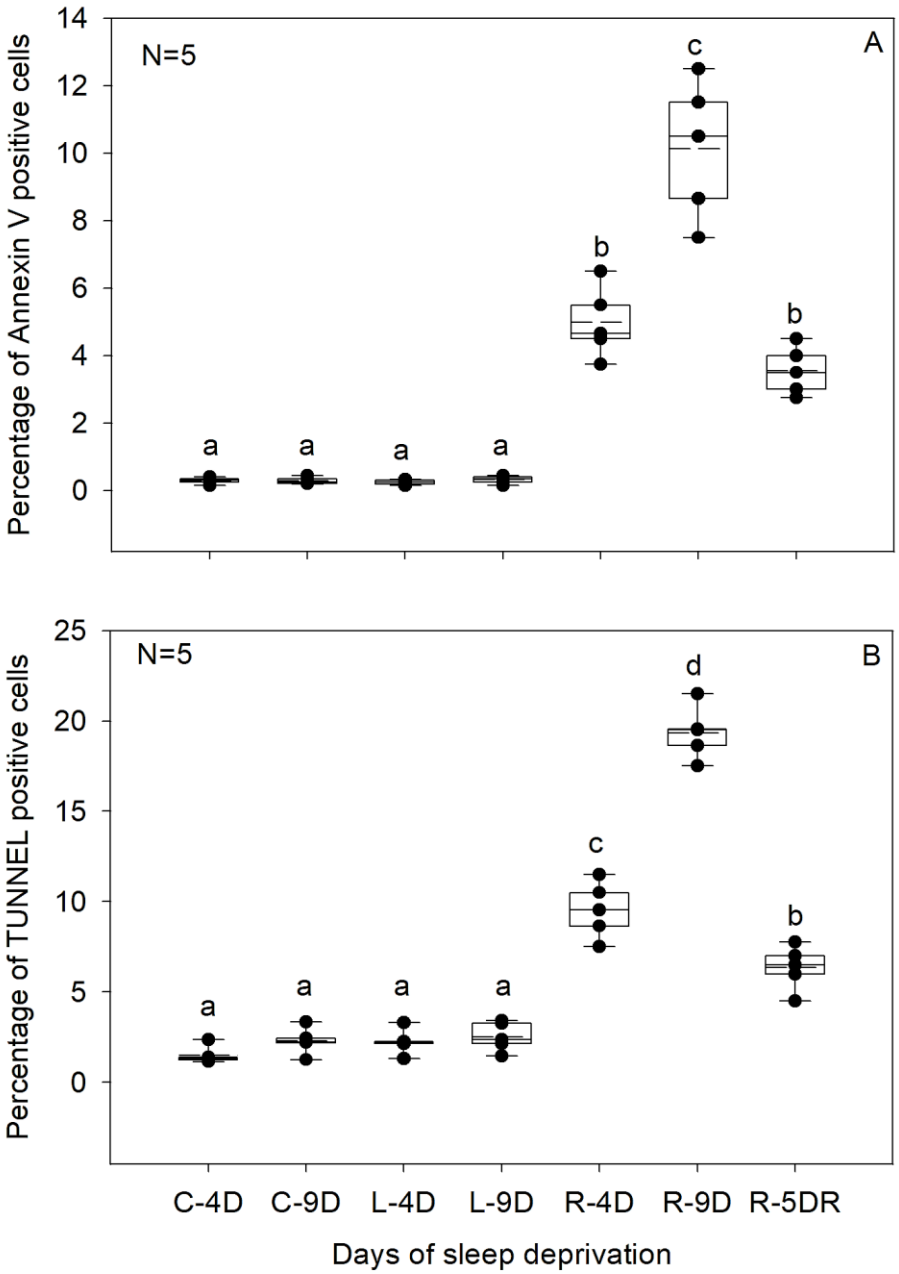
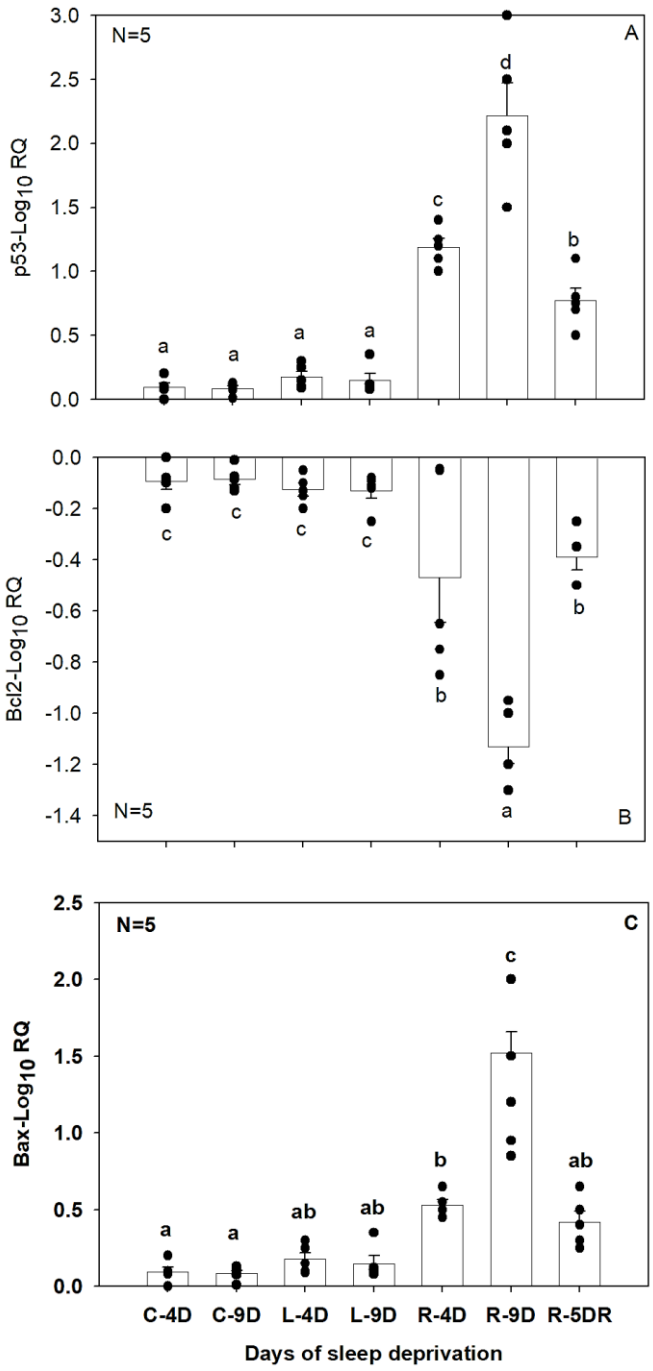


Figure 1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2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
a
c
d
b
N=5
B
Bcl2-Log10 RQ
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
c
c
c
c
b
a
b
N=5
C
Bax-Log10 RQ
2.5
2.0
1.5
1.0
0.5
0.0
a
a
ab
ab
b
c
ab
C-4D
C-9D
L-4D
L-9D
R-4D
R-9D
R-5DR
Days of sleep deprivation

Figure 2

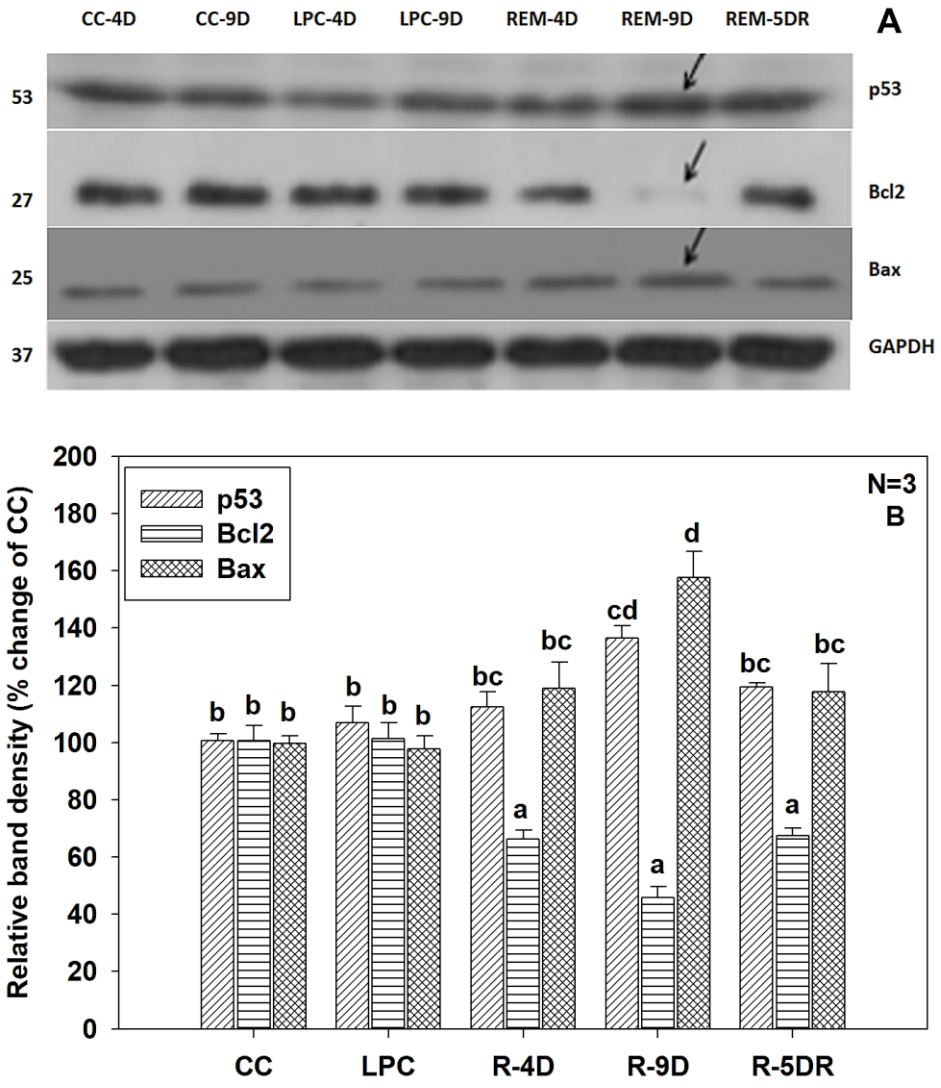
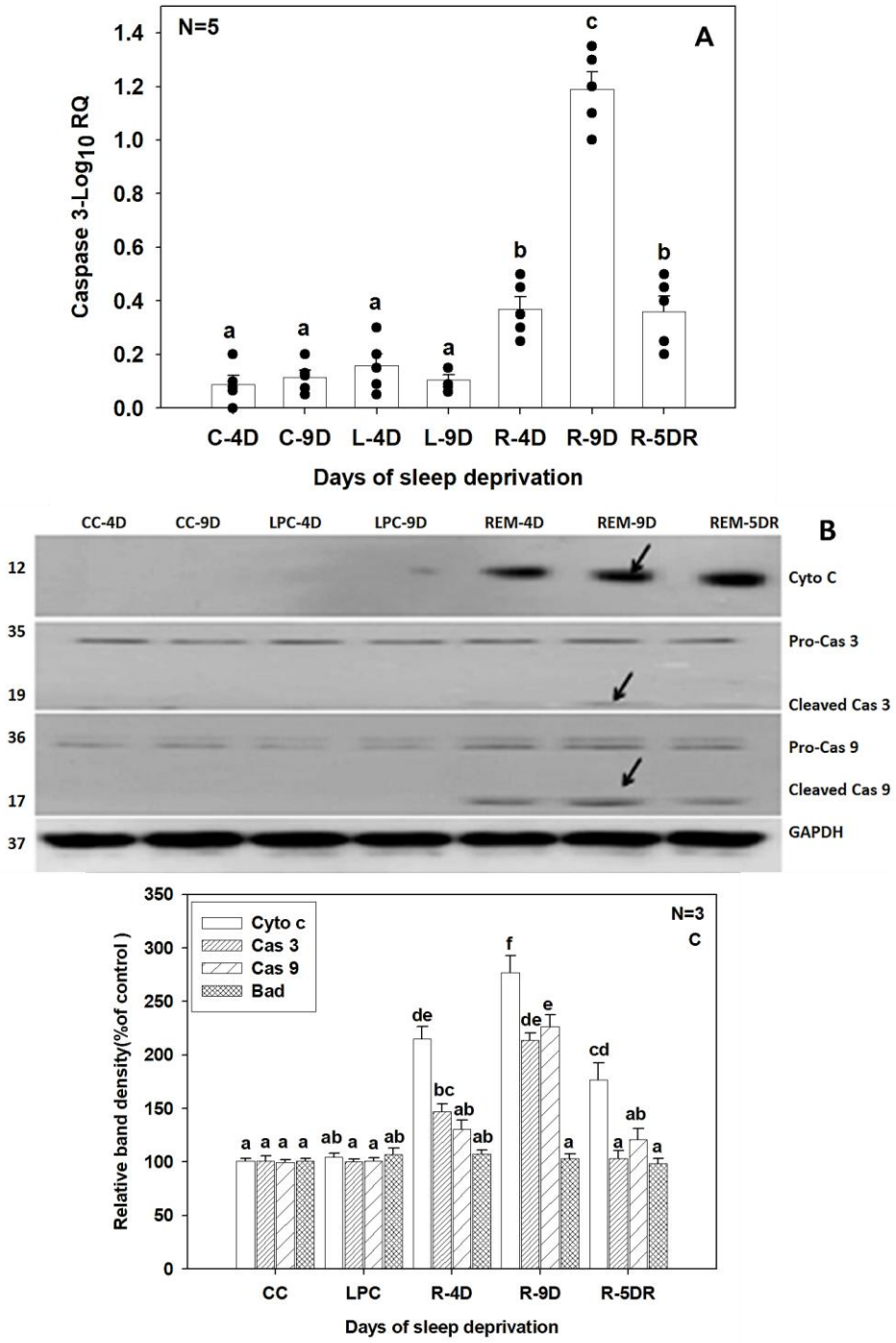

CC-4D
CC-9D
LPC-4D
LPC-9D
REM-4D
REM-9D
REM-5DR
A
53
27
25
37
p53
Bcl2
Bax
GAPDH
N=3
B
Relative band density (% change of CC)
200
180
160
140
120
100
80
60
40
20
0
p53
Bcl2
Bax
b b b
b b b
bc a bc
cd a d
bc a bc
CC
LPC
R-4D
R-9D
R-5DR

Figure 3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0.4
0.2
0.0
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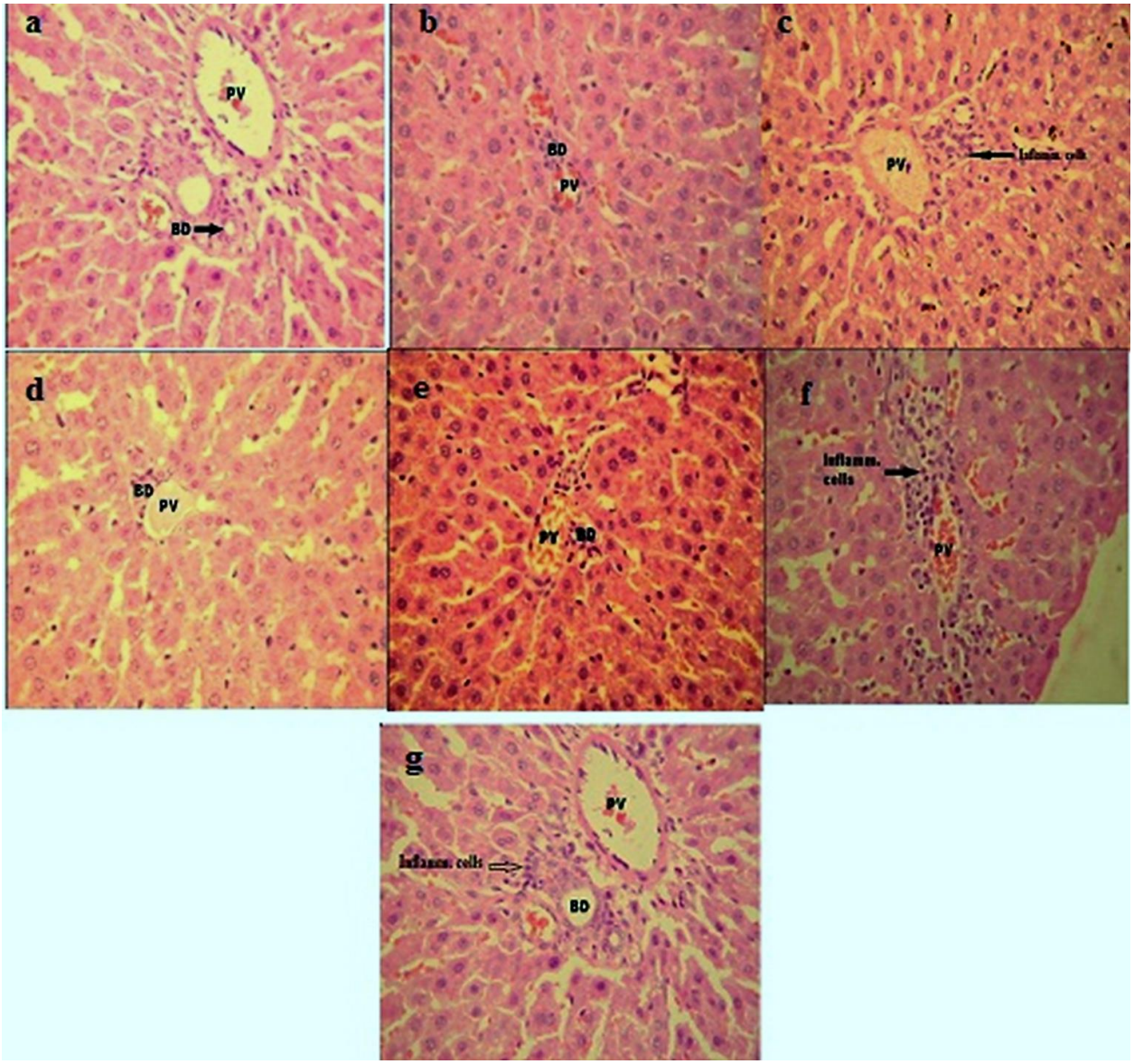
a
a
a
b
c
b
C-4D
C-9D
L-4D
L-9D
R-4D
R-9D
R-5DR
Days of sleep deprivation
CC-4D
CC-9D
LPC-4D
LPC-9D
REM-4D
REM-9D
REM-5DR
B
12
Cyto C
35
Pro-Cas 3
19
Cleaved Cas 3
36
Pro-Cas 9
17
Cleaved Cas 9
37
GAPDH
N=3
C
Cyto c
Cas 3
Cas 9
Bad
Relative band density(%of control)
350
300
250
200
150
100
50
0
CC
LPC
R-4D
R-9D
R-5DR
Days of sleep deprivation

Figure 4



Supplementary Figure 1

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