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RESEARCH ARTICLE

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Total Sleep Deprivation Reduces Quality of Spermatozoa in Male Albino Rat (*Rattus norvegicus*)

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Abstract:

Sleep deprivation-induced stress decreases the quality of spermatozoa due to the increase of glucocorticoid levels through the hypothalamic-pituitary-adrenal axis. Sleep recovery (SR) restores the effect of stress due to sleep deprivation by decreasing lipid peroxides and other free radicals and increasing glutathione antioxidants. This study aims to determine the spermatozoa quality in male albino rats (*Rattus norvegicus*) after the stress induction by diverse sleep deprivation protocols. This study is experimental research with posttest-only control group design in 30 male albino rats divided into five groups consisting of group I for negative control (no induction of sleep deprivation), group II for paradoxical sleep deprivation (PSD), group III for total sleep deprivation (TSD), group IV for PSD followed by SR, and group V for TSD followed by SR. PSD and TSD were performed by depriving rats sleep for 20 and 24 hours/day, respectively, for five days. SR was done for the next five days after PSD or TSD. Shapiro-Wilk test was used for normality of data distribution, and Levene's test was used for variance homogeneity. Comparative hypothesis was then analyzed with One Way Analysis of Variance (ANOVA) test, and significance between groups was analyzed with Tukey's Post-Hoc test. Group III (TSD) demonstrated the lowest mean of spermatozoa count (41,53±2,29), spermatozoa morphology (13,00±2,91), spermatozoa viability (31,00±7,39), and percentage of motility rate (37±8,58%) among all groups. In conclusion, total sleep deprivation (TSD) decreases the quality of spermatozoa in male albino rats (*Rattus norvegicus*).

Keywords: albino rat; quality of spermatozoa; total sleep deprivation

Introduction

Sleep deprivation (SD) is a sleep disorder characterized by a reduction of sleep duration. In modern society, reduction in duration and quality of sleep is prevalent, caused by the transformation in the social field, economy, lifestyle, and work. Sleep deprivation causes stress that disrupts the reproduction system by activating the hypothalamus-pituitary-adrenal (HPA) axis. An increase in the production of glucocorticoid suppresses gonadotropin-releasing hormone (GnRH) and subsequently decreases the secretion of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone [1]. Moreover, glucocorticoid increases the production of reactive oxygen species (ROS), generating oxidative stress and damaging testis, leading to a disruption of spermatogenesis and reduction of spermatozoa quality [2].

Paradoxical sleep deprivation (PSD) is a reduction of sleep duration that depletes almost all sleep lengths or phases of deep sleep and keeps in a conscious state due to the high intensity of brain activity. Meanwhile, total sleep deprivation (TSD) is an elimination of the entire sleep duration passing through one or more sleep periods providing stress induction by depleting sleep length for complete 24 hours [3]. On the other hand, sleep recovery (SR) is a restitution of the sleep period to the initial state. Sleep recovery for five days enhances testosterone levels and increases glutathione, which protects the spermatozoa from death [4]. This study compares the impact of various sleep deprivation stress models on the quality of spermatozoa.

Research Method

This study is an experimental study with posttest-only control group design carried out from December 2018 to January 2019. Thirty male albino rats (*Rattus norvegivus*) were acclimatized for seven days and randomly classified into five groups: group I (healthy control, no sleep deprivation), group II or PSD (20 hours/day of SD between 11.00 a.m. - 07.00 a.m. with 4 hours rest between 07.00 a.m. - 11.00 a.m.), group III or TSD (24 hours/day of SD between 04.00 a.m. - 04.00 a.m.), group IV or PSD+SR (PSD followed by SR), and group V or TSD+SR (TSD followed by SR). PSD and TSD were performed for consecutive five days, and SR was done for the next five days after PSD or TSD. The rats were placed in a container measuring 123x44x44 cm equipped with 14 platforms with 6.5 diameters and 10 cm distance between each platform. The base of the container was filled with water to 10 cm in depth. A shocking device called muscle atonia is installed and connected between platforms which automatically switched on every 10 minutes [5].

After the experiments were completed, the rats were terminated with cervical dislocation and dissected to obtain the epididymis. The cauda of epididymis was then incised to release the spermatozoa, placed in Petri dishes containing 15 mL of physiological NaCl, and chopped into sperm suspension. The right epididymis was used for spermatozoa analysis, and the left one was used for motility and morphology analysis. Spermatozoa viability was observed by two independent observers. One drop of 2% eosin and 10 µL of sperm suspension were applied onto object glass and homogenized. The samples were then covered with cover glass, and living spermatozoa were evaluated on the microscope with 40x magnification at five fields. A total of 200 spermatozoa were observed, and the number of live spermatozoa per total spermatozoa was calculated in percentage [6]. Spermatozoa count

was conducted using an Improved Neubauer hemacytometer on all 25 big squares in the central grid area with 100x magnification. After 200 spermatozoa were counted and summed, spermatozoa concentration was calculated and averaged. The volume of one big square on the hemacytometer is 4 nL (200 µm x 200 µm x 100 µm), so the total volume of 25 big squares is 25 x 4 nL = 100 nL. Spermatozoa concentration was calculated by dividing total spermatozoa count in million by total volume in mL (total spermatozoa/10^{-9} L = total spermatozoa x 10^{6}/10^{-3} L) [7].

For morphology analysis, 10 µL of sperm suspension were collected using a micropipette, smeared on a glass slide, and stained with Giemsa. The percentage of normal morphology of spermatozoa was defined by the normal cell count on 200 spermatozoa observed on a glass slide. Motility of spermatozoa was subjectively observed by classifying them into two categories: 1) motile, including (a) progressive, spermatozoa with active movement in linear or in big circle regardless of its speed (b) non-progressive, others movement excluded progressive movements, such as movement in small circle or tail movement only, and 2) immotile, spermatozoa without any movement. The mean of spermatozoa motility was calculated by dividing the total count of motile spermatozoa by 100 spermatozoa found in five fields in 200x magnification [7].

Reliability for interobserver microscopic observation was analyzed using a t-test but not a kappa test because the data were numeric. Obtained data were described and presented in mean and standard deviation. Data normality and homogeneity were analyzed using the Shapiro-Wilk test, continued by Levene's test. Significance among all groups was analyzed using One Way Analysis of Variance (ANOVA) with Post-Hoc Tukey to determine which groups have significant results. Research Ethical Committee of Faculty of Medicine, Jenderal Soedirman University, approved this study on the 13th of November 2018 (reference number: 5063/KEPK/IX/2018).

Results

Quantification results of spermatozoa count, morphology, viability, and motility in each group are shown in **table 1**.

One-way ANOVA analysis showed no significant result in spermatozoa count among all groups (p=0.081, p>0.05). On the other hand, spermatozoa morphology showed significant difference among all groups (ANOVA, p=0.0001). Post-hoc Tukey analysis demonstrated marked differences in multiple comparisons between groups, particularly group I and II, group I and III, group I and V, group II and III, group III and IV, and group IV and V. Moreover, spermatozoa viability also showed substantial difference among all groups (ANOVA, p=0.0001) with significant Post-hoc Tukey test between group I and group III, group I and V, group II and III, group II and V, group III and IV, and group IV and V. Furthermore, motility of spermatozoa showed notably difference among all groups (ANOVA, p=0.0001). Post-hoc Tukey comparison demonstrated significant results between group I and II, group I and III, group II and IV, group III and IV, and group III and V.

Table 1. Mean quantification of spermatozoa count, morphology, viability, and motility.

Groups	N	Count	Morphology	Viability	Motility
I (Control)	5	56.69 ± 10.80*	22.60 ± 1.14$^{\#}$	75.80 ± 4.56^	64.37 ± 8.58*
II (PSD)	5	46.51 ± 7.70^{*+}	18.00 ± 1.58$^{\#\neq}$	66.90 ± 4.08-	33.65 ± 11.61^{*+}
III (TSD)	5	41.53 ± 2.29^{*x}	13.00 ± 2.91$^{\#\neq=}$	31.00 ± 4.00$^{\wedge-=}$	28.36 ± 8.97^{*x}
IV (PSD+SR)	5	52.71 ± 7.18^{+x}	21.00 ± 1.58$^{=\dagger}$	67.60 ± 2.66=	57.35 ± 7.40^{+x}
V (PSD+SR)	5	44.00 ± 13.11^{x}	14.40 ± 2.70$^{\#\dagger}$	33.70 ± 7.04$^{\wedge-\dagger}$	50.34 ± 10.63*

Note:
* (Significant difference between groups: I, II, III)
# (Significant difference between groups: I, II, III, V)
^ (Significant difference between groups: I, III, V)
≠ (Significant difference between groups: II and III)
- (Significant difference between groups: II, III, V)
+ (Significant difference between groups: II and IV)
x (Significant difference between groups: III, IV, V)
= (Significant difference between groups: III and IV)
/ (Significant difference between groups: IV and V)
† (Significant difference between groups: IV and V)

Discussion

Spermatozoa count

This study showed mean spermatozoa count was lower in all experimental groups compared to the control, with the lowest count in group III (TSD, 41.53±2.29). However, these results were not statistically significant between two or among all groups. A study by Choi et al. demonstrated a similar result that sleep deprivation after four or seven days did not significantly reduce spermatozoa count. One possibility is that the testicular tissue starts exhibiting tubular atrophy on the fourth day of sleep deprivation, thus not yet affecting the spermatozoa quantity [3]. Group IV (PSD + SR) and group V (TSD + SR) showed better results than PSD or TSD alone, indicating sleep recovery restores stress conditions induced by sleep deprivation. Sleep recovery normalizes cortisol levels which reduces lipid peroxides and other free radicals. During the recovery periods, malondialdehyde (MDA) levels are decreased, whereas glutathione levels are increased and reach a plateau on the fifth day of recovery, lowering the levels of lipid peroxides and other free radicals [8]. It is speculated that mammals recover from stress conditions by secreting growth hormone-releasing hormone (GHRH) that inhibits corticotropic activity. Otherwise, another study showed three days of sleep recovery did not significantly improve testosterone levels after three days of sleep deprivation compared to sleep deprivation alone. There is a possibility that the storage of enzymes that protect cytoplasm in spermatozoa is limited, causing damage to the cell membrane that can not be repaired [4].

Spermatozoa morphology

In this study, the percentage of normal spermatozoa morphology was significantly different between groups, with the lowest percentage in group III (TSD; 13.00±2.91). These results indicate that 120 hours of TSD remarkably reduce the percentage of normal spermatozoa morphology. Otherwise, sleep recovery improved spermatozoa morphology after sleep deprivation compared to those without sleep recovery. Group II (PSD) and group III (TSD) showed a lower percentage of normal spermatozoa morphology than control, indicating sleep deprivation induces biological stress. In the human body, biological stressors are responded by activating stress responses that interfere HPA axis. Disruption of the HPA axis causes

oxidative stress, which damages endothelium and induces microangiopathy that interferes with the nutrition distribution into testicular tissue. Consequently, spermatogenesis within testis is imperfect, causing primary or secondary abnormality in the spermatozoa morphology. Interestingly, sleep recovery (group IV and V) showed a higher percentage of normal morphology than sleep deprivation alone (group II and III), indicating sleep recovery restores testosterone levels and increases melatonin levels. Melatonin is an endogenous antioxidant that decreases apoptosis in spermatogenesis, prevents DNA damage, and repairs DNA damage in spermatozoa [9].

Spermatozoa viability

This study found that mean of spermatozoa viability was significantly different between groups, with the lowest viability in group III (TSD, 31.00±4.00). Spermatozoa viability is determined by damages to the spermatozoa membrane that protects and maintains electrolyte balance intra- and extracellular. The decrease in spermatozoa viability is the result of the interaction between the HPA axis and the hypothalamic-pituitary-gonadal (HPG) axis. This interaction starts from the neuron in paraventricular nucleus (PVN) of hypothalamus that secrets corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) which enters the hypophysis system. Adenohypophysis is then responded by secreting adrenocorticotropin hormone (ACTH) that induces the adrenal cortex to release cortisol into the circulatory system [10].

An increase of glucocorticoids induces the accumulation of reactive oxygen species (ROS), leading to the stress oxidative that impairs the lipid component in the testicular tissue. Testicular damages reduce the capacity of germinativum layer to produce spermatozoa, sensitivity of LH receptor, activity of steroidogenesis, and testosterone production [11]. A decrease in testosterone also attenuates glucose, fructose, and sucrose content in seminal plasma, further reduces ATP production and enforces spermatozoa to perform anaerobe metabolism. Accumulation of lactic acid caused by anaerobe metabolism increases osmotic pressure in seminal plasma, reduces the permeability of the spermatozoa membrane, and further damages the spermatozoa membrane causing disorders in the physiology and metabolism of spermatozoa [4].

The lowest spermatozoa viability was observed in the TSD group, indicating that TSD damages reproductive function more severely than other groups. Sleep recovery after PSD improved the spermatozoa viability close to the control group. It is assumed that the glutathione levels are increased after sleep recovery and further prevent the death of spermatozoa [4]. Intriguingly, sleep recovery did not improve spermatozoa viability in the TSD group. MDA levels likely remain high after sleep recovery, indicating the damage to the spermatozoa membrane is still occurring. Besides, the effect of sleep recovery is not yet developed, in which testosterone just starts to increase on the fifth day of sleep recovery [12].

Spermatozoa motility

In this study, TSD and PSD altered progressive and non-progressive motility of the spermatozoa. Sleep deprivation causes stress and increases ROS, followed by the damage of spermatozoa through the reaction of lipid peroxides and cholesterol that disturb spermatozoa motility [13]. Lipid peroxides covalently bind to the enzyme or receptor

within the cell membrane and change membrane activity and function, decreasing membrane permeability and flexibility. Another mechanism is that lipid peroxides covalently bind to cell membrane components and change their structure, function, and character, causing the cell membrane is recognized as an antigen. These mechanisms inhibit acrosome reaction and destroy spermatozoa tail which can be measured by the increase of MDA levels in the testis that is significantly rising after three- or five days of sleep deprivation [4].

Group III (TSD, 28.36±8.97) exhibited the lowest spermatozoa progressive and non-progressive motility among all groups. It is proposed that sleep deprivation for three or five days decreases LH and serum testosterone levels, which alter spermatogenesis in the metamorphosis phase of spermatid into spermatozoa. Disturbance of spermatogenesis causes impairment of acrosome and mitochondria formation, thus affecting the motility of spermatozoa in the epididymis [4]. Moreover, glucocorticoids suppress testosterone produced by Leydig cells causing apoptosis of Leydig cells and disrupting spermatozoa maturation and motility. A study by Hampl and Starka showed that sleep deprivation increases serum glucocorticoid levels after one day, three days, and five days compared to control [14].

This study showed sleep recovery restored spermatozoa motility after sleep deprivation. Sleep recovery in groups IV and V only slightly reduced the percentage of spermatozoa motility compared to control, indicating sleep recovery restores the motility of spermatozoa. This result is consistent with the previous study demonstrating that 24 hours of sleep recovery improved spermatozoa motility by around 42% to 72% after being induced by 24 or 48 hours of sleep deprivation [15]. Evidence showed mammals secrete growth hormone-releasing hormone (GHRH) in response to stress conditions that are important for corticotropic inhibition, thus recovering the HPA axis physiological function [16]. This study is also in agreement with the previous study reporting that five days of sleep recovery significantly improved serum testosterone levels (5.8±0.9 ng/mL), serum LH levels (6.8±0.7 ng/mL), and glutathione levels (305.0±19.4 nmol/mg protein), and decreased MDA levels (8.79±0.9 µmol/g tissue) [4]. A decrease in MDA indicates a reduction of lipid peroxides and other free radicals after sleep recovery [17].

Furthermore, sleep recovery also increases testosterone and LH levels, indicating that sleep recovery protects the spermatozoa from alteration of proliferation, maturation, ATP production, and apoptosis caused by free radicals [17]. Sleep recovery induces the secretion of melatonin hormone, an endogenous antioxidant that gives electrons to free radicals, preventing the free radical from drawing electrons from cells and DNA. By terminating the oxidation chain, melatonin inhibits the formation of new free radicals or changes their forms into a stable molecule, protecting the nucleus, DNA, mitochondria, lipid membrane, and protein from oxidant damage. Melatonin also induces the secretion of antioxidant enzymes including glutathione peroxidase (GPx) and glutathione reductase (GR) that protect the mitochondria membrane from free radicals and prevent spermatozoa cells from death. Moreover, melatonin also stimulates superoxide dismutase (SOD) secretion that catalyzes the conversion of superoxide into oxygen and H2O, preventing lipid peroxides formation and improving spermatozoa motility [18].

Conclusions

Sleep deprivation significantly reduces the quality of spermatozoa in male albino rats

(*Rattus Norvegicus*). Total sleep deprivation (TSD) lowers the quality of spermatozoa more than other sleep deprivation-induced stress models.

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