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Paradoxical sleep deprivation decreases serum testosterone and Leydig cells in male rats

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ABSTRACT

BACKGROUND

Chronic stress increases glucocorticoid levels and accelerates reduction in Leydig cells functions and numbers. Chronic stress models in the working place comprise sleep deprivation, sedentary stress, and physical stress. The aim of this study was to evaluate the effect of various work stress models, such as stress from paradoxical sleep deprivation (PSD), immobilization, and footshock, on serum testosterone levels and number of Leydig cells in male albino rats.

METHODS

This study was of experimental randomized post-test only with control group design using 24 male Wistar albino rats (*Rattus norvegicus*). The sample was divided into 4 groups: K1 (control), K2 (PSD), K3 (immobilization) and K4 (footshock), receiving treatment for 25 days. Measured parameters were serum testosterone level and Leydig cell number. Analysis of variance (ANOVA) was used for statistical analysis, followed by post hoc LSD.

RESULTS

Mean serum testosterone levels (0.07 ± 0.08 ng/ml) and Leydig cell numbers (4.22 ± l0.96) were lowest in the PSD stress model. Serum testosterone levels differed significantly between controls and PSD group (p=0.014), while there was a significant difference in numbers of Leydig cells between footshock stress and PSD (p=0.011) and between the three stress groups and controls (p=0.006).

CONCLUSION

This study demonstrated that PSD, immobilization and footshock stress significantly decreased serum testosterone levels and number of Leydig cells in male albino rats *(Rattus norvegicus)*. The mechanism by which PSD affects serum testosterone is still unclear.

Keywords: Leydig cells, serum testosterone, work stress, male albino rats

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Paradoxical sleep deprivation *menurunkan kadar testosteron dan sel Leydig pada tikus jantan*

ABSTRAK

LATAR BELAKANG

Stres kronik meningkatkan glukokortikoid dan mempercepat penurunan fungsi dan jumlah sel Leydig. Model stres kronik di dunia kerja meliputi pengurangan waktu tidur, pola sedentarian dan stres fisik. Tujuan penelitian adalah untuk menilai pengaruh berbagai model stress kerja, yaitu paradoxical sleep deprivation *(PSD), imobilisasi, dan* footshock *kronik terhadap jumlah sel Leydig dan kadar testosteron pada tikus putih (Rattus norvegicus) jantan.*

METODE

Sebuah penelitian eksperimental post test only *dengan menggunakan kontrol digunakan pada 24 ekor tikus putih jantan galur Wistar. Tikus ini dibagi menjadi 4 kelompok: KI (kontrol), KII (PSD), KIII (imobilisasi), dan KIV (*footshock*) dan diberikan perlakuan selama 25 hari. Parameter yang diperiksa adalah kadar testosteron serum dan jumlah sel Leydig. Analisis statistik menggunakan* analysis of variance *(ANOVA) dengan* Post Hoc-*LSD.*

HASIL

Rata-rata kadar testosteron besarnya 0,07 ± 0,08 ng/mL dan rata-rata jumlah sel Leydig 4,22 ± 0,96 dengan jumlah terendah pada stres jenis PSD. Ada perbedaan rata-rata kadar testosteron serum yang bermakna antara kontrol dan PSD (p=0,014) dan stres footshock *dengan PSD (p=0,011) dan ada perbedaan jumlah sel Leydig yang bermakna pada kelompok perlakuan dibanding kontrol (p=0,006).*

KESIMPULAN

Model stres PSD menurunkan kadar testosteron dan jumlah sel Leydig pada tikus putih jantan. Mekanisme kerja PSD terhadap kadar serum testoesteron masih belum jelas.

***Kata kunci** : Sel Leydig, stres kerja, testosteron serum, tikus putih jantan*

INTRODUCTION

In the projected numbers of Indonesian workers for the year 2013, there is a predominance of males, accounting for around 66.8 million out of 107.7 workers. Therefore the impact of stress in male workers has the potential to cause huge business losses in the form of decreased performance, efficiency and work productivity.[1] Work stress connected with male reproduction follows the psychogenic hypothesis that work stress is part of the cause of infertility problems, where work stress levels are higher in infertile men than in fertile men.[2]

Chronic stress accelerates the reduction in functions and numbers of Leydig cells[3] through the hormonal system and oxidative stress by increasing the levels of glucocorticoids, which trigger an increase in reactive oxygen species (ROS) and initiate Leydig cell apoptosis, resulting in decreased levels of testosterone hormones.[4] The plasma membrane of Leydig cells has a high phospholipid content and is therefore susceptible to ROS, which is a lipid-, protein- and DNA-oxidizer,[5] causing damage to Leydig cells and decreased testosterone levels.[6] Glucocorticoid can directly inhibit testosterone biosynthesis by suppression of 11-beta-

hydroxysteroid dehydrogenase-1 (11âHSD1), a unique enzyme in Leydig cells that controls intracellular active glucocorticoid concentration, thus protecting Leydig cells from the negative effects of glucocorticoids.[7] The decreased level of the 11âHSD1 enzyme increases intracellular glucocorticoid levels, subsequently inhibiting the intracellular steroidogenic function of Leydig cells by suppressing testosterone enzyme biosynthesis.[4]

Stress in experimental animals comprises several models, namely paradoxical sleep deprivation (PSD), representing the physical and psychological stress of sleep deprivation;[8] immobilization, representing physical, psychological and social stress, which form the sedentary stress pattern;[9] and footshock stress, which is a reflection of physical stress.[10] These stress models were shown to be able to decrease testosterone levels. Paradoxical sleep deprivation and footshock stress led to lowered testosterone levels of 13.7 ng/dl and 31.7 ng/dl, respectively, as compared with 371.4 ng/dl in the control group,[11] while immobilization stress decreased testosterone levels.[12] However, there have been no studies comparing the effect of these stress models on serum testosterone levels and numbers of Leydig cells, which is therefore the objective of our present study conducted on male rats.

Figure 1. PSD models using the modified multiple platform method

METHODS

Research design

This experimental study used a completely randomized post-test only design with control group and was conducted from October 2012 to January 2013 at the Animal Laboratory, Medical and Health Sciences Faculty, Jenderal Soedirman University, and the Medico Labora Clinical Laboratory.

Experimental animals

The experimental animals were 24 male Wistar albino rats (*Rattus norvegicus*), aged 3-4 months and weighing 200-300 grams. The sample size was determined by means of Federer's formula (t-1)(r-1) ≥15 where t= number of treatments and r=number of replications.

Intervention

Experimental animals were divided equally into 4 treatment groups. K1 was the control group, in which the rats were left free in the cage and had normal sleep patterns; K2 was the stress model of PSD, where the rats were treated using the modified multiple platform method (MMPD). In this method the animals were put in a tank measuring 123 x 44 x 44 cm and filled with water to a height of 1 cm. filled with water to a level 1 cm below the surface of the platforms. The tank had two rows of 14 platforms located at 10-cm distances from each other, and muscle atonia tools that were switched on automatically every 10 minutes to cause the sleeping animals to fall into the water and thus re-awaken. Two days prior to the experiments, the rats underwent a social adaptation and interaction process with each other. The MMPD test was carried out for 18 hours from 04.00-22.00, after which the animals were returned to their cages and allowed to sleep for 6 hours from 22.00-04.00.[13] K3 was the group subjected to immobilization stress, by placing the animals in a 21-cm long transparent cylinder with a diameter of 6 cm for 1 hour/day. K4 was the footshock stress model group, in which the rats were placed on an acrylic box of 14x25x28 cm with an electrified base. The animals were subjected to 0.1 sec shocks of 5 mA for 1 hour/day at variable intervals every 5 minutes to avoid anticipation by the rats.[11] The shock treatment was given 4-6 times per day. All stress treatments were given at 09.00 am every day for 25 days to produce chronic stress.[14]

Hormonal assays

Blood samples were collected from the orbital plexus by means of hematocrit pipettes, put into individual test tubes and centrifuged at 4000 RPM for about 20 minutes to separate the blood serum, after which the serum-containing tubes were kept at -20°C until required for analysis. Measurement of serum testosterone levels was by means of an enzyme-linked immunosorbent assay (ELISA) reagent kit from Rodent Testosterone Endocrine Technologies Inc.

Isolation of Leydig cells

The number of Leydig cells was counted in 50 fields of view of the interstitial tissue in the left testis of the male albino rats (*Rattus norvegicus*) at a magnification of 400 x. Observations were made using a Nikon Eclipse E100® microscope equipped with an Optilab® digital camera and Image Raster software.

Data analysis

Results of univariate analysis of the frequency distribution were given as mean ± standard deviation. Normality of data distribution was tested with the Shapiro Wilk test, while homogeneity of variance was tested using the Levene test. The analysis was continued with one-way analysis of variance (ANOVA), and for determining different of means in each treatment the post hoc LSD test was used for testosterone levels and number of Leydig cells.[15]

Ethical clearance

All experimental procedures were approved by the Medical and Health Research Ethics

Table 1. Mean serum testosterone level and number of Leydig cells by treatment groups

	Treatment groups				p value
	Control (n=6)	PSD (n=6)	Immobilization (n=6)	Footshock (n=6)	
Serum testosterone (ng/mL)	0.26 ± 0.22	0.07 ± 0.08	0.09 ± 0.06	0.20 ± 0.07	0.032
Number of Leydig cells	10.96 ± 0.17	4.22 ± 0.96	6.69 ± 0.90	6.23 ± 0.35	0.001

Values are mean ± SD; PSD = paradoxical sleep deprivation

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RESULTS

Table 1 shows that the mean serum testosterone level (0.07 ± 0.08) ng/ml) and the mean number of Leydig cells (4.22 ± 0.96) were lowest in the PSD stress model. The ANOVA statistical test on serum testosterone levels (p=0.032) and the number of Leydig cells (p=0.001) showed a significant difference in at least two treatment groups. Figure 2 shows that the numbers of Leydig cells decreased to their lowest value in the PSD stress group.

The results of post hoc LSD ($p<0.05$) showed a significant difference in the mean serum testosterone level between control and footshock groups on the one hand, and the PSD group on the other. There was no significant difference

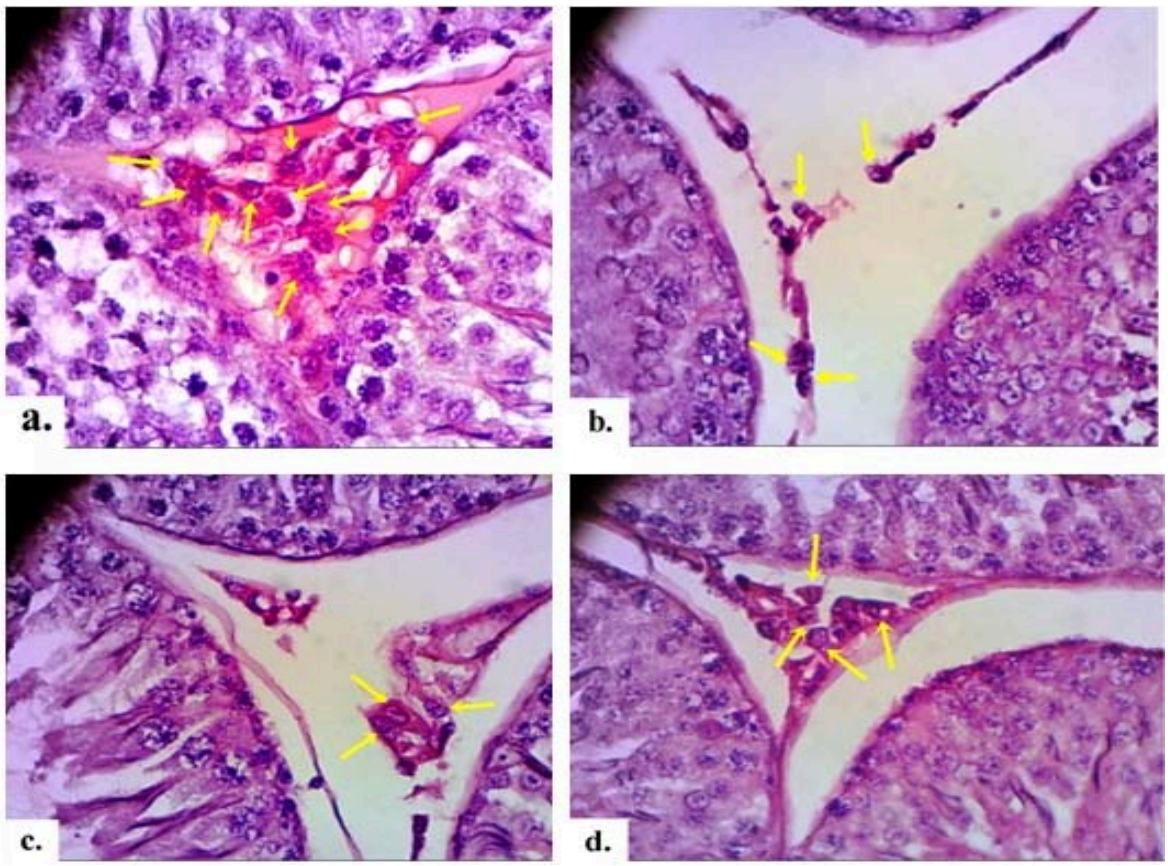

Figure 2. Leydig cells of *Rattus norvegicus*, HE stain, 400 X.
Leydig cells (yellow arrows) with large round nuclei and eosinophilic cytoplasm. a) K1 (control); b) K2 (paradoxical sleep deprivation); c) K3 (immobilization); d) K4 (footshock)

Table 2. Result post hoc test LSD of testosterone level and number of Leydig cells

Treatment group	Serum testosterone level	Number of Leydig cells
Control vs PSD	0.014*	0.001*
Control vs immobilization	0.128	0.001*
Control vs footshock	0.985	0.001*
PSD vs immobilization	0.275	0.001*
PSD vs footshock	0.011*	0.001*
Immobilization vs footshock	0.109	0.263

PSD = paradoxical sleep deprivation; * significantly different ($p<0.05$)

($p>0.05$) between control and immobilization versus footshock, PSD versus immobilization, and immobilization versus footshock. The post-hoc LSD test showed that the mean number of Leydig cells in the three treatment groups was lower than in the control group, with the lowest number being in the PSD group (Table 2).

DISCUSSION

The effect of PSD on number of Leydig cells has never been studied, although the effect on testosterone levels has frequently been investigated. Previous studies showed significant differences in plasma testosterone levels in rats treated with a variety of work stress models, such as PSD, footshock, cold, and swimming, with the lowest plasma testosterone level being found in the PSD stress test.[11] This was presumably due to the duration of the applied stress, since the PSD test was applied for around 18 hours/day, whereas the duration of the immobilization and footshock tests was only 1 hour/day.[16] Other studies also showed that serum testosterone levels in rats given PSD stress were lower than in those given food limitation stress.[17]

The mean decrease in serum testosterone levels in the stress groups presumably led to suppressed testosterone production through several mechanisms, viz. suppression of the hypothalamus-pituitary-testis axis (HPT) by the hypothalamus-pituitary-adrenal axis (HPA) through inhibition of gonadotropin releasing hormone (GnRH) secretion and inhibition of enzyme activity that plays a role in testosterone biosynthesis and the Leydig cell apoptosis induction process.[18]

The initiation of Leydig cell apoptosis by increased glucocorticoid levels occurs through several mechanisms, namely Fas, procaspase-3 cleavage, loss of mitochondrial membrane potential (ÄØm) and increased ROS. High glucocorticoid levels cause changes in mitochondrial membrane permeability through the intrinsic pathway as a result of activation of pro-apoptotic molecules (Bax, Bak, Bad) and suppression of anti-apoptotic molecules (Bcl-2, Bcl-xL, Bcl-W), causing cytochrome C release and activation of caspase-9, active caspase-8 and caspase-9 activate caspase-3. Activation of this cascade leads to apoptosis of mature Leydig cells due to the high number and sensitivity of their glucocorticoid receptors.[4]

High glucocorticoid levels also increase ROS by suppressing the production of nicotinamide adenine dinucleotide phosphate (NADPH) which is important in the regeneration of the antioxidant glutathione (GSH) from oxidized glutathione. The decrease in antioxidants and increase in free radicals lead to oxidative stress,[19] which induces an increase in lipid peroxidation. The latter results in changes in cell membrane permeability, so that cytochrome C releases [2] and oxidizes DNA bases that produce 8-hydroxy-2-deoxiguanosine (causing mutations and deletions of mitochondrial DNA), while cytochrome C also leads to apoptosis through p53 activation.[20] The increase in glucocorticoids inhibits steroidogenic enzyme secretion due to the excessive oxidative

capacity of 11âHSD enzymes. Another result of increased intracellular glucocorticoids is that the Leydig cells cannot be protected from the adverse effects of glucocorticoids.[18]

The lowest mean serum testosterone level in the PSD stress group signifies that oxidative stress caused by high glucocorticoid levels leads to an increase in ROS and also a decrease in the antioxidant glutathione (GSH).[21] In footshock stress, the increase in ROS is caused by the electric shocks given to the animals, and reduces the effectiveness of antioxidant utilization in the body.[22]

Physical sleep deprivation is analogous to sleep deprivation in humans that increases the melatonin hormone levels, especially at night after sleep deprivation.[23] Melatonin acts directly on neuronal gonadotropin-releasing hormone (GnRH) receptors to induce gonadotropin inhibiting hormone (GnIH) expression in the hypothalamic paraventricular nuclei and the testis.[24] At central nervous system level, increases in GnIH is effected by inducing GnIH expression in GnIH neurons of the paraventricular nuclei through its melatonin subtype IC (Mel1C) receptors. Increased GnIH inhibits GnRH and decreases the sensitivity of the pituitary to GnRH, resulting in lowered luteinizing hormone (LH) and follicle stimulating hormone (FSH). In the testis, binding of melatonin to its binding site on Leydig cells suppresses steroidogenic acute regulatory (StAR) expression [25] thus decreasing the phosphorylation and activity of P450 side chain cleavage enzyme that converts cholesterol to pregnenolone, and ultimately to testosterone. The reduction in StAR protein expression is the key to steroidogenesis and decreases testosterone levels after 24 and 48 hours of sleep deprivation.[26]

In this study, the numbers of Leydig cells in the PSD, immobilization, and footshock stress groups were lower than in the control group, the lowest being in the PSD stress group. The duration of the applied PSD stress was 18 hours, while the duration of the immobilization and footshock stresses was 1 hour per day, showing that stress is influenced by the duration of stressor exposure.[16] The increased glucocorticoid levels decrease the number of Leydig cells through the inhibition of mitosis and differentiation of Leydig cell precursors or through an increase in the rate of Leydig cell apoptosis, starting with the inhibition of hypothalamic GnRH synthesis and secretion. The decrease in LH and FSH secretion[27] further influences Leydig cell function and differentiation.[28] Stress also causes macrophages to produce hydrogen peroxide, resulting in oxidative stress in Leydig cells,[29] causes Leydig cell apoptosis through the extrinsic and intrinsic pathways, and increases p53 protein as a marker of DNA damage.[30]

A limitation of this study was that several blood samples underwent hemolysis on aspiration, and could not be read with the microtiter well reader (ELISA reader), thus necessitating repeat aspiration to obtain validated data. Another limitation was that this study did not evaluate other types of stress in rats, such as starvation and social stress. The results of this study may be of use to company doctors, psychiatrists and andrologists in promoting health status, particularly fertility problems in male workers. In future, we will investigate the effect of a traditional medicine known as *purwoceng (Pimpinella pruatjan* Molk*)* as an aphrodisiac and antioxidant to improve the fertility status of rats with PSD.

CONCLUSION

After PSD the serum testosterone levels and the Leydig cells decreased in male rats. The mechanism by which PSD affects serum testosterone is still unclear.

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