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Morphology and DNA fragmentation spermatozoa in animal models with sleep deprivation-induced stress

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ABSTRACT

Background: A stress, sleep deprivation, can cause imbalance of reactive oxygen species (ROS) that can damage DNA, lipids and proteins of spermatozoa, consequently leading to infertility.
Objective: The aim of this study is to investigate differences of morphology and DNA fragmentation with some models of sleep deprivation in spermatozoa of white male Wistar rat strain (*Rattus novergicus*).
Methods: This study was an experimental study with post-test-only with control group design. There were 30 male Wistar rats which were randomly divided into 5 groups: control group, paradoxical sleep deprivation (PSD) group, total sleep deprivation (TSD) group, sleep recovery (SR) group, PSD group + SR group, and TSD group + SR group. Stress induction was conducted for 5 days. Morphological abnormality of spermatozoa was observed by creating sperm removal tinged by eosin and nigrosine. DNA fragmentation was performed by determining DNA fragmentation index (DFI). Morphological data of spermatozoa were analysed by One-Way Annova and Post-Hoc Turkey test, and DNA fragmentation of spermatozoa was analysed by Kruskal Wallis and Mann Whitney test to determine differences among the groups.
Results: The highest mean of the abnormal morphology was in PSD Group (89.5 %, p=0.004). The normal mean of DNA fragmentation was found in control group, PSD+SR group and TSD+SR group, PSD and TSD groups. Bad DNA fragmentation index was found in PSD and TSD groups (30.16% and 31.4%, p=0.023).
Conclusion: There were differences of morphology and DNA fragmentation of the spermatozoa in the male Wistar rat strain induced by various models of sleep deprivation.

Latar Belakang: *Stres* sleep deprivation *menimbulkan ketidakseimbangan* reactive oxygen species *(ROS) yang merusak DNA, lipid, dan protein pada spermatozoa sehingga mengakibatkan infertilitas.*
Tujuan: *Mengetahui perbedaan morfologi dan fragmentasi DNA spermatozoa dengan berbagai metode stres* sleep deprivation *pada tikus putih galur wistar (*Rattus norvegicus*) jantan.*
Metode: *Penelitian ini merupakan penelitian eksperimental dengan* post-test only with control group design*. Hewan coba sebanyak 30 ekor tikus putih* Rattus norvegicus *galur wistar dibagi secara acak menjadi 5 kelompok yaitu kontrol,* paradoxical sleep deprivation *(PSD),* total sleep deprivation *(TSD),* sleep recovery *(SR), kelompok PSD+SR, dan kelompok TSD+SR. Induksi stres dilaksanakan selama 5 hari. abnormalitas morfologi spermatozoa diamati dengan membuat hapusan sperma yang diwarnai dengan eosin dan*

nigrosin, fragmentasi DNA ditentukan berdasarkan DNA fragmentation index (DFI)*, Data morfologi spermatozoa dianalisis dengan menggunakan* One-Way *ANOVA dan uji* Post-Hoc Tukey*, sedangkan data fragmentasi DNA spermatozoa dianalisis dengan uji Kruskal Wallis dan Mann Whitney untuk perbedaan antar kelompok.*

Hasil: *Rerata morfologi spermatosoa yang abnormal tertinggi terdapat pada kelompok TSD (89,5%, p=0.004). Rerata nilai fragmentasi DNA dengan kriteria sedang terdapat pada kelompok kontrol, PSD+SR dan TSD+SR. Kriteria indeks fragmentasi DNA buruk terdapat pada kelompok PSD dan TSD sebesar 30,16% dan 31,4% (p=0.023).*

Kesimpulan: *Terdapat perbedaan morfologi dan fragmentasi DNA spermatozoa pada tikus putih galur wistar jantan pasca induksi berbagai model stres* sleep deprivation*.*

INTRODUCTION

Sleep is a recovery process in body that plays an important role in physical and psychological health. Needs for sleep can vary and can be classified based on age. Average needs for sleep for a person in middle age range between 7-9 hours per day, and a person at the age of 65 years and more needs sleep for 7-8 hours. However, since a person is getting older, needs for sleep are decreased due to a variety of activities that are characterized by decreased sleep duration at the night. The amount of sleep that loses for a certain period of a required sleep time and causes disruption of the sleep cycle is also called sleep deprivation (SD).[1]

Sleep deprivation can cause stress which further affects health of the body. Decrease of sleep duration can cause an oxidative stress mechanism due to an imbalance between formation and elimination of ROS. An oxidative stress in a long exposure can affect fertility, especially in men with an erectile function and a spermatogenesis pathway. In Indonesia, there are 40% of couples of childbearing ages, and 10% of them experience infertility. Disorder factors in men are 40% of the causes of infertility. One of techniques to determine diagnostic values of male infertility is by using a spermatozoa analysis. 25% of spermatozoa analysis shows abnormality in a morphology of spermatozoa.[2–4]

Sleep deprivation can induce a stress that affects the quality of spermatozoa through the activation pathways of the hypothalamus pituitary adrenal (HPA) axis and can initiate glucocorticoid production. Excessive glucocorticoid production can decrease production of gonadotropin releasing hormone (GnRH) by the hypothalamus, and then it can downregulate production of luteinizing hormone (LH) and follicle stimulating hormone (FSH).[5] Decrease of FSH and LH tends to suppress testosterone secretion which reduces nutrition during spermatogenesis. Increased glucocorticoids induce an oxidative stress which upregulates production of ROS. Effects of decreased testosterone and increased production of ROS play a role in interfering a spermatogenesis process.[6,7]

Men with high DNA fragmentation tend to experience greater infertility that will affect the quality of spermatozoa, especially the morphology of spermatozoa. Sleep deprivation usually causes a stress through the HPA axis activation pathway and influences increasing glucocorticoids which in turn increases the production of ROS. High ROS levels will damage spermatozoa because plasma membranes of the spermatozoa are rich in unsaturated fatty acids so that they easily bind to ROS and the spermatozoa has a limited number of antioxidants in accordance with the small cytoplasmic volume. The source of ROS can come from leukocytes (extrinsic) in forms of decreased concentration, motility, and normal morphology of spermatozoa and immature spermatozoa cells (intrinsic) associated with DNA fragmentation. High spermatozoa DNA fragmentation due to ROS affects sperm quality, especially the morphology of the spermatozoa head, namely an integrity of the DNA nucleus, making ability of the spermatozoa to fertilize ovum cells decreased.[8] There is a positive correlation between DNA fragmentation and spermatozoa morphology and it has been recommended to perform a DNA integrity testing as a complement to sperm analysis in cases of male infertility.[9]

Stress due to sleep deprivation in humans can be compared to various stress models in

experimental animals, namely PSD and TSD.[10] There is a decrease in quality of spermatozoa in groups of experimental animals treated with various sleep deprivation models.[6] The impact of sleep deprivation can be relieved by sleep recovery, a sleep time given after sleep deprivation.[11] Sleep recovery is a method that makes animals try to make normal sleep time return. It reduces lipid peroxidase and free radical production, and then it increases antioxidant glutathione so that it restores production and maturation of spermatozoa as previous condition.[6] There is a limited amount of research about differences of various parameters of fertility test on sleep deprivation, so the authors focus on differences of spermatozoa morphology and DNA fragmentation of spermatozoa in the white rats induced by various sleep deprivation models.

METHODS

Study Design

This was a true experimental study with post-test only with control group design. This study was approved by the Medical Research Ethics Commission, Faculty of Medicine, Universitas Jenderal Soedirman No. Ref: 4815/KEPK/X/2018.

Research Subjects

30 males of white Wistar rats (Rattus norvegicus) aged 3-4 months with body weight of 200-300 grams obtained from the Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada were divided into 5 groups. Group I was a healthy control group (without a stress treatment). Group II was treated with PSD stress treatment for 5 days (20 hours of sleep deprivation at 11:00 to 07:00 West Indonesia Time and 4 hours of rest at 7:00 to 11:00 West Indonesia Time). Group III was treated with TSD stress for 5 full days without resting time. Group IV was treated with PSD stress for 5 days and followed by SR for 5 days. Group V was treated with TSD stress for 120 hours without sleep followed by SR for 5 days. Stress induction with paradoxical sleep deprivation and total sleep deprivation models were performed for 5 days based on the estimated duration of spermiogenesis of the male white rats that play roles in morphological transformation of spermatozoa.

Sleep deprivation instruments

Tools to made sleep deprivations were a modified multiple platform method (MMPM) tank with a size of 123 x 44 x 35 cm which contained water and was equipped with 12 platforms with a size of 6.5 cm with a distance of 10 cm among the platforms. The tank was equipped with a muscle atony device that provided an automatic shock effect about 0.15 mA every 10 minutes to experimental animals always made in awake condition.[10]

Spermatozoa morphological abnormality

To get some samples of the spermatozoa, a termination of the animal models was conducted. The termination of animal models was performed by administering aesthetic inhalation of ether until their consciousness was lost and was terminated mechanically with cervical dislocation.[12] Spermatozoa suspension was formed from fine pieces of cauda epididymis in a proximal portion of the animal models and stirred with 0.9% NaCL. Morphological examination was conducted by dripping one drop of spermatozoa suspension, adding 2 drops of eosin and 3 drops of nigrosine, and flattening to make a smear with a feathering technique. After the smear was dry, observation was held by using a Motic-B2 microscope. The abnormal morphological amount was seen by using 100x magnification in 5 fields of view to obtain 200 spermatozoa. Determination of abnormal spermatozoa morphology were performed by observing abnormalities in the head, tail and neck of the spermatozoa.[10]

DNA fragmentation

DNA fragmentation was conducted by using the Sperm Chromatin Dispersion (SCD) method using the Halomax® Halotech kit. The amount of DNA of spermatozoa which was unfragmented

or damaged was calculated based on the presence of halo. The results of examination of DNA spermatozoa fragmentation are called spermatozoa DNA fragmentation index (DFI), which is the percentage of total spermatozoa with damaged DNA compared to the number of observed spermatozoa and are divided into 3 classifications: good (DFI 0-15%), average (DFI> 15 - <30%) and poor (DFI> 30%).[10]

Statistical Analysis

Morphological data of the spermatozoa were analysed by using One-Way ANOVA and Post-Hoc Tukey test, while spermatozoa DNA fragmentation data were analysed by using Kruskal Wallis and Mann Whitney tests to determine differences among the groups. All the data were analysed by using 95% CI and p = 0.005

RESULTS

Morphology of normal spermatozoa consisted of hook-shaped head, neck as connector and long slender tail for movement. Abnormal pictures of spermatozoa of white male Wistar strain in each treatment group could be seen in Figure 1.

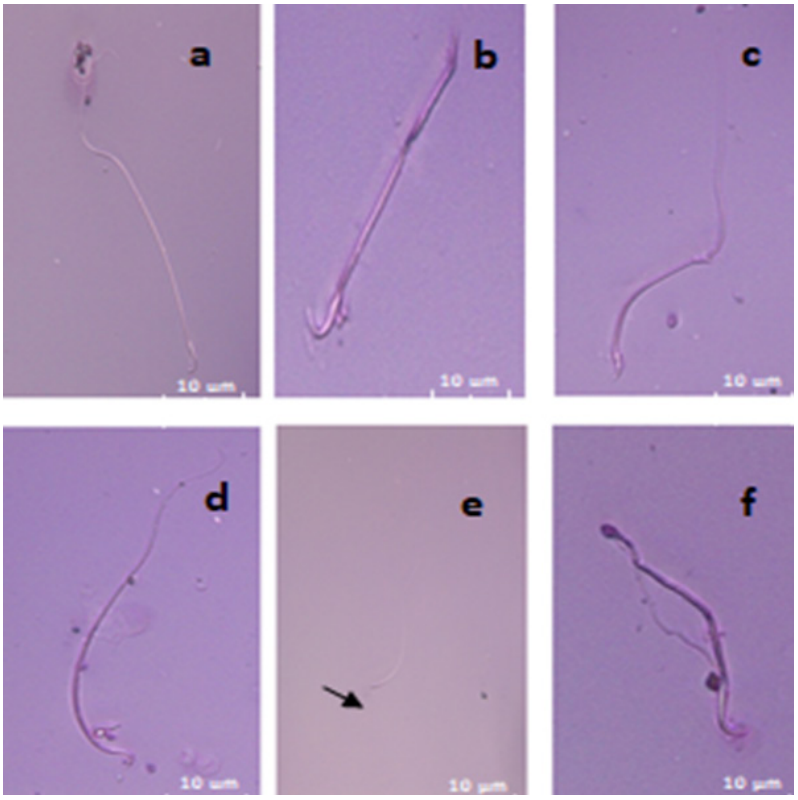

Figure 1. Abnormal spermatozoa after PSD: (a) coiled tail; (b) thickened and short tail; (c) neck lengthening and tail appearing damaged in the middle; (d) hollow in the neck; (e) spermatozoa having no head and neck (arrow), only consisting of the tail; (f) thickening in the middle, lumpy and short tail (100x magnification, eosin-nigrosine staining).

Data analysis of the mean of the spermatozoa morphology was presented in Table 1. The highest normal morphology of the animal models was in the PSD + SR group (47.0 ± 15.58), and the highest mean morphology of the highest spermatozoa of the animal models was in the TSD group (179.00 ± 7.21)

The one-way ANOVA test showed a significant difference (p=0.002) between the sleep deprivation groups on normal spermatozoa morphology (Table 1). Post hoc LSD test was conducted to determine any significant

differences between each treatment in the sample groups. A group with significant results, p = 0.000 (p <0.05) was the control group to the TSD group, the PSD group to the TSD group, the PSD + SR group to TSD, the PSD + SR group and TSD + SR and the TSD group to PSD + SR. Significant differences were found in the TSD group (Table 2)

Table 1. Mean percentages of spermatozoa in cauda epididymis of white male rats (rattus norvegicus) wistar rat strain with normal morphology after induction of some sleep deprivation

Groups	N	Normal Morphology				
		Mean ± SD	%	Min	Max	p
Control	5	39.2 ± 5.40	19.6%	34	48	0.002*
PSD	5	38.8 ± 5.58	19.4%	34	48	
TSD	5	21.0 ± 7.21	10.5%	10	30	
PSD + SR	5	47.0 ± 15.58	23.5%	20	60	
TSD + SR	5	38.0 ± 1.67	19%	36	40	

* p<0.05

Table 2. Post hoc test of mean of normal spermatozoa morphology in cauda epididymis white male rats (rattus norvegicus) wistar strain after induction of some sleep deprivation

Groups	p value				
	Control	PSD	TSD	PSD+SR	TSD+SR
Control		0.941	0.003*	0.161	0.912
PSD	0.941		0.003*	0.141	0.971
TSD	0.003*	0.003*		0.000**	0.132
PSD+SR	0.161	0.141	0.000**		0.004*
TSD+SR	0.912	0.971	0.004*	0.132	

In the abnormal spermatozoa morphology, the one-way ANOVA test found a significant difference (p=0.004) among the sleep deprivation groups (Table 3). Post Hoc Test of mean of abnormal spermatozoa morphology in Table 4 indicated that there were significant differences of morphology in the abnormal spermatozoa treated by sleep deprivation in the TSD, PSD and PSD + SR groups. A group with significant results p = 0.000 was the PSD group to the TSD group and the PSD + SR group to the TSD group. Significant differences were found in the TSD group.

Table 3. Mean percentage of spermatozoa in cauda epididymis of white male rat (rattus norvegicus) wistar strain with abnormal morphology after induction of some sleep deprivation

Groups	N	Abnormal Morphology				
		Mean ± SD	%	Min	Max	p
Control	5	160.80 ± 5.40	80.4%	152	166	0.004*
PSD	5	159.20 ± 9.85	79.6%	142	166	
TSD	5	179.00 ± 7.21	89.5%	170	190	
PSD + SR	5	153.00 ± 15.58	76.5%	140	180	
TSD + SR	5	161.40 ± 1.67	80.7%	160	164	

Table 4. Post-hoc test of mean of abnormal spermatozoa morphology in cauda epididymis of white male rats (rattus norvegicus) wistar strain after induction of some sleep deprivation.

Groups	p value				
	Control	PSD	TSD	PSD+SR	TSD+SR
Control		0.786	0.005	0.196	0.919
PSD	0.786		0.003*	0.300	0.710
TSD	0.005	0.003*		0.000**	0.007
PSD+SR	0.196	0.300	0.000**		0.165
TSD+SR	0.919	0.710	0.007	0.165	

** p<0.01

Fragmentation of Spermatozoa

From all samples, there were 4 criteria: large halo, medium halo, small halo and invincible halo, as can be seen in Figure 2. Table 5 presented the mean group with DFI classification. Poor DNA fragmentation index criteria were found in the PSD and TSD groups (30.16% and 31.4%). The Kruskal Wallis test found a significant result, p = 0.023 (p <0.05) in the spermatozoa DNA fragmentation, while the Mann-Whitney test (Table 6) showed that there was a significant difference in the spermatozoa DFI (p <0.05) in the control group to the PSD group, the control group to the TSD group, the control group to the TSD + SR group.

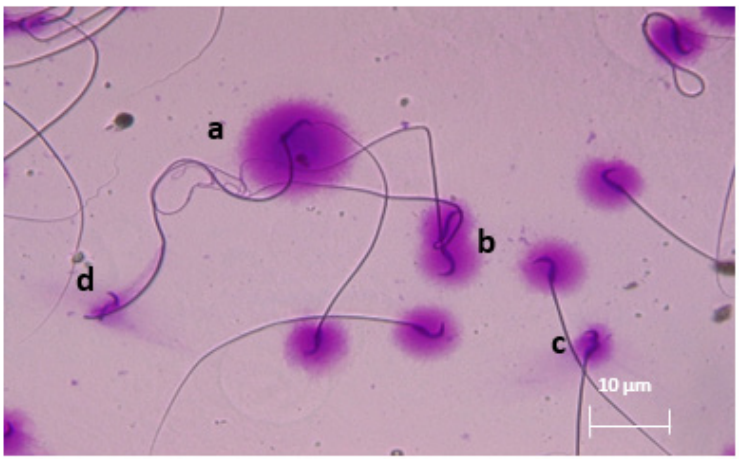

Figure 2. Spermatozoa DNA fragmentation after sleep deprivation. (a) large halo; (b) medium halo; (c) small halo and (d) non-visible halo.

Table 5. Mean of spermatozoa DNA fragmentation index in sleep deprivation-induced stress group

Groups	N	Mean	DFI	p
Control	5	15.8%	Average	
PSD	5	30.16%	Poor	
TSD	5	31.4%	Poor	0.023
PSD+SR	5	25.15%	Average	
TSD+SR	5	23.48%	Average	

Table 6. Mann-whitney test for DNA fragmentation of spermatozoa.

Groups	p-value				
	Control	PSD	TSD	PSD+SR	TSD+SR
Control		0.031*	0.020*	0.050	0.042*
PSD	0.031*		0.594	0.134	0.513
TSD	0.020*	0.020*		0.050	0.221
PSD+SR	0.050	0.134	0.050		0.317
TSD+SR	0.042*	0.513	0.221	0.317	

DISCUSSION

Mean of the morphology of the rat spermatozoa in the sleep deprivation groups was decreased compared to the control group because sleep deprivation was a type of physical and psychological stress that interfered the hypothalamic-pituitary-testicular axis.[13] In the control group, abnormal morphological results mostly found spermatozoa without head, spermatozoa without tail, spermatozoa with a broken tail, so the results obtained by 80.4%. This finding is consistent with previous studies that the abnormal morphology of spermatozoa obtained in the control group with criteria: no head, no tail, broken tail, coiled tail, and there is a possibility of error during a treatment after spermatozoa are removed from the cauda epididymis.[14]

Morphological abnormalities of spermatozoa can be developed after spermatozoa are ejaculated out of the testis through a reproductive tract, which generally results in spermatozoa morphological abnormalities in the tail.[15] Factors causing infertility are also inseparable from spermatozoa production, hormonal disorders and lack of sleep quality.[16] This study showed that morphology of normal spermatozoa with the highest mean was found in the PSD + SR group with sleep recovery for 5 days, while the lowest morphology of normal spermatozoa was found in the TSD group, a group that had sleep disturbance for 24 hours. Stress due to sleep disorders impacts on decreased activity of the HPG axis. GnRH secretion in the hypothalamus initiates activation of the HPG axis and HPA axis activity, thereby stimulating the adrenal cortex to excrete cortisol.

This study also found a significant difference in the morphology of normal spermatozoa in the TSD group in most of all treatment groups. Lack of sleep will trigger hormonal abnormalities because of metabolic syndrome which can change the sexual behaviour of animal models, that lack of sleep reduces testosterone levels thereby affecting the adrenal cortex to stimulate cortisol release.[17] Sleep disorders are known to cause changes in endocrine secretion patterns because they play role as stressors.[18] The treatment group treated with sleep recovery experienced improved morphology of normal spermatozoa. Testosterone and LH hormone levels after treated with sleep deprivation and sleep recovery during 5 days could increase compared to the only sleep deprivation treatment group.

Based on this study, PSD + SR and TSD + SR groups indicated significant differences compared to the TSD group. In the PSD group, although they did not receive sleep recovery treatment but given a rest time of 4 hours, there was a process of homeostasis improvement due to the effects of sleep disturbance compensation experienced for 20 hours. The 5-day recovery period was chosen because it showed that 24 hours of sleep were sufficient to fully normalize sleep parameters after 5 days of sleep deprivation treatment; however this is not enough to completely reverse the normal hours of sleep.[19]

In this study, there were significant differences in spermatozoa DNA fragmentation after induced by the sleep deprivation treatment in all groups. There was a significant difference

in the spermatozoa DNA fragmentation in rats that experienced sleep deprivation. The increase of DFI was significantly related to the increase of ROS in spermatozoa affecting on poor DNA replication, DNA damage and protein synthesis errors that can produce infertility.[5,7] TSD and PSD groups have poor DNA fragmentation index; this means sleep deprivation has a direct impact on infertility in men due to DNA fragmentation that will determine a success of pregnancy.

Damaged DNA caused by free radicals influences decomposition of unsaturated fatty acids into very unstable lipid peroxide. Lipid peroxidation can also be decomposed by free radicals into malondialdehyde (MDA) compounds.[6,8] Lipid peroxidation leads to structural damage and disruption of spermatozoa metabolism producing dead spermatozoa. Oxidative stress is originated from leakage of sperm mitochondrial electrons.[5,20] Oxidative stress induces cross-linking of DNA-chromatin oxidation and broken DNA bonds. Mature spermatozoa have almost no cytoplasm so that spermatozoa DNA is more sensitive to damage caused by ROS.[9]

Oxidative stress induces fat peroxidation in membranes that contain lots of unsaturated fatty acids, thereby reducing their dilution and resulting damage to mitochondria and DNA.[20] In spermatozoa DNA, oxidation of 2-deoxsyguanosine to 8-OH-2-deoxyguanosine makes DNA damaged resulting in nucleotides that were previously bound, with cytidine paired with thymine during DNA replication.[5] The control group when compared with the PSD and TSD groups showed a significant difference, that DNA damage due to oxidative stress would increase in sleep deprivation even though it only lasted for one day.[21] Sleep deprivation is responsible for neurobiological mechanisms which is related to stress as well as oxidative processes. DNA damage due to oxidative stress will increase in sleep deprivation even though it only lasts for one day. Sleep deprivation is responsible for the neurobiological mechanisms associated with stress and oxidative processes.[22] Sleep recovery for 4 hours did not provide significant improvement in DNA fragmentation.[23] This is consistent with this study that DNA fragmentation group II did not differ significantly with group III ,and both were included in the poor DFI category even though they were given a rest period with sleep recovery.

CONCLUSION

There were differences between morphology and DNA fragmentation of spermatozoa in the male Wistar rats after induction of some sleep deprivation models. Sleep recovery significantly increased normal morphology of spermatozoa and DNA fragmentation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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