Global Medical and Health Communication

Online submission: https://ejournal.unisba.ac.id/index.php/gmhc
DOI: https://doi.org/10.29313/gmhc.v11i2.10743

GMHC. 2023;11(2):102–108
pISSN 2301-9123 | eISSN 2460-5441

RESEARCH ARTICLE

Effect of Sleep Deprivation on the Number of Prefrontal Cortex Neuroglia Cells in Male White Rats (*Rattus norvegicus*)

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Abstract

Stress induced by sleep deprivation can increase inflammation and oxidative stress, destroying the pyramidal and neuroglia cells in the prefrontal cerebral cortex and interrupting cognitive and behavioral functions. This study aims to observe the difference in the number of pyramidal and neuroglia cells in the prefrontal cortex of male white rats (*Rattus norvegicus*) after stress induction by paradoxical sleep deprivation (PSD) and total sleep deprivation (TSD). This study was conducted in the Anatomy Laboratory of the Faculty of Medicine, Universitas Jenderal Soedirman, from November 2019 to February 2020. The method of this study was a posttest-only design with a control group approach using ten rats for each group; that was control (K.I.), PSD (KII), and TSD (K.I.). PSD and TSD groups received sleep deprivation treatment for eight days for 20 hours/day and 24 hours/day, respectively. The mean pyramidal cell number decreased in the PSD (66.67±24.55) and TSD (65.90±34.91) compared to the control (77.10±26.11) group, but no significant differences were found between all groups ($p>0.05$). The mean neuroglial cell number was lower in the PSD (97.78±28.17) and TSD (75.80±22.39) compared to the control (126.00±48.81). Post-hoc Bonferroni test showed a significant difference between control and TSD ($p<0.05$) but not between control and PSD or PSD and TSD ($p>0.05$). In conclusion, there was a significant difference in the number of neuroglial cells but not pyramidal cells in the prefrontal cortex of male white rats (*Rattus norvegicus*) after stress induction with total sleep deprivation (TSD).

Keywords: Male white rat, neuroglial cells, prefrontal cortex, pyramidal cells, sleep deprivation

Introduction

Sleep is a basic human need to maintain normal body function and support physical and psychological recovery.[1] A good sleep cycle is measured by observing the deepness and quantity of sleep.[2] In adults, the standard sleep duration is 7–9 hours, whereas teenagers need 8–10 hours, and children require 9–11 hours of sleep. Epidemiological data shows that 30% of men and women aged 30 to 64 years sleep less than 6 hours per day,[3] and the survey of the healthy lifestyle index in Indonesia by Taylor Nelson Sofres (TNS) in 2013 in Indonesia, shows that Indonesian people have an average sleep time of 6.8 hours every day due to a lot of activity.[4]

Shorter sleep duration causes sleep deprivation, which can induce disruption of decision-making, repetition of error, and reduction of spontaneity, speed, and motivation to communicate.[3] Sleep deprivation-induced stress in humans can be investigated using animal models by paradoxical sleep deprivation (PSD) or total sleep deprivation (TSD). Sleep deprivation can damage the brain's prefrontal cortex and interrupt cognitive function and behavioral control.[4] Pyramidal and neuroglial cells are primary excitatory neurons in the prefrontal cortex (PFC); therefore, the stress in PFC can be measured by calculating pyramidal and neuroglial cell reduction.[5]

Sleep deprivation can reduce brain glucose metabolism by inducing the hypothalamus-pituitary axis (HPA) and sympatho-adrenomedullary system, further releasing corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP), increasing adrenocorticotropic hormone (ACTH) and glucocorticoid secretion, and reducing glucocorticoid receptor expression.[6]

Received: 10 October 2022; Revised: 24 July 2023; Accepted: 8 August 2023; Published: 25 August 2023

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Glucocorticoids increase the release of glutamate in the brain, N-methyl-d-aspartic acid (NMDA) and á-amino-3-hydroxy-5-methyl-4 isoxazole propionic acid (AMPA) in the postsynaptic membrane, magnesium (Mg^{2+}) ion blockade from NMDA receptor, and mobilize plenty of Ca^{2+} ion in the cytosol as an apoptotic signal in a neuron.[7] Neuroglia cells consisting of microglia, astrocytes, and oligodendrocytes are supporting cells in the central nervous system (CNS) that function as a provider of nutrition, protection, and support for neurons and mediate brain metabolism, nerve detoxification, repair, and synaptic plasticity so that they play an essential role in sleep homeostasis.[8] Pyramidal cells are the central neuronal cells that make up the brain and play a role in sensory and motor coordination, especially those related to behavior control and cognition.[9] No research has confirmed whether PSD and TSD can induce pyramidal and neuroglial cell deaths in the prefrontal cortex.

Rahmadhani's[10] research showed that there was a decrease in the number of pyramidal cells in the cerebral cortex of rats induced by monosodium glutamate, with the ecotoxicity mechanism of glutamate similar to that which occurs during exposure to PSD and TSD. Research by Arjadi et al.[11] also showed a decrease in the number of pyramidal cells in the hippocampus as a memory center due to exposure to stress, one of which is PSD. None of the studies can confirm that PSD and TSD can cause neuronal death in the area of the cerebral prefrontal cortex, which is dominated by pyramidal cells. This study aims to observe the difference in the number of pyramidal and neuroglia cells in the prefrontal cortex of male white rats (*Rattus norvegicus*) after stress induction by PSD and TSD.

Methods

This research design is experimental with a posttest-only control group design with a 4-month duration (November 2019–February 2020) conducted in the Anatomy Laboratory of the Faculty of Medicine, Universitas Jenderal Soedirman. Research ethics was approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Jenderal Soedirman, No. 8395/KEPK/XII/2019.

This study utilized 30 male white rats (*Rattus norvegicus*) of Wistar strain with 3–4 months of age and 100–200 grams of weight from the Animals Laboratory of the Faculty of Medicine, Universitas Jenderal Soedirman. Federer's formula was used for sample size determination; that is (t−1) (n−1) ≥15, in which t is the number of interventions and n is the number of repetitions. Based on this formula, the required sample for each group was nine rats. Since this experiment employed three intervention groups, the total required animal subjects were 27 rats. Ten percent of the total animals were added to avoid dropout, so the samples used in this study were 30 or 10 rats for each group.

The modified multiple platform method (MMPM) was applied for sleep deprivation induction using water-filled tanks measuring 123×44×35 cm containing twelve platforms with 6.5 cm width and 10 cm distance between each platform.[12] The pyramidal and neuroglial cells were measured with a light microscope (Motic) paired with Optilab. Other tools used in this study were a digital weight scale (Dragon 303®), black marker (Snowman®), and surgery set. Experimental materials used in this study were animal feed (Comfeed AD II), water (Aqua®), ether, xylol, alcohol (90% and 95%), ethanol (95%, 90%, 80%, and 70%), gelatin 0.5%, standard formalin buffer 10%, paraffin, aqua dest, and hematoxylin-eosin (HE) staining kit.

Experimental animals were weighed, randomly grouped, acclimatized for seven days, and placed in cages measuring 60×30×30 cm with a temperature of 28±2°C and humidity of 75±5%. The animals were given the same type, amount, and composition of feeds *ad libitum*, namely Comfeed AD II, consisting of protein (19%), water (12%), fat (7%), calcium (1.1%), phosphorus (0.5%), and the rest is crude fiber, then reweighed at the end of acclimatization. The animals were then divided into three groups with a completely randomized design. The animals were grouped by labeling each animal with numbers 1 to 30, and 30 lottery papers were drawn for each group (K.I., KIII, and KIII) and tabulated. The lottery papers were taken one by one and put onto the table to determine control and experiment groups; that is, Group K.I. (control group, without any sleep deprivation intervention); Group KII (PSD, 192 hours total, 20 hours/day, sleep deprivation at 11.00 a.m.–07.00 a.m. local time, and break at 07.00 a.m.–11.00 a.m. local time continuously); and group K.I. (TSD, 192 hours total, 24 hours/day, sleep deprivation at 11.00 a.m.–11.00 a.m. local time continuously).[8]

MMPM equipped with muscle atonia was used to induce sleep deprivation in KII (PSD) and KIII

(TSD) groups.[12] When the animals enter the sleep phase of rapid eye movement, they will fall into the water and wake up. The electric shocks were given every 10 minutes to maintain the animal's awake state.

Animal termination was done after eight days of intervention with ether inhalation. After the rats were dissected, the prefrontal cortex was taken within 2–3 minutes, starting with a skin incision in the middle of the rat head with scissors, followed by a bone incision of the head from the parietal into the frontal bone. Calvaria was then opened using forceps, and the brain was released from its membrane and cranial nerve by cutting to liberate the brain from the cranium base. The brain was fixated using 10% of the standard formalin buffer for at least 24 hours. The fixated brain was then cut coronally to obtain the prefrontal cortex. Darker areas of the prefrontal cortex, the medial prefrontal cortex, were stained with HE.[11]

Pyramidal and neuroglial cells were observed and counted using Image Raster v.2.1 software with 400 times magnification in 10 fields of view. The normal neuroglial cell was defined as a neuroglia cell without pycnotic, karyorrhexis, and karyolysis appearance. The pyramidal cell was described as a cell that has a big cell body with a triangular shape and basophilic cytoplasm.[9] Two independent observers performed the observation, and the interobserver reliability was analyzed by the Bland-Altman test. Specimens were processed in the Anatomic Pathology Laboratory of the Faculty of Medicine, Universitas Jenderal Soedirman.

Univariate data analysis results were presented in maximum and minimum values, mean, median, and standard deviation. Shapiro-Wilk test was used for analyzing data normality, and Levene's test was used for homogeneity. The parametric test was performed with a one-way analysis of variance (ANOVA), followed by post-hoc Bonferroni with a 95% confidence interval (α=0.05).

Results

The interobserver reliability test used the Bland Altman test and showed a range limit of agreement (–5 and 5) between –0.71–0.58 and –1.29–1.36 for the pyramidal and neuroglial cells data that were predominantly distributed around the mean of difference, indicating interobserver reliability is good. The number of neuroglia and pyramid cells was observed using a binocular light microscope, and the number was counted in 10 fields of view for one preparation. The neuroglia cells counted are normal cells with the criteria of not experiencing pycnotic (nucleus condenses, darkens, and shrivels), cariorexis (nucleus tears and forms fragments), karyolysis (the nucleus is pale because it cannot absorb color). Pyramidal cells are neurons with the characteristics of a large cell body, triangular in shape, with basophilic cytoplasm and basophilic cytoplasm (black arrows) and granular cells (red arrows). Histological pictures of neuroglia cells and prefrontal cortex pyramidal cells (Figure 1 and Figure 2) have in same results in all study groups, showing that K.I. (control) had the highest number of pyramidal cells (A), followed by KII (PSD) (B), and the least was owned by

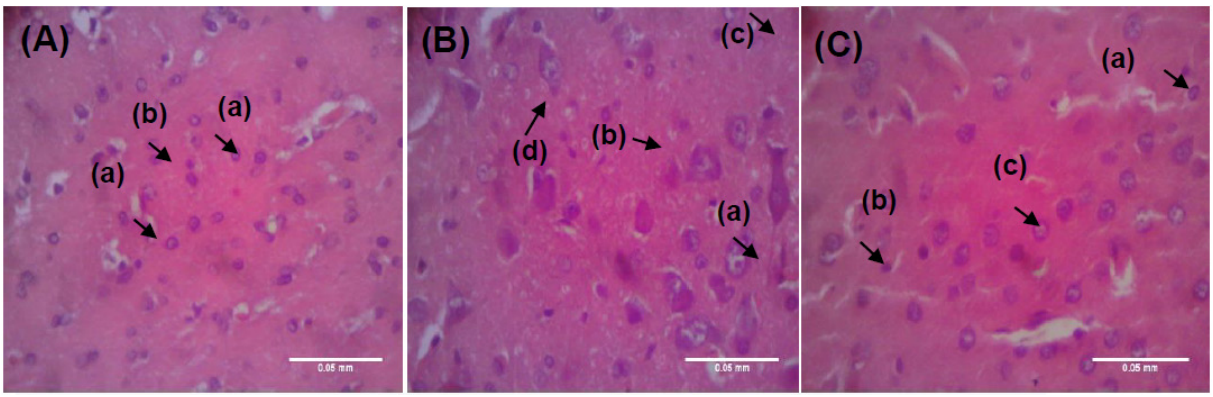

Figure 1 Medial Prefrontal Cortex of the Rat Brain Stained with HE (400× Magnification)
(**A**) control group, (**B**) PSD, (**C**) TSD. Black arrows indicated (**a**) normal neuroglia cell, (**b**) apoptotic neuroglia cell, (**c**) normal granular cell, and (**d**) normal pyramidal cell. Scale bars 0.05 mm

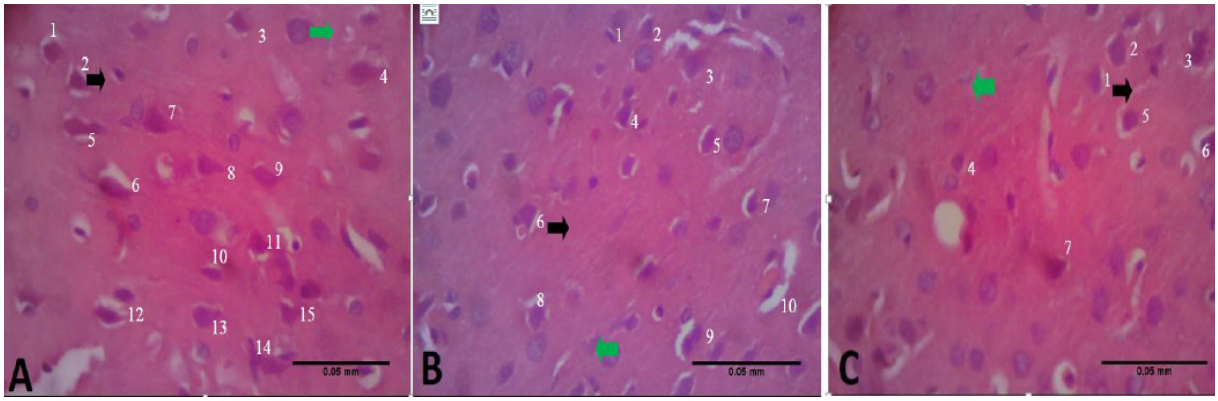

Figure 2 Pyramidal Cell Number in Prefrontal Cortex Pyramidal Stained with HE (400× Magnification)

(**A**) KI (control) showed 15 cells per field, (**B**) KII (PSD) showed 10 cells per field, and (**C**) K III (TSD) showed 7 cells per field. Pyramidal cells exhibited triangular-shaped with basophilic cytoplasm in black arrow and granular cell in green arrow. Scale bars 0.05 mm

group K III (TSD) (C).

Table 1 shows that the highest pyramidal cell number was in the control group (77.10±26.11), and the lowest was in the TSD group (65.90±34.91). Meanwhile, the highest number of normal neuroglia cells was in the control group (126.00±48.81), and the lowest was in the TSD group (75.80±22.39), as shown in Table 2. Shapiro-Wilk analysis showed the data was normally distributed (p>0.05), and Levene's test for homogeneity showed that all data were homogenous in all groups. ANOVA analysis for pyramidal cells showed no significant difference among all groups (p=0.638, p>0.05); therefore, post-hoc analysis was not further performed. Neuroglial cell analysis exhibited a significant difference in at least two groups (p=0.014). Post-hoc Bonferroni test in the mean normal neuroglial cell number showed a significant difference between the control and TSD (p=0.011), while no significant differences between the control and PSD (p=0.280) and between PSD and TSD (p=0.559).

Discussion

Paradoxical sleep deprivation (PSD) is the reduction of human sleep time, which can result

Table 1 Mean Pyramidal Cell Number in Each Group

Groups	n	Pyramidal Cell Number			
		Mean±SD	Min	Max	p
K.I. (Control)	10	77.10±26.11	45	131	0.638*
KII (PSD)	9	66.67±24.55	36	100	
KIII (TSD)	10	65.90±34.91	23	119	

Note: *one-way ANOVA

Table 2 Mean Neuroglia Cell Number in Each Group

Groups	n	Neuroglia Cell Number			
		Mean±SD	Min	Max	p
K.I. (Control)	10	126.00±48.81	50	209	0.014*
KII (PSD)	9	97.78±28.17	57	131	
KIII (TSD)**	10	75.80±22.39	40	113	

Note: *one-way ANOVA, post-hoc Bonferroni test showed a significant result compared to the control (p<0.05)

in the loss of almost all sleep time, and total sleep deprivation (TSD) is a condition in which a person does not sleep at all through one or more periods of actual sleep.[13,14] Paradoxical and total sleep deprivation harm decision-making control and trigger repetition of working memory errors and loss of individual spontaneity in communicating, making them appear humans experience lazy, lethargic, and unmotivated.[15] Sleep deprivation stress, so this research can be used as a preliminary study to determine the effect of sleep deprivation on neuroglia cells as brain neuron support cells and influences various brain functions.

The results of the study indicated a concordance between the hypothesis and the results of the study that there were significant differences in the number of neuroglia cells in male white rats (*Rattus norvegicus*) in the control group, the paradoxical sleep deprivation (PSD) group and the total sleep deprivation (TSD) group. The K1 (control) group showed that the mean pyramidal cell number was higher than that of KII (PSD) and K.I. (TSD) groups, indicating stress induces pyramidal cell death. In addition, the pyramidal cell number of the *cornu ammonis* three hippocampi after PSD was lower than the control. However, there was no statistically significant difference in pyramidal cell number between the control and sleep deprivation groups.

This result did not match recent research, which found a marked difference in pyramidal cell numbers between the control and the stress group induced by immobilization. Immobilization-induced stress is complex, containing physical, psychological, and social stress, while PSD only causes physical and psychological stress.[8] This insignificant result is probably due to the neuroprotective effect of neuroglia activity and brain-derived neurotrophic factor (BDNF) secretion that counter glutamate toxicity caused by cortisol and overactivated glutamate receptors (GluRs) that start neuroglia cell death.[10] Therefore, stress exposure in these experiments reduced apical dendrite complexity and cell size without any pyramidal cell deaths.[5]

Sleep deprivation for 2–4 hours/day can increase the size of mitochondria without damaging their structure.[16] Neuron deaths begin with neuroglia deaths because of neuroglia's role in protecting neurons. For example, an astrocyte, one type of neuroglia, releases BDNF, which regulates neuron proliferation and differentiation to maintain life continuity and neuron defense. The homeostasis mechanism disrupted by 12 hours of sleep deprivation can be preserved by BDNF release for at least one month.[5] Another neuroprotective neuroglia is microglia, which have macrophage-like roles in the central nervous system, clear dead cells and debris, increase neurogenesis, and reduce inflammation.[7]

This study found that the difference in pyramidal cell number between the control and sleep deprivation groups was insignificant. It is speculated that glucocorticoid release due to stress can disrupt the pyramidal cell and neuroprotector functions of neuroglia.[11] Glucocorticoid secretion inhibits glucose uptake and increases glutamate at the extracellular gap, producing excitotoxicity.[12] Glutamate release is responded to by cystine efflux via glutamate-cystine exchanger in neuroglial membrane cells, reducing intracellular glutathione level and elevation of reactive oxygen species (ROS). Glutamate excitotoxicity can induce glutamate receptor (NMDA) overactivity in the pyramidal cell membrane and affect alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainite receptors in the neuroglial cell membrane.[7] The glutamate receptor overactivity releases Na^+ influx and activates voltage-gated calcium channel (VGCC) and sodium-calcium exchanger (NCX), causing intracellular calcium ions overload.[17] Calcium overload is absorbed rapidly by mitochondria, causing rapid depolarization and activation of apoptotic cell caspase, ROS elevation, and glutathione reduction.[5]

On the other hand, neuroglia has a role in maintaining glutathione levels and clearing ROS directly. Change in neuroglial cell number in chronic sleep deprivation is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, which elevates plasma cortisol levels. Elevation of cortisol level caused by sleep deprivation inhibits glucose transport in the brain and reduces basal ATP energy. Glucocorticoids also inhibit glucose uptake and increase glutamate levels at the extracellular gap.[8]

The mean neuroglia cell number difference after sleep deprivation treatment was significant in K.I. (TSD) but not in KII (PSD) compared to K.I. (control) group. This result is not consistent with previous studies that demonstrated the notable increase of glutamate and aspartate levels in the hippocampus and brain cortex, reduction of cortex glutathione reductase, and elevation of

malondialdehyde (MDA) after 72 hours of PSD.[18] Elevation of MDA and reduced glutathione reductase make mitochondrial and cytoplasmic antioxidant systems unable to fight free radicals. When animals are awake, their neuron activity is high, causing the oxygen demand to maintain the membrane potential is rising.[19] This condition triggers ROS accumulation, disrupting neuroglia membrane cells in the cortex and hippocampus.[13] Neuroglia acts as a neuroprotector by maintaining glutathione levels and eradicating free radicals directly, but its action does not completely inhibit the effects of oxidative stress on pyramidal cells; even repeated oxidative stress causes >80% damage to neurons that are not protected by neuroglia and 45% to neurons with neuroglial protection.[14]

Paradoxical sleep deprivation implemented for 96 hours can reduce lipid peroxidase by 38% in the cerebral cortex.[15] Oxidative stress reduction in PSD causes an elevation of superoxide dismutase (SOD) level for 40% without glutathione peroxidase (GPx) change. SOD and GPx are antioxidants for transforming free radicals into safe substances for neurons and neuroglia. SOD elevation after PSD indicates compensating responses to control anion superoxide due to oxidation-reduction reaction imbalance in mitochondria.[20]

A study by Inoue et al.[21] showed an elevation of SOD after PSD (68.92±19.08 U/mL) and TSD (82.70±12.93 U/mL) for five days. An elevation of SOD can induce apoptotic signals because of SOD transformation to superoxide reductase (SOR). SOR plays a role as superoxide oxide (SOO) to generate free radicals using alternative pathways. The PSD group had 4 hours of sleep time, so the difference in SOD between the control and PSD group was not as much as between the control and TSD groups. Metabolism is reduced during sleeping, while residual metabolism product inhibits oxidative stress and induces cellular apoptotic. The sleeping period in PSD also inhibits excitatory neuron activity and glutamate neurotransmitters by glutathione disulfide (GSSD). This inhibition reduces extracellular glutamate concentration and minimizes its excitotoxic effect on neuroglia cells.[18]

People who experience sleep deprivation were reported to have a disruption of cognition and behavioral function conducted by the brain's prefrontal cortex, such as a decrease in concentration, reduction of precision work, unsystematic thought, inaccurate decision-making, and emotional disturbance. Continuous disruption of these functions can cause work accidents and decreased performance. Further study by measuring the prefrontal cortex thickness after the induction of PSD and TSD needs to be accomplished to determine the effect of sleep deprivation on the organ level. In addition, sleep recovery needs to be added to the experiment procedure since sleep recovery can improve the condition caused by sleep deprivation.

Conclusions

Total sleep deprivation significantly reduces the neuroglial but not pyramidal cell number in the prefrontal cortex of male white rats (*Rattus norvegicus*). The number of pyramidal and neuroglial cells in the prefrontal cortex of a male white rat (*Rattus norvegicus*) is not significantly reduced after induction of paradoxical sleep deprivation.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

The author would like to express gratitude to the Research and Community Services Center of Universitas Jenderal Soedirman for funding through the Hibah Riset Unggulan Terapan scheme in 2020 and the Research Laboratory Faculty of Medicine of Universitas Jenderal Soedirman for the facilities.

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