- Current Journal:Molekul fitranto1971

Submission Title:Levels of Cortisol and Inflammatory Cytokines after The Induction of Various Sleep Deprivation Stress Models in Male Wistar Rats

Author(s)Fitranto Arjadi, Sindhu Wisesa, Nor Sri Inayani, Prasetyo Tri Kuncoro, Catharina Widiartini

- **Submission**

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.Pre-Review Discussions

Revisi zusfahair
Jun/27

Participants

zusfahair zusfahair (zusfahair)
Fitranto Arjadi (fitranto1971)

Messages

From

Dear Fitranto

On the Molecul Journal guide the number of words from introduction to conclusion is at least around 3000 words, your article

is still around 2500 words. Please add more data and discussion. Abstract only in English form.

Regards

Editor

Revisi artikel pre review

- Fitranto Arjadi (fitranto1971)

Messages

Note

Editor Jurnal Molekul

We hereby submit a revised pre-review article

Thank you

Sincerely

Fitranto Arjadi
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Participants

zusfahair zusfahair (zusfahair)
Fitranto Arjadi (fitranto1971)

Messages

Note	From
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Dear editor

Thank you for your kind commentary and suggestion. We sent the edited manuscript according to your suggestions.

best regards

Fitranto Arjadi

fitranto1971, Author, 6218-99Z_Article Text-25485-1-18-20220916 - edited 18-9-2022.docx

[jm] Editor Decision

2022-08-21 11:33 PM

Fitranto Arjadi, Sindhu Wisesa, Nor Sri Inayani, Prasetyo Tri Kuncoro, Catharina Widiartini:

We have reached a decision regarding your submission to Molekul, " LEVELS OF INFLAMMATORY CYTOKINES (IL-6, IL-10, TNF-α, IFN-γ) AFTER THE INDUCTION OF VARIOUS STRESS MODELS OF SLEEP DEPRIVATION IN MALE WISTAR RATS".

Our decision is: Revisions Required

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Molekul

[jm] Editor Decision

2022-08-28 05:36 AM

Fitranto Arjadi, Sindhu Wisesa, Nor Sri Inayani, Prasetyo Tri Kuncoro, Catharina Widiartini:

We have reached a decision regarding your submission to Molekul, " LEVELS OF INFLAMMATORY CYTOKINES (IL-6, IL-10, TNF-α, IFN-γ) AFTER THE INDUCTION OF VARIOUS STRESS MODELS OF SLEEP DEPRIVATION IN MALE WISTAR RATS".

Our decision is: Revisions Required

Coment Reviewer"

This study showed difference in Cortisol protein level, but not inflammatory mediators in many sleep deprivation models.
1. In the abstract, please state clearly the sample for ELISA analysis.
2. In the introduction, please elaborate more about the relation between sleep deprivation, cortisol and inflammation. Author had explained, however interconnection between is not so

clear.
3. Do you had many basic data about body weight? or functional analysis to see the effect of the SD to organ injury? What is the effect of the systemic inflammation to organ injury?
4. Please state the limitation of the study, and the future perspective of this field.

zusfahair zusfahair
Unsoed
zusfahair@unsoed.ac.id

Revisi Molekul

Dear Editor

Thank you for your kind commentary and suggestion. We will respond as below:
1. In the abstract, please state clearly the sample for ELISA analysis.

Author's response:
Sample of the ELISA analysis is plasma from the animal subject. This information was added on page 1, Abstract, paragraph 1, line 27, and page 2, Abstrak, paragraph 1, line 4.

2. In the introduction, please elaborate more about the relation between sleep deprivation, cortisol and inflammation. Author had explained, however interconnection between is not so clear.

Author's response:
Sleep deprivation induces the release of adrenocorticotropin hormone (ACTH) through the HPA axis and further induces the release of cortisol in the adrenal gland. Cortisol upregulates the expression of pro-inflammatory cytokine in the brain and immune cells via the transcriptional regulator gene, leading to inflammatory reactions. This information was added on page 3, Introduction, paragraph 4, lines 28-33, and page 4, Introduction, paragraph 1, lines 1-4.

3. Do you had many basic data about body weight? or functional analysis to see the effect of the SD to organ injury? What is the effect of the systemic inflammation to organ injury?

Author's response:
We apologize that we did not record the animal's body weight or conduct functional analysis because those parameters were not our focus in this study. We agreed that inflammation caused by sleep deprivation potentially causes organ injury. We will consider this idea for further research.

4. Please state the limitation of the study, and the future perspective of this field.

Author's response:
This study has several limitations that need to be clarified for further investigation, including not analyzing tissue samples for inflammatory cytokine levels and insufficient stress induction protocols using sleep deprivation. Although insignificant, sleep deprivation increased inflammatory cytokine levels, and sleep recovery restored the inflammatory cytokine level similar to the control. Defining the quantity of sleep deprivation and sleep recovery that affected the cortisol and inflammatory cytokine levels is important for future studies. This information was added on page 13, Discussion, paragraphs 2-3, lines 3-29.

Thank you
Sincerely yours
--

Dr dr Fitranto Arjadi MKes
Anatomy Department - Medical Faculty
Jenderal Soedirman University

Round 2 Status **Submission accepted.**

[jm] Editor Decision

2022-09-20 04:13 AM

Fitranto Arjadi, Sindhu Wisesa, Nor Sri Inayani, Prasetyo Tri Kuncoro, Catharina Widiartini:

We have reached a decision regarding your submission to Molekul, " LEVELS OF INFLAMMATORY CYTOKINES (IL-6, IL-10, TNF-α, IFN-γ) AFTER THE INDUCTION OF VARIOUS STRESS MODELS OF SLEEP DEPRIVATION IN MALE WISTAR RATS".

Our decision is to: Accept Submission

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Molekul

[jm] Editor Decision

2022-09-20 04:13 AM

Fitranto Arjadi, Sindhu Wisesa, Nor Sri Inayani, Prasetyo Tri Kuncoro, Catharina Widiartini:

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Our decision is to: Accept Submission

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[jm] Editor Decision

2022-09-20 04:13 AM

Fitranto Arjadi, Sindhu Wisesa, Nor Sri Inayani, Prasetyo Tri Kuncoro, Catharina Widiartini:

The editing of your submission, " LEVELS OF INFLAMMATORY CYTOKINES (IL-6, IL-10, TNF-α, IFN-γ) AFTER THE INDUCTION OF VARIOUS STRESS MODELS OF SLEEP DEPRIVATION IN MALE WISTAR RATS," is complete. We are now sending it to production.

Submission URL: http://jos.unsoed.ac.id/index.php/jm/authorDashboard/submission/6218

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Unsoed
zusfahair@unsoed.ac.id

Molekul

Fwd: [jm] Editor Decision

Inbox

fitranto arjadi <fitrantoarjadi1971@gmail.com> Sun, Aug 28, 2022, 3:02 PM

to me

---------- Forwarded message ---------
Dari: **zusfahair zusfahair** <josunsoed@unsoed.ac.id>
Date: Min, 28 Agu 2022 12:36
Subject: [jm] Editor Decision
To: Fitranto Arjadi <fitrantoarjadi1971@gmail.com>, Sindhu Wisesa <sindhu.wisesa@unsoed.ac.id>, Nor Sri Inayani <nor.sri.inayani@unsoed.ac.id>, Prasetyo Tri Kuncoro <prast.neuro@unsoed.ac.id>, Catharina Widiartini <catharina.widiartini@unsoed.ac.id>

Fitranto Arjadi, Sindhu Wisesa, Nor Sri Inayani, Prasetyo Tri Kuncoro, Catharina Widiartini:

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Coment Reviewer"

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2. In the introduction, please elaborate more about the relation between sleep deprivation, cortisol and inflammation. Author had explained, however interconnection between is not so clear.
3. Do you had many basic data about body weight? or functional analysis to see the effect of the SD to organ injury? What is the effect of the systemic inflammation to organ injury?
4. Please state the limitation of the study, and the future perspective of this field.

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Molekul

Revisi Molekul

FK Unsoed - Fitranto Arjadi <fitranto.arjadi@unsoed.ac.id>, Sep 5, 2022, 11:05 AM

to josunsoed

Thank you for your kind commentary and suggestion. We will respond as below:

1. Kindly mention the novelty of this research.

Author's response:
Many studies have shown that cortisol and inflammatory cytokines are affected by stress induced by sleep deprivation. In contrast, the effect of sleep recovery after sleep deprivation is still uncertain. In this study, we compare the level of cortisol and inflammatory cytokines in sleep-deprived condition and after sleep recovery. This information was added on page 4, Introduction, paragraph 3, lines 11-18.

2. What relevance does this study have? Since we already know that the research's findings have no influence on the causes (not inflammatory cytokines levels of IL-6, IL-10, TNF-α, and IFN-γ)?

Author's response:
In this study, we found that sleep deprivation did not significantly affect the levels of inflammatory cytokines. Stress induced by sleep deprivation may have different mechanisms from other stress conditions in affecting mammal physiology. Therefore, inflammatory cytokines of IL-6, IL-10, TNF-α, and IFN-γ may not be used as indicators of stress caused by sleep deprivation. On the other hand, sleep recovery has the ability to restore the level of inflammatory cytokines to a nearly physiological level highlighting the benefit of sleep recovery at the molecular level. This information was added on page 14, Conclusion, paragraph 1, lines 4-10.

Thank you
Sincerely yours
Dr dr Fitranto Arjadi MKes
Anatomy Department - Medical Faculty
Jenderal Soedirman University

Revisi Molekul

FK Unsoed - Fitranto Arjadi <fitranto.arjadi@unsoed.ac.id> Mon, Sep 5, 2022, 11:16 AM

to josunsoed

Dear Editor

Thank you for your kind commentary and suggestion. We will respond as below:
1. In the abstract, please state clearly the sample for ELISA analysis.

Author's response:
Sample of the ELISA analysis is plasma from the animal subject. This information was added on page 1, Abstract, paragraph 1, line 27, and page 2, Abstrak, paragraph 1, line 4.

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Thank you
Sincerely yours
--
Fitranto Arjadi

Proof Read - Jurnal MOLEKUL Vol 17 No 3

Inbox

Jurnal Molekul <j.molekul@gmail.com>

to me

Dear **Dr. Fitranto Arjadi,**

Sehubungan dengan artikel yang telah diterima untuk diterbitkan pada Jurnal Molekul Volume 17 No 3, November 2022, der

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Salam,

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Yours sincerely,

MOLEKUL Editorial Team.

FK Unsoed - Fitranto Arjadi <fitranto.arjadi@unsoed.ac.id>

Dear Editor MOLEKUL
Hereby we attach the declaration form that had been fulfilled.
Thank you for the kindness.

Best regards
-**Fitranto Arjadi**

LEVELS OF CORTISOL AND INFLAMMATORY CYTOKINES AFTER THE INDUCTION OF VARIOUS SLEEP DEPRIVATION STRESS MODELS IN MALE WISTAR RATS

KADAR KORTISOL DAN SITOKIN INFLAMASI PASCA INDUKSI BERBAGAI MODEL STRES *SLEEP DEPRIVATION* PADA TIKUS PUTIH JANTAN

Fitranto Arjadi[1*], Sindhu Wisesa[1], Nor Sri Inayani[2], Catharina Widiartini[1], Prasetyo Tri Kuncoro[1]

[1]Departement of Anatomy, Faculty of Medicine, Universitas Jenderal Soedirman Purwokerto 53112, Indonesia
[2]Departement of Biochemistry, Faculty of Medicine, Universitas Jenderal Soedirman Purwokerto 53112, Indonesia

*Corresponding author email: fitranto.arjadi@unsoed.ac.id

ABSTRACT

Sleep deprivation (SD) can modulate the production of various cytokines, including pro-inflammatory cytokines such as IL-6, TNF-α, and IFN-γ, and anti-inflammatory cytokines such as IL-10. Paradoxical sleep deprivation (PSD) increases the risk of inflammation but can be relieved by sleep recovery (SR). This study aimed to determine the differences in levels of cortisol and inflammatory cytokines (IL-6, IL-10, TNF-α, dan IFN-γ) in male Wistar rats (*Rattus norvegicus*) after induction of various sleep deprivation stress models. Twenty-five of male Wistar rats were randomly divided into five groups: control, PSD (20 hours of SD/day for five days), Total Sleep Deprivation or TSD (24 hours of SD/day for five days), PSD+SR (PSD followed by SR), and TSD+SR (TSD followed by SR). The cortisol levels were measured with ELISA, and inflammatory cytokine levels were measured with immunoassay and calculated with fold change. Mean cortisol levels were significantly increased in treatment groups compared to the control group (p=0.029). Multivariate analysis showed no statistically significant difference in inflammatory cytokine levels of IL-6 (p=0.658), IL-10 (p=0.065), TNF-α (p=0.399), and IFN-γ (p=0.283) in all groups. In conclusion, various sleep deprivation stress models affect cortisol levels but not inflammatory cytokine levels of IL-6, IL-10, TNF-α, and IFN-γ among male Wistar rats.

Keywords: cortisol, inflammatory cytokines, sleep deprivation, sleep recovery

ABSTRAK

Sleep deprivation dapat memodulasi produksi berbagai sitokin dalam tubuh diantaranya yaitu sitokin pro-inflamasi seperti IL-6, TNF-α, IFN-γ, dan sitokin anti-inflamasi seperti IL-10. *Paradoxical sleep deprivation (PSD)* berhubungan dengan peningkatan risiko inflamasi, tetapi dapat diredakan dengan *sleep recovery (SR)*. Tujuan penelitian adalah mengetahui perbedaan kadar kortisol dan sitokin inflamasi (IL-6, IL-10, TNF-α, dan IFN-γ) pada tikus putih (*Rattus norvegicus*) jantan pasca induksi berbagai model stres *sleep deprivation*.

Sebanyak 25 ekor tikus putih jantan galur Wistar dibagi secara acak menjadi 5 kelompok: kontrol, PSD (20 jam SD/hari selama 5 hari), TSD (24 jam SD/hari selama 5 hari), PSD+SR (Induksi PSD dilanjutkan SR), dan TSD+SR (Induksi TSD dilanjutkan SR). Pengukuran kadar kortisol dilakukan dengan ELISA, sedangkan pengukuran kadar sitokin inflamasi dilakukan dengan *immunoassay* yang dibandingkan kadarnya menggunakan *fold change*. Rerata kadar kortisol meningkat pada kelompok perlakuan dibandingkan dengan kelompok kontrol (p=0,029). Hasil analisis multivariat menunjukkan perbedaan yang tidak signifikan pada kadar sitokin inflamasi IL-6 (p=0,658), IL-10 (p=0,065), TNF-α (p=0,399), dan IFN-γ (p=0,283) pada seluruh kelompok. Kesimpulannya, berbagai model stress *sleep deprivation* mempengaruhi kadar kortisol tetapi tidak mempengaruhi kadar sitokin inflamasi IL-6, IL-10, TNF-α, dan IFN-γ pada tikus putih jantan galur Wistar.

Kata kunci: sitokin inflamasi, kortisol, *sleep deprivation, sleep recovery*

INTRODUCTION

Sleep plays a vital role in life by maintaining the optimal physiological function of the human body. It combines stimulation of afferent nerve delivery to the brain and activation of functional neurons in specific brain areas (Chokroverty & Ferini-Strambi, 2017). Good sleep is determined by the quality of sleep, how deep a person sleeps, and the quantity of sleep, amount of time as a person sleeps (Patrick et al., 2017). According to the National Sleep Foundation, a young adult (18-25 years) or adult (26-64 years) needs 7-9 hours of sleep daily, but not less than 6 hours or more than 11 hours for young adults or 10 hours for adults. Otherwise, an older adult (65 years and over) requires at least 7-8 hours of sleep daily, but not less than 5 hours or more than 9 hours (Lichtenstein, 2015). People who live in developed countries are chronically sleep-deprived because of their cultural and socioeconomic environments. Incidence of symptoms related to sleep deprivation has recently increased, suggesting its long-term detrimental health effects are more abundant than expected (Chokroverty & Ferini-Strambi, 2017).

A study by Hirshkowitz et al. (2015) showed that > 30% of men and women between the ages of 30-64 years sleep less than 6 hours per day, and 5-15% of the world population suffer from sleep disorders. Another study indicated that 40-70% of older adults have chronic sleep disorders that are more prominent in people with medical or psychiatric comorbidity (Praharaj et al., 2018). The National Sleep Foundation stated that 70% of Indonesian people experience sleep disturbances at least once a week, and 30 million people have sleeping difficulty every night (Lestarianto, 2014). A survey of the healthy lifestyle index by American International Assurance (AIA) in 2013 demonstrated that Indonesian people have an average sleep duration of 6.8 hours per day due to increased activity and

decreased sleep duration from its normal range, indicating many Indonesian people are suffered from sleep deprivation (Putri et al., 2017).

The relationship between sleep duration and the inflammation process has not been studied extensively, despite showing a similar correlation between sleep duration and mortality. People with long sleep duration (>8 hours/night) and short sleep duration (<7 hours/night) have a 30% and 12% greater risk of death, respectively, compared to people with moderate sleep duration (7-8 hours/night). Physiologically, sleep affects two major effector systems, the Hypothalamus-Pituitary-Adrenal (HPA) axis and the sympathetic nervous system, which simultaneously increases the release of pro-inflammatory cytokines and markers of systemic inflammation through β-adrenergic activation. The activity of sympathetic nervous system decreases when a person is sleeping, which may explain the relationship between sleep disorder, short sleep duration, and elevation of inflammatory markers (Chen et al., 2017). Patients that pathologically tend to fall asleep during daytime and easily get tired exhibit an increase in IL-6, a pro-inflammatory cytokine, in the circulatory system due to activation of the HPA Axis. People suffering from sleep deprivation produce more IL-6 during the day and less IL-6 at night, whereas good sleep reduces pro-inflammatory exposure to tissues. Furthermore, sleep deprivation upregulates pro-inflammatory cytokines through NF-kB activation, generating a pro-inflammatory state. Overexposure to the pro-inflammatory cytokines caused by sleep deprivation will increase tissue damage, decrease brain function, and increase the expression of IL-6 (Vgontzas et al., 2000).

Sleep deprivation correlates with the increase in TNF-α, indicating a pathological condition caused by sleep disorders (Chennaoui et al., 2011). Moreover, TNF-α is expressed in neurons and plays a role in brain neuroplasticity (Rockstrom et al., 2018). On the other hand, a clinical trial showed that IFN-γ was found in cases of fever, flu, and drowsiness, whereas IFN-γ can be somnogenic in the presence of TNF-α. Therefore, IFN-γ has a significant role in sleep regulation during viral infection (Van Dongen et al., 2011).

Studies suggest that cortisol levels as a physiological stress marker may increase in sleep-deprived conditions. HPA axis activation triggered by sleep deprivation can induce cortisol release from adrenal glands mediated by adrenocorticotropic hormone (ACTH) (Simpson & Dinges, 2007). One of the outcomes of this study is to evaluate the effect of sleep deprivation and sleep recovery on plasma cortisol levels.

Sleep deprivation (SD) can be categorized into paradoxical sleep deprivation (PSD) and total sleep deprivation (TSD), and studied using the Modified Multiple Platform Method (MMPM). While both types can induce stress, total sleep deprivation causes poor decision-making control, triggers repetitive errors in working memory, and abolishes individual spontaneity in communication, making the subjects appear lazy, lethargic, and unmotivated (Gunn et al., 2017). Considering that many people have experienced sleep deprivation, this study is essential as initial research to determine the effect of sleep deprivation on the levels of cortisol and inflammatory cytokines that impairs various metabolic functions in mammals.

Commented [RM1]: Kindly mention the novelty of this research.

EXPERIMENTAL SECTION

Research Design

This study was an experimental study using a post-test only with control group design.

Subject

Wistar rats (*Rattus norvegicus)* with characteristics of male, 8-12 weeks old, and 200-300 grams of weight were obtained from the Laboratorium Penelitian dan Pengujian Terpadu (LPPT) 4 Universitas Gajah Mada. Sleep deprivation experiments were performed using the Modified Multiple Platform Method (MMPM) to prevent the rats from sleeping. A total of 25 rats were randomly divided into five groups containing five rats in each group: control (no SD), PSD (20 hours of SD per day at 11.00 a.m. - 07.00 a.m. for 5 days and 4 hours rest at 07.00 a.m. - 11.00 a.m.), TSD (24 hours of SD per day at 11.00 a.m. - 11.00 a.m. for 5 days without rest), PSD+SR (PSD treatment for 5 days followed by sleep recovery (SR) for 5 days at 07.00 a.m. - 07.00 a.m.), and TSD+SR (TSD treatment for 5 days followed by sleep recovery for 5 days at 11.00 a.m. - 11.00 a.m.).

Research Procedure

Modified Multiple Platform Method (MMPM) tanks measuring 123 x 44 x 35 cm were used for the sleep deprivation stress model. The tanks were filled with water and equipped with 12 platforms of 6.5 cm in size with a distance of 10 cm between the platforms as a foothold for the rats that are instinctively avoiding water. Each tank contains five experimental rats equipped with a muscle atonia device, an instrument that gives an

automatic shock effect every 10 minutes to keep the rats awake. The experiments were conducted in the Anatomy Laboratory of the Faculty of Medicine of Jenderal Soedirman University from August to September 2021.

Observation

Cortisol levels were measured by drawing 2 ml of rat blood for each group at 07.00-09.00 a.m. via retroorbital vein using a microhematocrit pipette into an EDTA tube, centrifuging for 15 minutes at 2,000-3,000 rpm, and storing in -20°C. A total of 50 ml of collected plasma was examined with the Enzyme-Linked Immunosorbent Assay (ELISA) method and read by the ELISA reader in 450 nm absorbance (Zabidi et al., 2015).

For inflammatory cytokines level measurement, rat blood plasma samples were collected from the retroorbital plexus as much as 55 ml. The levels of inflammatory cytokines of IL-6, IL-10, TNF-α, and IFN-γ were then measured using multiplex bead-based immunoassay from MILLIPLEX® MAP kit with Luminex technology in the Integrated Laboratory of Faculty of Medicine, Universitas Indonesia. Briefly, the frozen samples were thawed, stirred thoroughly with a vortex, and centrifuged to remove particulates before the immunoassay. The processed samples were then placed on the plate, added with conjugated beads from the MILLIPLEX® MAP kit, and incubated for 2 hours at 20-24˚C or overnight at 4-8˚C. After incubation, the beads were washed, incubated in biotinylated-detection antibodies, added with Streptavidin-PE (SAPE), and further incubated. The beads were then washed, resuspended in an appropriate buffer, and placed on Luminex technology to measure cytokine levels. The plasma cytokine levels were analyzed based on the fold change value by comparing the treatment and the control group.

Statistical analysis

Normality analysis was performed by the Shapiro-Wilk test, and data homogeneity was analyzed by Levene's test. The multivariate analysis was then performed by the One-Way ANOVA and Post-hoc Least Significant Difference (LSD) to determine differences between groups. Kruskal-Wallis and Mann-Whitney tests were performed for non-parametric analysis.

All procedures were approved by The Medical Research Ethics Commission of The Faculty of Medicine of Jenderal Soedirman University on May 10, 2021 (reference number: 097/KEPK/V/2021).

RESULT AND DISCUSSION

Measurement and analysis results of mean cortisol levels among all groups are shown in **Figure 1**. Since the plasma cortisol levels were not normally distributed, non-parametric tests were used for further analysis.

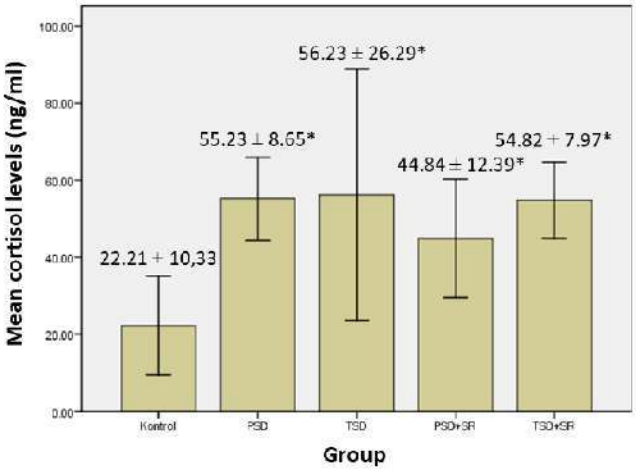

Figure 1. Mean cortisol levels in male Wistar rat (*Rattus norvegicus*). Control: no SD; PSD: 20 hours of SD with 4 hours rest for five days; TSD: 24 hours of SD for five days; PSD+SR: PSD followed by SR; TSD+SR: TSD followed by SR. SD: sleep deprivation, SR: sleep recovery, PSD: paradoxical sleep deprivation, TSD: total sleep deprivation. Error bars represent 95% confidence intervals. Statistical analysis was performed using Kruskal-Wallis test ($p = 0.029$) and Mann-Whitney test for post-hoc analysis. *Post-hoc Mann-Whitney test compared to control, $p < 0.05$.

Kruskal-Wallis analysis showed substantial differences in cortisol levels between at least two groups for the assessed variables ($p=0.029$). Further post-hoc analysis with the Mann-Whitney test obtained significant results between every intervention group and the control ($p<0.05$). PSD for 20 hours/day increased cortisol levels in the afternoon and evening in rats by two times compared to control (Olayaki et al., 2015). Cortisol levels increased in the first six hours after PSD induction caused by HPA axis activation to maintain metabolism changes that were occurred during sleep deprivation (Galvão et al., 2009). Consistent with a study by Olayaki et al. (2015) demonstrating that TSD for five days increased cortisol levels compared to the control, this study pointed out that the TSD group had the highest mean cortisol levels among all groups (56.23±26.29 ng/ml).

The PSD group showed lower mean cortisol levels than the TSD group because PSD has a sleep period daily, thus the negative impact of sleep deprivation can be relieved. Two hours of an afternoon nap after one night of sleep deprivation decreased the secretion of cortisol hormone caused by inhibition of the HPA axis at the slow wave sleep (SWS) phase that reduces cortisol levels (Pejovic et al., 2013). The effects of sleep deprivation that impair HPA function cause an increase in evening cortisol levels as a result of an elevation in its secretory pulses amplitude, suggesting that the negative feedback of glucocorticoid mediated by hippocampal function may be affected by sleep-deprived condition (Hirotsu et al., 2015). Cortisol levels were higher in the subjects who stayed awake than in subjects who slept sufficiently, indicating that sleep deprivation affects the cortisol levels as a result of stress responses (Bassett et al., 2018). Cauter et al. (2008) emphasized that the initial consequence of partial sleep loss is a cortisol level increase in the evening. While under normal conditions, cortisol levels decrease rapidly and reach the minimum level just before sleeping time. Kantasa et al. (2016) stated that cortisol can suppress the immune system and induce an inflammatory pathway, making the body vulnerable to various diseases.

PSD + SR group exhibited lower cortisol levels than TSD + SR, and both PSD + SR and TSD + SR showed lower cortisol levels than PSD and TSD. A study by Mattice et al. (2011) showed that subjects induced by TSD for 24 hours and 48 hours followed by sleep recovery for 24 hours relieved their sleep deprivation effect for 72% and 42%, respectively. Sleep recovery for three days decreases cortisol levels and ameliorates the impact of sleep deprivation by restoring the HPA axis interaction (Pejovic et al., 2013). Sleep recovery also decreases lipid peroxidase and free radical production by increasing glutathione and other enzymatic antioxidants (Hirotsu et al., 2015). Glutathione increases after sleep recovery for two days, indicated by the elevation of 6-PGD, an enzyme that acts on the pentose phosphate pathway in the carbohydrate metabolism that protects cells from oxidative stress in the form of NADPH, thus inhibiting stress oxidative that has a role in cortisol increase (Kim et al., 2022). Taken together, these results indicate that sleep recovery is able to decrease cortisol levels which increase after induction of PSD or TSD.

Results of data analysis showing the value of minimum, maximum, mean, median, and standard deviation for each group are shown in **Table 1.**

Table 1. Levels of inflammatory cytokine in male Wistar rat (pg/mL).

Group	N	Min.				Max.				Mean±SD			
		IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ
Control	5	311,14	15,14	2,89	736,59	1706,25	83,31	12,06	1375,77	974± 504	34,42±11,92	6,91±3,28	1038,08±272,26
PSD+SR	5	452,10	17,52	4,54	754,54	3103,86	93,76	23,89	1001,67	1281±1056	53,07±12,98	9,75±8,05	847,45±102,73
TSD+SR	5	311,14	57,89	6,54	718,80	2518,29	317,89	26,13	1001,67	1578±928	87,06±47,99	14,17±8,17	865,83±112,95
PSD	5	520,98	44,84	6,90	781,54	1858,94	74,98	12,24	1385,65	1192±544	59,47±5,44	9,39±2,62	1046,28±239,32
TSD	5	382,22	33,52	1,76	700,85	1365,64	178,02	14,17	1824,71	952±373	68,06±25,53	8,01±4,39	1167,39±432,52

Table 2. P-value of normality, homogeneity, and One-Way ANOVA test in the levels of inflammatory cytokines in male Wistar rat.

Group	Shapiro-Wilk (p-value)				Levene's (p-value)				One-Way Anova (p-value)			
	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ
Control	0,832	0,941	0,389	0,640								
PSD+SR	0,884	0,482	0,081	0,409								
TSD+SR	0,812	0,909	0,096	0,921	0,256	0,352	0,697	0,075	0,658	0,065	0,399	0,283
PSD	0,067	0,845	0,200	0,610								
TSD	0,622	0,500	0,615	0,750								

Normality analysis by the Shapiro-Wilk test and homogeneity analysis by the Levene's test showed a p-value >0.05 in all groups, indicating data of inflammatory cytokine levels were normally distributed and homogenous. Multivariate analysis using the One-Way ANOVA test demonstrated a p-value >0.05 in all groups, indicating no significant difference in inflammatory cytokine levels among the treatment groups, as shown in **Table 2**. The inflammatory cytokine levels were then compared among the treatment and control groups using fold change. The results demonstrated the highest increase in inflammatory cytokine levels was in the TSD treatment group (**Figure 2**). However, the multivariate analysis of the fold change of inflammatory cytokine levels using the One-Way ANOVA test did not show a significant difference ($p > 0.05$) in IL-6 ($p=0.658$), IL-10 ($p=0.085$), TNF-α ($p= 0.313$), and IFN-γ ($p=0.283$).

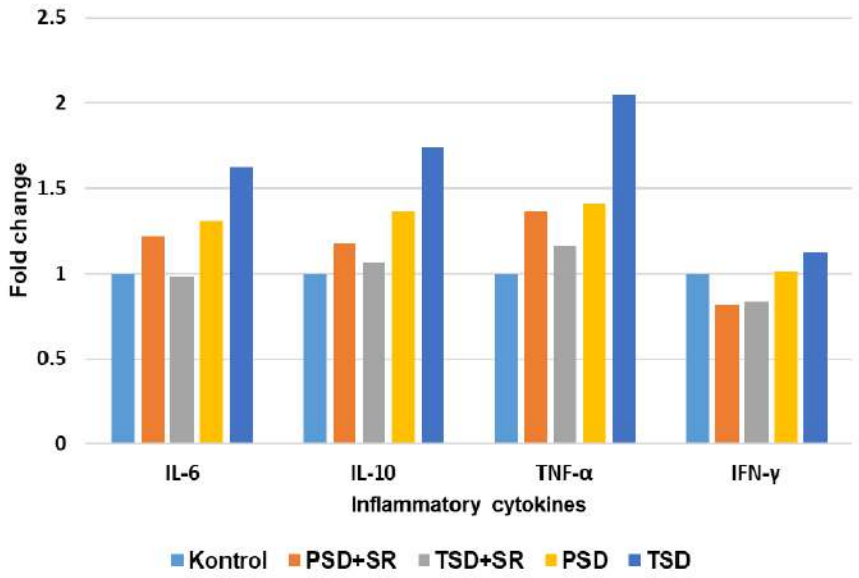

Figure 2. Mean of the fold change of inflammatory cytokine levels of IL-6, IL-10, TNF-α, and IFN-γ in male Wistar rat (*Rattus norvegicus*). Control group: no SD; PSD+SR: PSD followed by SR; TSD+SR: TSD followed by SR; PSD: 20 hours of SD with 4 hours rest for five days; TSD: 24 hours of SD for five days. SD: sleep deprivation, SR: sleep recovery, PSD: paradoxical sleep deprivation, TSD: total sleep deprivation.

Sleep deprivation triggers a stress response in animal models by increasing pro-inflammatory cytokines and activates the main stress axis in humans, the HPA axis. IL-6 can stimulate cortisol secretion directly in the adrenal gland or via activation of the hypothalamus

that induces the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. On the other hand, a slight elevation of IL-6 level in plasma induces the anti-inflammatory cytokines, IL-1ra and IL-10, along with c-reactive protein (CRP). Recent studies stated that apart from injury and infection, sleep deprivation triggers the pro-inflammatory response through the increase of pro-inflammatory cytokines secretion (Medic et al., 2017; Simpson & Dinges, 2007; Sukendra, 2015).

Activation of inflammatory responses caused by sleep deprivation can increase the levels of anti-inflammatory cytokines to prevent excessive inflammatory responses. IL-10, a potent anti-inflammatory cytokine, inhibits the activation of macrophages and dendritic cells, leading to the reduction of cytokine levels produced by T-helper1 (Th1) cells (Welsh et al., 2011). IL-10 also potently inhibits the production of IL-10 itself, IL-12, L-1α, IL-1β, IL-6, macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), leukemia inhibitory factor (LIF), and platelet-activating factor (PAF) from activated monocytes and macrophages (Saraiva & O'Garra, 2010).

This study showed that sleep deprivation did not significantly affect levels of IL-10 in each variable ($p = 0.065$). This result is consistent with the study of Neto et al. (2010), showing that sleep deprivation did not impair levels of anti-inflammatory cytokines, including IL-10. Otherwise, they also observed sleep deprivation increased IL-10 levels in different adipose tissue depots and decreased the TNF-α levels in the brain. Therefore, the profile of systemic anti-inflammatory cytokines after sleep deprivation remains controversial.

A study by Patel et al. (2009) also demonstrated that sleep deprivation did not affect levels of anti-inflammatory cytokines such as IL-1 and IL-10. They explained that the different responses of sleep deprivation on the levels of anti-inflammatory cytokines may depend on the differences in the effect of sleep on inflammatory components in each individual, the half-life of each inflammatory component, and the instrument performance for cytokine measurement. Intriguingly, anti-inflammatory cytokine levels increase in chronic sleep deprivation that lasts over weeks, months, or years.

Wright Jr et al. (2015) demonstrated that sleep deprivation significantly increased levels of IL-10 on day 26 to day 28 measured with ELISA. Sleep deprivation induces circadian rhythm impairment that, over weeks, will increase levels of anti-inflammatory cytokine (IL-10), the pro-inflammatory protein (TNF-α), and CRP. Otherwise, cortisol levels

increase during acute sleep deprivation, indicating that acute and chronic sleep deprivation provoke different responses of circadian rhythm impairment. Therefore, acute sleep deprivation is associated with physiological stress or metabolic response of increased cortisol levels, whereas chronic sleep deprivation is associated with physiological adaptation as a response to decreased cortisol levels followed by increased pro-inflammatory and anti-inflammatory cytokines.

No significant difference in TNF-α levels after induction of various sleep deprivation stress models in this study ($p = 0.366$) is consistent with the study by Ruiz et al. (2012), showing that total sleep deprivation for two days or paradoxical sleep deprivation for four days followed by sleep recovery for three days did not alter TNF-α levels. Other studies also demonstrated that ten days of sleep deprivation did not increase TNF-α or its receptors, regardless of sex characteristics (Shearer et al., 2001; Xu et al., 2015). In contrast, a study by Irwin et al. (2006) exhibited that sleep deprivation increased TNF-α production. Sleep deprivation probably induced TNF-α cellular expression through the activation of nuclear factor NF-kB, a key component in controlling the cellular expression of pro-inflammatory cytokines.

In agreement with the study by Crooks et al. (2019) that demonstrated no significant result between sleep quality and IL-6 levels, this study approved that there was no significant result between the levels of pro-inflammatory cytokines, IL-6, with various sleep deprivation stress models ($p = 0.658$). Conversely, a study by Siregar (2017) showed a significant result between sleep quality and IL-6 levels. Sleep deprivation may affect components of the immune system through modification of CD4+ cells, CD8+ cells, NK cells, and levels of pro-inflammatory cytokines such as TNF-α, INF-γ, and IL-6 (Ibarra-Coronado et al., 2015). Collectively, these results indicate that pure TSD and PSD or TSD and PSD followed by sleep recovery did not affect plasma cytokine levels of IL-6 and TNF-α.

Multivariate analysis among the treatment groups showed no significant difference between sleep deprivation and IFN-γ levels ($p=0.283$). This result is consistent with the study by Hirotsu et al. (2012), demonstrating that there was no significant difference in IFN-γ levels in psoriasis rats model induced by paradoxical sleep deprivation for 48 hours. IFN-γ production is generally unaffected by sleep deprivation, instead is increased at the end of sleep recovery. These insignificant results may be affected by sample processing that caused cytokine stability disruption, cytokine degradation by protease, or cytokine binding to its

soluble cellular receptors. These alterations may also explain the decrease in cytokine levels over time during sample storage (Hennø et al., 2017).

To date, studies concerning sleep deprivation and inflammatory cytokines remain elusive. Some studies indicated that people with sleep disorders showed an alteration in circulating levels of TNF-α and IL-6. However, other studies failed to demonstrate the change in TNF-α and IL-6 levels between normal and sleep-deprived subjects. We speculate that most cytokines are released in tissue locally, leading to low systemic concentrations under normal physiological conditions. Furthermore, variation in the circadian cycle may also affect the production of various cytokines, which confounds the levels of circulating cytokines in this study (Ruiz et al., 2012).

CONCLUSION

Stress induction by the various models of sleep deprivation modifies cortisol levels but not inflammatory cytokines levels of IL-6, IL-10, TNF-α, and IFN-γ in male Wistar rats (*Rattus norvegicus*).

Commented [RM2]: What relevance does this study have? Since we already know that the research's findings have no influence on the causes (not inflammatory cytokines levels of IL-6, IL-10, TNF-α, and IFN-γ)?

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REFERENCES

Bassett, S. M., Lupis, S. B., Gianferante, D., Rohleder, N., & Wolf, J. M. (2018). Sleep quality but not sleep quantity effects on cortisol responses to acute psychosocial stress. *Revista Del Colegio Americano de Cardiologia*, *72*(23), 2964–2979. https://doi.org/10.3109/10253890.2015.1087503.Sleep

Cauter, E. V., Spiegel, K., Tasali, E., & Leproult, R. (2008). Metabolic consequences of sleep and sleep loss. *Physiology & Behavior*, *176*(3), 139–148. https://doi.org/10.1016/S1389-9457(08)70013-3

Chen, L., Huang, J., Yang, L., Zeng, X. A., Zhang, Y., Wang, X., Chen, M., Li, X., Zhang, Y., & Zhang, M. (2017). Sleep deprivation accelerates the progression of alzheimer's disease by influencing Aβ-related metabolism. *Neuroscience Letters*, *650*, 146–152. https://doi.org/10.1016/j.neulet.2017.04.047

Chennaoui, M., Sauvet, F., Drogou, C., Van Beers, P., Langrume, C., Guillard, M., Gourby, B., Bourrilhon, C., Florence, G., & Gomez-Merino, D. (2011). Effect of one night of sleep loss on changes in tumor necrosis factor alpha (TNF-α) levels in healthy men. *Cytokine*, *56*(2), 318–324. https://doi.org/10.1016/j.cyto.2011.06.002

Chokroverty, S., & Ferini-Strambi, L. (2017). Oxford textbook of sleep disorders. In *Oxford University Press*.

Crooks, E., Hansen, D. A., Satterfield, B. C., Layton, M. E., & Van Dongen, H. P. A. (2019). Cardiac autonomic activity during sleep deprivation with and without caffeine administration. *Physiology & Behavior*, *210*, 112643. https://doi.org/10.1016/j.physbeh.2019.112643.Cardiac

Galvão, M. de O. L., Sinigaglia-Coimbra, R., Kawakami, S. E., Tufik, S., & Suchecki, D. (2009). Paradoxical sleep deprivation activates hypothalamic nuclei that regulate food intake and stress response. *Psychoneuroendocrinology*, *34*(8), 1176–1183. https://doi.org/10.1016/j.psyneuen.2009.03.003

Gunn, B. G., Cox, C. D., Chen, Y., Frotscher, M., Gall, C. M., Baram, T. Z., & Lynch, G. (2017). The endogenous stress hormone CRH modulates excitatory transmission and network physiology in hippocampus. *Cerebral Cortex*, *27*(8), 4182–4198. https://doi.org/10.1093/cercor/bhx103

Hennø, L. T., Storjord, E., Christiansen, D., Bergseth, G., Ludviksen, J. K., Fure, H., Barene, S., Waage-Nielsen, E., Mollnes, T. E., & Brekke, O. L. (2017). Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines – Consequences for defining reference values in healthy humans. *Cytokine*, *97*(May), 86–95. https://doi.org/10.1016/j.cyto.2017.05.014

Hirotsu, C., Rydlewski, M., Araújo, M. S., Tufik, S., & Andersen, M. L. (2012). Sleep Loss and Cytokines Levels in an Experimental Model of Psoriasis. *PLoS ONE*, *7*(11), 1–9. https://doi.org/10.1371/journal.pone.0051183

Hirotsu, C., Tufik, S., & Andersen, M. L. (2015). Interactions between sleep, stress, and metabolism: From physiological to pathological conditions. *Sleep Science*, *8*(3), 143–152. https://doi.org/10.1016/j.slsci.2015.09.002

Hirshkowitz, M., Whiton, K., Albert, S. M., Alessi, C., Bruni, O., DonCarlos, L., Hazen, N., Herman, J., Katz, E. S., Kheirandish-Gozal, L., Neubauer, D. N., O'Donnell, A. E., Ohayon, M., Peever, J., Rawding, R., Sachdeva, R. C., Setters, B., Vitiello, M. V., Ware, J. C., & Adams Hillard, P. J. (2015). National sleep foundation's sleep time duration recommendations: Methodology and results summary. *Sleep Health*, *1*(1), 40–43. https://doi.org/10.1016/j.sleh.2014.12.010

Ibarra-Coronado, E. G., Pantaleón-Martínez, A. M., Velazquéz-Moctezuma, J., Prospéro-García, O., Méndez-Díaz, M., Pérez-Tapia, M., Pavón, L., & Morales-Montor, J. (2015). The Bidirectional Relationship between Sleep and Immunity against Infections. *Journal of Immunology Research*, *2015*, 678164. https://doi.org/10.1155/2015/678164

Irwin, M. R., Wang, M., Campomayor, C. O., Collado-Hidalgo, A., & Cole, S. (2006). Sleep deprivation and activation of morning levels of cellular and genomic markers of inflammation. *Archives of Internal Medicine*, *166*(16), 1756–1762. https://doi.org/10.1001/archinte.166.16.1756

Kantasa, V. D., Kusumawardani, B., & Herniyati. (2016). Efek Stresor Rasa Sakit Renjatan Listrik terhadap Limfosit dan Makrofag pada Gingiva Tikus Sprague Dawley (The Effect of Electrical Shock Stressor on Lymphocytes and Macrophages in Gingival Tissue of Sprague Dawley Rats). *Pustaka Kesehtan*, *4*(1), 48–54.

Kim, J., Yun, K. sang, Cho, A., Kim, D. H., Lee, Y. K., Choi, M. J., Kim, S. hyung, Kim, H., Yoon, J. W., & Park, H. C. (2022). High cortisol levels are associated with oxidative stress and mortality in maintenance hemodialysis patients. *BMC Nephrology*, *23*(1), 1–9. https://doi.org/10.1186/s12882-022-02722-w

Lestarianto, J. A. (2014). Hubungan antara internet addiction dan tingkat stres dengan kejadian insomnia pada mahasiswa keperawatan fakultas ilmu kesehatan universitas muhammadiyah. *Diss. Universitas Muhammadiyah Purwokerto*. http://repository.ump.ac.id/id/eprint/5503

Lichtenstein, G. R. (2015). The Importance of Sleep. *Gastroenterology & Hepatology*, *11*(12), 790.

Mattice, C. D., Brooks, R., & Lee-Chiong, T. (2011). Fundamentals of Sleep Technology: Second edition. *Philadelphia: Lippincott Williams & Wilkins.*

Medic, G., Wille, M., & Hemels, M. E. H. (2017). Short- and long-term health consequences of sleep disruption. *Nature and Science of Sleep*, *9*, 151–161. https://doi.org/10.2147/NSS.S134864

Neto, J. C. R., Lira, F. S., Venancio, D. P., Cunha, C. A., Oyama, L. M., Pimentel, G. D., Tufik, S., Do Nascimento, C. M. O., Santos, R. V. T., & De Mello, M. T. (2010). Sleep deprivation affects inflammatory marker expression in adipose tissue. *Lipids in Health and Disease*, *9*(1), 125. https://doi.org/10.1186/1476-511X-9-125

Olayaki, L. A., Sulaiman, S. O., & Anoba, N. B. (2015). Vitamin C Prevents Sleep Deprivation-induced Elevation in Cortisol and Lipid Peroxidation in the Rat Plasma. *Nigerian Journal of Physiological Sciences : Official Publication of the Physiological Society of Nigeria*, *30*(1–2), 5–9.

Patel, S. R., Zhu, X., Storfer-Isser, A., Mehra, R., Jenny, N. S., Tracy, R., & Redline, S. (2009). Sleep duration and biomarkers of inflammation. *Sleep*, *32*(2), 200–204. https://doi.org/10.1093/sleep/32.2.200

Patrick, Y., Lee, A., Raha, O., Pillai, K., Gupta, S., Sethi, S., Mukeshimana, F., Gerard, L., Moghal, M. U., Saleh, S. N., Smith, S. F., Morrell, M. J., & Moss, J. (2017). Effects of sleep deprivation on cognitive and physical performance in university students. *Sleep and Biological Rhythms*, *15*(3), 217–225. https://doi.org/10.1007/s41105-017-0099-5

Pejovic, S., Basta, M., Vgontzas, A. N., Kritikou, I., Shaffer, M. L., Tsaoussoglou, M., Stiffler, D., Stefanakis, Z., Bixler, E. O., & Chrousos, G. P. (2013). Effects of recovery sleep after one work week of mild sleep restriction on interleukin-6 and cortisol secretion and daytime sleepiness and performance. *American Journal of Physiology - Endocrinology and Metabolism*, *305*(7), 890–896. https://doi.org/10.1152/ajpendo.00301.2013

Praharaj, S. K., Gupta, R., & Gaur, N. (2018). Clinical practice guideline on management of sleep disorders in the elderly. *Indian Journal of Psychiatry*, *60*(7), S383–S396. https://doi.org/10.4103/0019-5545.224477

Putri, D. N. E., Nasrul, E., & Masri, M. (2017). Pengaruh Kurang Tidur terhadap Berat Badan pada Tikus Wistar Jantan. *Jurnal Kesehatan Andalas*, *6*(1), 78–82. https://doi.org/10.25077/jka.v6i1.655

Rockstrom, M. D., Chen, L., Taishi, P., Nguyen, J. T., Gibbons, C. M., Veasey, S., & Krueger, J. M. (2018). Tumor necrosis factor alpha in sleep regulation. *Sleep Medicine Reviews*, *40*, 69–78. https://doi.org/10.1016/j.smrv.2017.10.005.Tumor

Ruiz, F. S., Andersen, M. L., Martins, R. C. S., Zager, A., Lopes, J. D., & Tufik, S. (2012). Immune alterations after selective rapid eye movement or total sleep deprivation in healthy male volunteers. *Innate Immunity*, *18*(1), 44–54. https://doi.org/10.1177/1753425910385962

Saraiva, M., & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature Reviews Immunology*, *10*(3), 170–181. https://doi.org/10.1038/nri2711

Shearer, W. T., Reuben, J. M., Mullington, J. M., Price, N. J., Lee, B. N., O'brian Smith, E., Szuba, M. P., Van Dongen, H. P. A., & Dinges, D. F. (2001). Soluble TNF-α receptor 1 and IL-6 plasma levels in humans subjected to the sleep deprivation model of spaceflight. *Journal of Allergy and Clinical Immunology*, *107*(1), 165–170. https://doi.org/10.1067/mai.2001.112270

Simpson, N., & Dinges, D. F. (2007). Sleep and Inflammation. *Nutrition Reviews*, *65*(SUPPL.3), S244–S252. https://doi.org/10.1111/j.1753-4887.2007.tb00371.x

Siregar, E. M. H. (2017). Korelasi Kualitas Tidur dengan Kadar Interleukin-6 Serum pada Pasien Hemodialisis Regular. In *Thesis Universitas Sumatera Utara*.

Sukendra, D. M. (2015). Efek Olahraga Ringan Pada Fungsi Imunitas Terhadap Mikroba Patogen: Infeksi Virus Dengue. *Media Ilmu Keolahragaan Indonesia*, *5*(2), 57–65.

Van Dongen, H. P. A., Belenky, G., & Krueger, J. M. (2011). A Local, Bottom-Up Perspective on Sleep Deprivation and Neurobehavioral Performance. *Current Topics in Medicinal Chemistry*, *11*(19), 2414–2422. https://doi.org/doi: 10.2174/156802611797470286.

Vgontzas, A. N., Papanicolaou, D. A., Bixler, E. O., Hopper, K., Lotsikas, A., Lin, H. M., Kales, A., & Chrousos, G. P. (2000). Sleep apnea and daytime sleepiness and fatigue:

Relation to visceral obesity, insulin resistance, and hypercytokinemia. *Journal of Clinical Endocrinology and Metabolism*, *85*(3), 1151–1158. https://doi.org/10.1210/jcem.85.3.6484

Welsh, P., Murray, H. M., Ford, I., Trompet, S., De Craen, A. J. M., Jukema, J. W., Stott, D. J., McInnes, I. B., Packard, C. J., Westendorp, R. G. J., & Sattar, N. (2011). Circulating interleukin-10 and risk of cardiovascular events: A prospective study in the elderly at risk. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(10), 2338–2344. https://doi.org/10.1161/ATVBAHA.111.231795

Wright Jr, K. P., Drake, A. L., Frey, D. J., Fleshner, M., Desouza, C. A., Gronfier, C., & Czeisler, C. A. (2015). Influence of sleep deprivation and circadian misalignment on cortisol, inflammatory markers, and cytokine balance. *Physiology & Behavior*, *47*, 24–34. https://doi.org/10.1016/j.bbi.2015.01.004.Influence

Xu, C., Wu, X., Hack, B. K., Bao, L., & Cunningham, P. N. (2015). TNF causes changes in glomerular endothelial permeability and morphology through a Rho and myosin light chain kinase-dependent mechanism. *Physiological Reports*, *3*(12), 1–16. https://doi.org/10.14814/phy2.12636

Zabidi, L., Supriatna, M., Departemen, M. M., Kesehatan, I., Fk-Undip, A., Rsup, /, Semarang, K., & Mexitalia, M. (2015). Kadar Kortisol Serum sebagai Indikator Prognosis Sepsis pada Anak Serum Cortisol Level as Prognostic Indicator for Pediatric Sepsis. *Sari Pediatri*, *17*(2), 101–106.

LEVELS OF CORTISOL AND INFLAMMATORY CYTOKINES AFTER THE INDUCTION OF VARIOUS SLEEP DEPRIVATION STRESS MODELS IN MALE WISTAR RATS

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ABSTRACT

Sleep deprivation (SD) can modulate the production of various cytokines, including pro-inflammatory cytokines such as IL-6, TNF-α, and IFN-γ, and anti-inflammatory cytokines such as IL-10. Paradoxical sleep deprivation (PSD) increases the risk of inflammation but can be relieved by sleep recovery (SR). This study aimed to determine the differences in levels of cortisol and inflammatory cytokines (IL-6, IL-10, TNF-α, dan IFN-γ) in male Wistar rats (*Rattus norvegicus*) after induction of various sleep deprivation stress models. Twenty-five of male Wistar rats were randomly divided into five groups: control, PSD (20 hours of SD/day for five days), Total Sleep Deprivation or TSD (24 hours of SD/day for five days), PSD+SR (PSD followed by SR), and TSD+SR (TSD followed by SR). The plasma cortisol levels were measured with ELISA, and inflammatory cytokine levels were measured with immunoassay and calculated with fold change. Mean cortisol levels were significantly increased in treatment groups compared to the control group (p=0.029). Multivariate analysis showed no statistically significant difference in inflammatory cytokine levels of IL-6 (p=0.658), IL-10 (p=0.065), TNF-α (p=0.399), and IFN-γ (p=0.283) in all groups. In conclusion, various sleep deprivation stress models affect cortisol levels but not inflammatory cytokine levels of IL-6, IL-10, TNF-α, and IFN-γ among male Wistar rats.

Keywords: cortisol, inflammatory cytokines, sleep deprivation, sleep recovery

INTRODUCTION

Sleep plays a vital role in life by maintaining the optimal physiological function of the human body. It combines stimulation of afferent nerve delivery to the brain and activation of functional neurons in specific brain areas (Chokroverty et al., 2017). Good sleep is determined by the quality of sleep, how deep a person sleeps, and the quantity of sleep, amount of time as a person sleeps (Patrick et al., 2017). According to the National Sleep Foundation, a young adult (18-25 years) or adult (26-64 years) needs 7-9 hours of sleep daily, but not less than 6 hours or more than 11 hours for young adults or 10 hours for

adults. Otherwise, an older adult (65 years and over) requires at least 7-8 hours of sleep daily, but not less than 5 hours or more than 9 hours (Lichtenstein, 2015). People who live in developed countries are chronically sleep-deprived because of their cultural and socioeconomic environments. Incidence of symptoms related to sleep deprivation has recently increased, suggesting its long-term detrimental health effects are more abundant than expected (Chokroverty & Ferini-Strambi, 2017).

A study by Hirshkowitz et al. (2015) showed that > 30% of men and women between the ages of 30-64 years sleep less than 6 hours per day, and 5-15% of the world population suffer from sleep disorders. Another study indicated that 40-70% of older adults have chronic sleep disorders that are more prominent in people with medical or psychiatric comorbidity (Praharaj et al., 2018). The National Sleep Foundation stated that 70% of Indonesian people experience sleep disturbances at least once a week, and 30 million people have sleeping difficulty every night (Lestarianto, 2014). A survey of the healthy lifestyle index by American International Assurance (AIA) in 2013 demonstrated that Indonesian people have an average sleep duration of 6.8 hours per day due to increased activity and decreased sleep duration from its normal range, indicating many Indonesian people are suffered from sleep deprivation (Putri et al., 2017).

The relationship between sleep duration and the inflammation process has not been studied extensively, despite showing a similar correlation between sleep duration and mortality. People with long sleep duration (>8 hours/night) and short sleep duration (<7 hours/night) have a 30% and 12% greater risk of death, respectively, compared to people with moderate sleep duration (7-8 hours/night). Physiologically, sleep affects two major effector systems, the Hypothalamus-Pituitary-Adrenal (HPA) axis and the sympathetic nervous system, which simultaneously increases the release of pro-inflammatory cytokines and markers of systemic inflammation through β-adrenergic activation. The activity of sympathetic nervous system decreases when a person is sleeping, which may explain the relationship between sleep disorder, short sleep duration, and elevation of inflammatory markers (Chen et al., 2017). Patients that pathologically tend to fall asleep during daytime and easily get tired exhibit an increase in IL-6, a pro-inflammatory cytokine, in the circulatory system due to activation of the HPA Axis. People suffering from sleep deprivation produce more IL-6 during the day and less IL-6 at night, whereas good sleep reduces pro-inflammatory exposure to tissues. Furthermore, sleep deprivation upregulates pro-inflammatory cytokines through NF-kB activation, generating a pro-inflammatory state.

Overexposure to the pro-inflammatory cytokines caused by sleep deprivation will increase tissue damage, decrease brain function, and increase the expression of IL-6 (Vgontzas et al., 2000).

Sleep deprivation correlates with the increase in TNF-α, indicating a pathological condition caused by sleep disorders (Chennaoui et al., 2011). Moreover, TNF-α is expressed in neurons and plays a role in brain neuroplasticity (Rockstrom et al., 2018). On the other hand, a clinical trial showed that IFN-γ was found in cases of fever, flu, and drowsiness, whereas IFN-γ can be somnogenic in the presence of TNF-α. Therefore, IFN-γ has a significant role in sleep regulation during viral infection (Van Dongen et al., 2011).

Studies suggest that cortisol levels as a physiological stress marker and systemic inflammatory marker may increase in sleep-deprived conditions. Sleep deprivation triggers the release of adrenocorticotropic hormone (ACTH) via the HPA axis, inducing the adrenal gland to release cortisol. Along with other stimulations, including norepinephrine and radical oxygen species (ROS), cortisol triggers inflammatory activation in the brain and peripheral immune cells by increasing the expression of pro-inflammatory cytokine genes such as IL-1, IL-6, and TNF-α via transcriptional regulator of pro-inflammatory gene expression such as NF-κB. These pro-inflammatory cytokines then enter the systemic circulation inducing the increase of leukocytes, mainly neutrophils, CD4+ T-cells, B-cells, and monocyte leading to inflammatory reactions (Garbarino et al., 2021).

Sleep deprivation (SD) can be categorized into paradoxical sleep deprivation (PSD) and total sleep deprivation (TSD), and studied using the Modified Multiple Platform Method (MMPM). While both types can induce stress, total sleep deprivation causes poor decision-making control, triggers repetitive errors in working memory, and abolishes individual spontaneity in communication, making the subjects appear lazy, lethargic, and unmotivated (Gunn et al., 2017).

Previous studies showed that sleep deprivation affects cortisol and inflammatory cytokine levels in rats or humans by interfering with homeostatic sleep patterns and disrupting HPA axis activation (Chennaoui et al., 2011; Medic et al., 2017; Neto et al., 2010; Wright et al., 2015). While sleep recovery may restore rat deteriorating conditions caused by sleep deprivation, no studies clearly define the effect of sleep recovery on pro-inflammatory and anti-inflammatory cytokine levels. This study directly compared cortisol and inflammatory cytokine level among rats treated with PSD, TSD, and sleep recovery after PSD and TSD treatment. Considering that many people have experienced sleep deprivation,

this study is essential as initial research to determine the effect of sleep deprivation and sleep recovery on the levels of cortisol and inflammatory cytokines that impair various metabolic functions in mammals.

EXPERIMENTAL SECTION

Research Design

This study was an experimental study using a post-test only with control group design.

Subject

Wistar rats (*Rattus norvegicus)* with characteristics of male, 8-12 weeks old, and 200-300 grams of weight were obtained from the Laboratorium Penelitian dan Pengujian Terpadu (LPPT) 4 Universitas Gajah Mada. Sleep deprivation experiments were performed using the Modified Multiple Platform Method (MMPM) to prevent the rats from sleeping. A total of 25 rats were randomly divided into five groups containing five rats in each group: control (no SD), PSD (20 hours of SD per day at 11.00 a.m. - 07.00 a.m. for 5 days and 4 hours rest at 07.00 a.m. - 11.00 a.m.), TSD (24 hours of SD per day at 11.00 a.m. - 11.00 a.m. for 5 days without rest), PSD+SR (PSD treatment for 5 days followed by sleep recovery (SR) for 5 days at 07.00 a.m. - 07.00 a.m.), and TSD+SR (TSD treatment for 5 days followed by sleep recovery for 5 days at 11.00 a.m. - 11.00 a.m.).

Research Procedure

Modified Multiple Platform Method (MMPM) tanks measuring 123 x 44 x 35 cm were used for the sleep deprivation stress model. The tanks were filled with water and equipped with 12 platforms of 6.5 cm in size with a distance of 10 cm between the platforms as a foothold for the rats that are instinctively avoiding water. Each tank contains five experimental rats equipped with a muscle atonia device, an instrument that gives an automatic shock effect every 10 minutes to keep the rats awake. The experiments were conducted in the Anatomy Laboratory of the Faculty of Medicine of Jenderal Soedirman University from August to September 2021.

Observation

Cortisol levels were measured by drawing 2 ml of rat blood for each group at 07.00-09.00 a.m. via retroorbital vein using a microhematocrit pipette into an EDTA tube,

centrifuging for 15 minutes at 2,000-3,000 rpm, and storing in -20 °C. A total of 50 mL of collected plasma was examined with the Enzyme-Linked Immunosorbent Assay (ELISA) method and read by the ELISA reader in 450 nm absorbance (Zabidi et al., 2015).

For inflammatory cytokines level measurement, rat blood plasma samples were collected from the retroorbital plexus as much as 55 mL. The levels of inflammatory cytokines of IL-6, IL-10, TNF-α, and IFN-γ were then measured using multiplex bead-based immunoassay from MILLIPLEX® MAP kit with Luminex technology in the Integrated Laboratory of Faculty of Medicine, Universitas Indonesia. Briefly, the frozen samples were thawed, stirred thoroughly with a vortex, and centrifuged to remove particulates before the immunoassay. The processed samples were then placed on the plate, added with conjugated beads from the MILLIPLEX® MAP kit, and incubated for 2 hours at 20-24 ˚C or overnight at 4-8 ˚C. After incubation, the beads were washed, incubated in biotinylated-detection antibodies, added with Streptavidin-PE (SAPE), and further incubated. The beads were then washed, resuspended in an appropriate buffer, and placed on Luminex technology to measure cytokine levels. The plasma cytokine levels were analyzed based on the fold change value by comparing the treatment and the control group.

Statistical analysis

Normality analysis was performed by the Shapiro-Wilk test, and data homogeneity was analyzed by Levene's test. The multivariate analysis was then performed by the One-Way ANOVA and Post-hoc Least Significant Difference (LSD) to determine differences between groups. Kruskal-Wallis and Mann-Whitney tests were performed for non-parametric analysis.

All procedures were approved by The Medical Research Ethics Commission of The Faculty of Medicine of Jenderal Soedirman University on May 10, 2021 (reference number: 097/KEPK/V/2021).

RESULT AND DISCUSSION

Measurement and analysis results of mean cortisol levels among all groups are shown in **Figure 1**. Since the plasma cortisol levels were not normally distributed, non-parametric tests were used for further analysis.

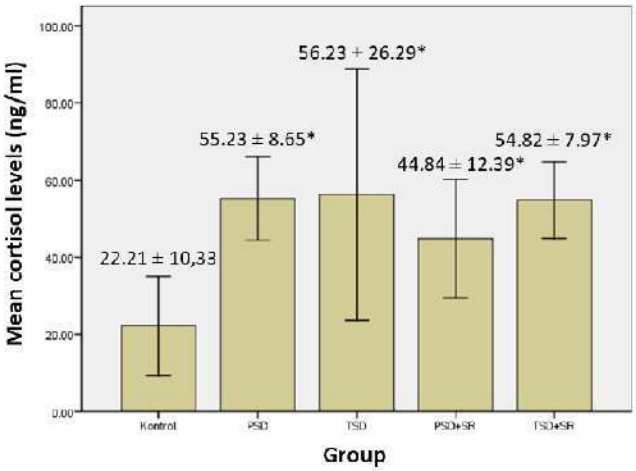

Figure 1. Mean cortisol levels in male Wistar rat (*Rattus norvegicus*). Control: no SD; PSD: 20 hours of SD with 4 hours rest for five days; TSD: 24 hours of SD for five days; PSD+SR: PSD followed by SR; TSD+SR: TSD followed by SR. SD: sleep deprivation, SR: sleep recovery, PSD: paradoxical sleep deprivation, TSD: total sleep deprivation. Error bars represent 95% confidence intervals. Statistical analysis was performed using Kruskal-Wallis test ($p = 0.029$) and Mann-Whitney test for post-hoc analysis. *Post-hoc Mann-Whitney test compared to control, $p < 0.05$.

Kruskal-Wallis analysis showed substantial differences in cortisol levels between at least two groups for the assessed variables (p=0.029). Further post-hoc analysis with the Mann-Whitney test obtained significant results between every intervention group and the control ($p<0.05$). PSD for 20 hours/day increased cortisol levels in the afternoon and evening in rats by two times compared to control (Olayaki et al., 2015). Cortisol levels increased in the first six hours after PSD induction caused by HPA axis activation to maintain metabolism changes that were occurred during sleep deprivation (Galvão et al., 2009). Consistent with a study by Olayaki et al. (2015) demonstrating that TSD for five days increased cortisol levels compared to the control, this study pointed out that the TSD group had the highest mean cortisol levels among all groups (56.23±26.29 ng/mL).

The PSD group showed lower mean cortisol levels than the TSD group because PSD has a sleep period daily, thus the negative impact of sleep deprivation can be relieved. Two hours of an afternoon nap after one night of sleep deprivation decreased the secretion of cortisol hormone caused by inhibition of the HPA axis at the slow wave sleep (SWS) phase that reduces cortisol levels (Pejovic et al., 2013). The effects of sleep deprivation that impair HPA function cause an increase in evening cortisol levels as a result of an elevation in its

secretory pulses amplitude, suggesting that the negative feedback of glucocorticoid mediated by hippocampal function may be affected by sleep-deprived condition (Hirotsu et al., 2015). Cortisol levels were higher in the subjects who stayed awake than in subjects who slept sufficiently, indicating that sleep deprivation affects the cortisol levels as a result of stress responses (Bassett et al., 2018). Cauter et al. (2008) emphasized that the initial consequence of partial sleep loss is a cortisol level increase in the evening. While under normal conditions, cortisol levels decrease rapidly and reach the minimum level just before sleeping time. Kantasa et al. (2016) stated that cortisol can suppress the immune system and induce an inflammatory pathway, making the body vulnerable to various diseases.

PSD + SR group exhibited lower cortisol levels than TSD + SR, and both PSD + SR and TSD + SR showed lower cortisol levels than PSD and TSD. A study by Mattice et al. (2011) showed that subjects induced by TSD for 24 hours and 48 hours followed by sleep recovery for 24 hours relieved their sleep deprivation effect for 72% and 42%, respectively. Sleep recovery for three days decreases cortisol levels and ameliorates the impact of sleep deprivation by restoring the HPA axis interaction (Pejovic et al., 2013). Sleep recovery also decreases lipid peroxidase and free radical production by increasing glutathione and other enzymatic antioxidants (Hirotsu et al., 2015). Glutathione increases after sleep recovery for two days, indicated by the elevation of 6-PGD, an enzyme that acts on the pentose phosphate pathway in the carbohydrate metabolism that protects cells from oxidative stress in the form of NADPH, thus inhibiting stress oxidative that has a role in cortisol increase (Kim et al., 2022). Taken together, these results indicate that sleep recovery is able to decrease cortisol levels which increase after induction of PSD or TSD.

Results of data analysis showing the value of minimum, maximum, mean, median, and standard deviation for each group are shown in **Table 1.**

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Table 1. Levels of inflammatory cytokine in male Wistar rat (pg/mL).

Group	N	Min.				Max.				Mean±SD			
		IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ
Control	5	311,14	15,14	2,89	736,59	1706,25	83,31	12,06	1375,77	974± 504	34,42±11,92	6,91±3,28	1038,08±272,26
PSD+SR	5	452,10	17,52	4,54	754,54	3103,86	93,76	23,89	1001,67	1281±1056	53,07±12,98	9,75±8,05	847,45±102,73
TSD+SR	5	311,14	57,89	6,54	718,80	2518,29	317,89	26,13	1001,67	1578±928	87,06±47,99	14,17±8,17	865,83±112,95
PSD	5	520,98	44,84	6,90	781,54	1858,94	74,98	12,24	1385,65	1192±544	59,47±5,44	9,39±2,62	1046,28±239,32
TSD	5	382,22	33,52	1,76	700,85	1365,64	178,02	14,17	1824,71	952±373	68,06±25,53	8,01±4,39	1167,39±432,52

Table 2. P-value of normality, homogeneity, and One-Way ANOVA test in the levels of inflammatory cytokines in male Wistar rat.

Group	Shapiro-Wilk (p-value)				Levene's (p-value)				One-Way Anova (p-value)			
	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ
Control	0,832	0,941	0,389	0,640								
PSD+SR	0,884	0,482	0,081	0,409								
TSD+SR	0,812	0,909	0,096	0,921	0,256	0,352	0,697	0,075	0,658	0,065	0,399	0,283
PSD	0,067	0,845	0,200	0,610								
TSD	0,622	0,500	0,615	0,750								

Normality analysis by the Shapiro-Wilk test and homogeneity analysis by the Levene's test showed a p-value >0.05 in all groups, indicating data of inflammatory cytokine levels were normally distributed and homogenous. Multivariate analysis using the One-Way ANOVA test demonstrated a p-value >0.05 in all groups, indicating no significant difference in inflammatory cytokine levels among the treatment groups, as shown in **Table 2**. The inflammatory cytokine levels were then compared among the treatment and control groups using fold change. The results demonstrated the highest increase in inflammatory cytokine levels was in the TSD treatment group (**Figure 2**). However, the multivariate analysis of the fold change of inflammatory cytokine levels using the One-Way ANOVA test did not show a significant difference ($p > 0.05$) in IL-6 ($p=0.658$), IL-10 ($p=0.085$), TNF-α ($p= 0.313$), and IFN-γ ($p=0.283$).

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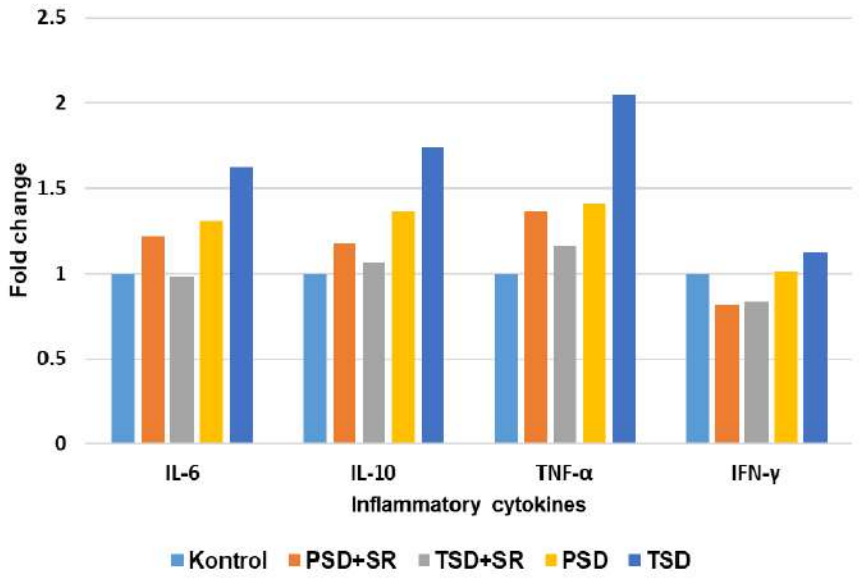

Figure 2. Mean of the fold change of inflammatory cytokine levels of IL-6, IL-10, TNF-α, and IFN-γ in male Wistar rat (*Rattus norvegicus*). Control group: no SD; PSD+SR: PSD followed by SR; TSD+SR: TSD followed by SR; PSD: 20 hours of SD with 4 hours rest for five days; TSD: 24 hours of SD for five days. SD: sleep deprivation, SR: sleep recovery, PSD: paradoxical sleep deprivation, TSD: total sleep deprivation.

Sleep deprivation triggers a stress response in animal models by increasing pro-inflammatory cytokines and activates the main stress axis in humans, the HPA axis. IL-6 can stimulate cortisol secretion directly in the adrenal gland or via activation of the hypothalamus

that induces the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. On the other hand, a slight elevation of IL-6 level in plasma induces the anti-inflammatory cytokines, IL-1ra and IL-10, along with c-reactive protein (CRP). Recent studies stated that apart from injury and infection, sleep deprivation triggers the pro-inflammatory response through the increase of pro-inflammatory cytokines secretion (Medic et al., 2017; Simpson & Dinges, 2007; Sukendra, 2015).

Activation of inflammatory responses caused by sleep deprivation can increase the levels of anti-inflammatory cytokines to prevent excessive inflammatory responses. IL-10, a potent anti-inflammatory cytokine, inhibits the activation of macrophages and dendritic cells, leading to the reduction of cytokine levels produced by T-helper1 (Th1) cells (Welsh et al., 2011). IL-10 also potently inhibits the production of IL-10 itself, IL-12, L-1α, IL-1β, IL-6, macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), leukemia inhibitory factor (LIF), and platelet-activating factor (PAF) from activated monocytes and macrophages (Saraiva & O'Garra, 2010).

This study showed that sleep deprivation did not significantly affect levels of IL-10 in each variable ($p = 0.065$). This result is consistent with the study of Neto et al. (2010), showing that sleep deprivation did not impair levels of anti-inflammatory cytokines, including IL-10. Otherwise, they also observed sleep deprivation increased IL-10 levels in different adipose tissue depots and decreased the TNF-α levels in the brain. Therefore, the profile of systemic anti-inflammatory cytokines after sleep deprivation remains controversial.

A study by Patel et al. (2009) also demonstrated that sleep deprivation did not affect levels of anti-inflammatory cytokines such as IL-1 and IL-10. They explained that the different responses of sleep deprivation on the levels of anti-inflammatory cytokines may depend on the differences in the effect of sleep on inflammatory components in each individual, the half-life of each inflammatory component, and the instrument performance for cytokine measurement. Intriguingly, anti-inflammatory cytokine levels increase in chronic sleep deprivation that lasts over weeks, months, or years.

Wright et al. (2015) demonstrated that sleep deprivation significantly increased levels of IL-10 on day 26 to day 28 measured with ELISA. Sleep deprivation induces circadian rhythm impairment that, over weeks, will increase levels of anti-inflammatory cytokine (IL-10), the pro-inflammatory protein (TNF-α), and CRP. Otherwise, cortisol levels increase

during acute sleep deprivation, indicating that acute and chronic sleep deprivation provoke different responses of circadian rhythm impairment. Therefore, acute sleep deprivation is associated with physiological stress or metabolic response of increased cortisol levels, whereas chronic sleep deprivation is associated with physiological adaptation as a response to decreased cortisol levels followed by increased pro-inflammatory and anti-inflammatory cytokines.

No significant difference in TNF-α levels after induction of various sleep deprivation stress models in this study ($p = 0.366$) is consistent with the study by Ruiz et al. (2012), showing that total sleep deprivation for two days or paradoxical sleep deprivation for four days followed by sleep recovery for three days did not alter TNF-α levels. Other studies also demonstrated that ten days of sleep deprivation did not increase TNF-α or its receptors, regardless of sex characteristics (Shearer et al., 2001; Xu et al., 2015). In contrast, a study by Irwin et al. (2006) exhibited that sleep deprivation increased TNF-α production. Sleep deprivation probably induced TNF-α cellular expression through the activation of nuclear factor NF-kB, a key component in controlling the cellular expression of pro-inflammatory cytokines.

In agreement with the study by Crooks et al. (2019) that demonstrated no significant result between sleep quality and IL-6 levels, this study approved that there was no significant result between the levels of pro-inflammatory cytokines, IL-6, with various sleep deprivation stress models ($p = 0.658$). Conversely, a study by Siregar (2017) showed a significant result between sleep quality and IL-6 levels. Sleep deprivation may affect components of the immune system through modification of CD4+ cells, CD8+ cells, NK cells, and levels of pro-inflammatory cytokines such as TNF-α, INF-γ, and IL-6 (Ibarra-Coronado et al., 2015). Collectively, these results indicate that pure TSD and PSD or TSD and PSD followed by sleep recovery did not affect plasma cytokine levels of IL-6 and TNF-α.

Multivariate analysis among the treatment groups showed no significant difference between sleep deprivation and IFN-γ levels ($p=0.283$). This result is consistent with the study by Hirotsu et al. (2012), demonstrating that there was no significant difference in IFN-γ levels in psoriasis rats model induced by paradoxical sleep deprivation for 48 hours. IFN-γ production is generally unaffected by sleep deprivation, instead is increased at the end of sleep recovery. These insignificant results may be affected by sample processing that caused cytokine stability disruption, cytokine degradation by protease, or cytokine binding to its

soluble cellular receptors. These alterations may also explain the decrease in cytokine levels over time during sample storage (Hennø et al., 2017).

The main limitation of this study is that it only examined the level of inflammatory cytokine levels in plasma but not in the tissue resulting in an insignificant increase in inflammatory cytokine levels after sleep deprivation treatment. Induction of inflammatory cytokine release is likely more prominent in the brain or other tissues, which are initially released by immune and glial cells in the brain after activation of the HPA axis (Garbarino et al., 2021). Another limitation is that the stress induction protocol by sleep deprivation in this study was not enough to induce an increase in the cytokine level in plasma. Various sleep deprivation protocols may also cause different results in inflammatory cytokine levels among studies. Furthermore, variation in the circadian cycle may also affect the production of various cytokines, which confounds the levels of circulating cytokines in this study (Ruiz et al., 2012).

To date, studies concerning sleep deprivation and inflammatory cytokines remain elusive. Sleep deprivation indeed increases the main stress hormone, cortisol, which plays a role in various disease development, such as hypertension and cardiovascular disease (Sá Gomes e Farias et al., 2022). On the other hand, some studies indicated that people with sleep disorders showed an alteration in circulating levels of TNF-α and IL-6. However, other studies failed to demonstrate the change in TNF-α and IL-6 levels between normal and sleep-deprived subjects (Crooks et al., 2019; Irwin et al., 2006; Shearer et al., 2001; Siregar, 2017). Although this study showed insignificant results, sleep deprivation also has a potency to increase the pro-inflammatory cytokine expression that is associated with inflammatory reaction-related diseases such as cancer, neurodegeneration, and cardiovascular disease (Garbarino et al., 2021). Establishing how far sleep deprivation can affect cortisol and inflammatory cytokine levels and how much sleep recovery is required to alleviate the physiological disruption caused by sleep deprivation is essential to making recommendations for a healthy lifestyle. Furthermore, in this modern era, night shift job is increasing in various sectors, and the future development of this study can be used to make recommendations for working regulation to create work-life balance.

CONCLUSION

Stress induction by the various models of sleep deprivation modifies cortisol levels but not inflammatory cytokines levels of IL-6, IL-10, TNF-α, and IFN-γ in male Wistar rats (*Rattus norvegicus*). These results indicate cortisol levels can be used as a stress indicator induced by sleep deprivation, but inflammatory cytokines levels of IL-6, IL-10, TNF-α, and IFN-γ may not be used for this purpose. Furthermore, although not statistically different, sleep recovery restores the levels of cortisol and inflammatory cytokines after PSD or TSD treatment, suggesting sleep recovery able to bring back homeostasis conditions after stress. Further exploration of sleep deprivation and sleep recovery protocols is required to get valuable outcomes.

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REFERENCES

Commented [Z3]: The title of the article in Indonesian is written in brackets in the English title..... 1. Lestarianto, J. A. (2014). 2. Putri, D. N. E. 3. Siregar, E. M. H 4. Sukendra, D. M. (2015). 5. Zabidi,
Judul artikel hanya huruf pertama yg besar. Kecuali hal2 tertentu. Sdh sy perbaiki

Bassett, S. M., Lupis, S. B., Gianferante, D., Rohleder, N., & Wolf, J. M. (2018). Sleep quality but not sleep quantity effects on cortisol responses to acute psychosocial stress. *Revista Del Colegio Americano de Cardiologia*, *72*(23), 2964–2979. https://doi.org/10.3109/10253890.2015.1087503.Sleep

Cauter, E. V., Spiegel, K., Tasali, E., & Leproult, R. (2008). Metabolic consequences of sleep and sleep loss. *Physiology & Behavior*, *176*(3), 139–148. https://doi.org/10.1016/S1389-9457(08)70013-3

Chen, L., Huang, J., Yang, L., Zeng, X. A., Zhang, Y., Wang, X., Chen, M., Li, X., Zhang, Y., & Zhang, M. (2017). Sleep deprivation accelerates the progression of alzheimer's disease by influencing Aβ-related metabolism. *Neuroscience Letters*, *650*, 146–152. https://doi.org/10.1016/j.neulet.2017.04.047

Chennaoui, M., Sauvet, F., Drogou, C., Van Beers, P., Langrume, C., Guillard, M., Gourby, B., Bourrilhon, C., Florence, G., & Gomez-Merino, D. (2011). Effect of one night of sleep loss on changes in tumor necrosis factor alpha (TNF-α) levels in healthy men. *Cytokine*, *56*(2), 318–324. https://doi.org/10.1016/j.cyto.2011.06.002

Chokroverty, S., & Ferini-Strambi, L. (2017). Oxford textbook of sleep disorders. In *Oxford*

University Press.

Crooks, E., Hansen, D. A., Satterfield, B. C., Layton, M. E., & Van Dongen, H. P. A. (2019). Cardiac autonomic activity during sleep deprivation with and without caffeine administration. *Physiology & Behavior*, *210*, 112643. https://doi.org/10.1016/j.physbeh.2019.112643.Cardiac

Galvão, M. de O. L., Sinigaglia-Coimbra, R., Kawakami, S. E., Tufik, S., & Suchecki, D. (2009). Paradoxical sleep deprivation activates hypothalamic nuclei that regulate food intake and stress response. *Psychoneuroendocrinology*, *34*(8), 1176–1183. https://doi.org/10.1016/j.psyneuen.2009.03.003

Garbarino, S., Lanteri, P., Bragazzi, N. L., Magnavita, N., & Scoditti, E. (2021). Role of sleep deprivation in immune-related disease risk and outcomes. *Communications Biology*, *4*(1). https://doi.org/10.1038/s42003-021-02825-4

Gunn, B. G., Cox, C. D., Chen, Y., Frotscher, M., Gall, C. M., Baram, T. Z., & Lynch, G. (2017). The endogenous stress hormone CRH modulates excitatory transmission and network physiology in hippocampus. *Cerebral Cortex*, *27*(8), 4182–4198. https://doi.org/10.1093/cercor/bhx103

Hennø, L. T., Storjord, E., Christiansen, D., Bergseth, G., Ludviksen, J. K., Fure, H., Barene, S., Waage-Nielsen, E., Mollnes, T. E., & Brekke, O. L. (2017). Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines – Consequences for defining reference values in healthy humans. *Cytokine*, *97*(May), 86–95. https://doi.org/10.1016/j.cyto.2017.05.014

Hirotsu, C., Rydlewski, M., Araújo, M. S., Tufik, S., & Andersen, M. L. (2012). Sleep loss and cytokines levels in an experimental model of psoriasis. *PLoS ONE*, *7*(11), 1–9. https://doi.org/10.1371/journal.pone.0051183

Hirotsu, C., Tufik, S., & Andersen, M. L. (2015). Interactions between sleep, stress, and metabolism: From physiological to pathological conditions. *Sleep Science*, *8*(3), 143–152. https://doi.org/10.1016/j.slsci.2015.09.002

Hirshkowitz, M., Whiton, K., Albert, S. M., Alessi, C., Bruni, O., DonCarlos, L., Hazen, N., Herman, J., Katz, E. S., Kheirandish-Gozal, L., Neubauer, D. N., O'Donnell, A. E., Ohayon, M., Peever, J., Rawding, R., Sachdeva, R. C., Setters, B., Vitiello, M. V., Ware, J. C., & Adams Hillard, P. J. (2015). National sleep foundation's sleep time duration recommendations: Methodology and results summary. *Sleep Health*, *1*(1), 40–43. https://doi.org/10.1016/j.sleh.2014.12.010

Ibarra-Coronado, E. G., Pantaleón-Martínez, A. M., Velazquéz-Moctezuma, J., Prospéro-García, O., Méndez-Díaz, M., Pérez-Tapia, M., Pavón, L., & Morales-Montor, J. (2015). The Bidirectional relationship between sleep and immunity against infections. *Journal of Immunology Research*, *2015*, 678164. https://doi.org/10.1155/2015/678164

Irwin, M. R., Wang, M., Campomayor, C. O., Collado-Hidalgo, A., & Cole, S. (2006). Sleep deprivation and activation of morning levels of cellular and genomic markers of inflammation. *Archives of Internal Medicine*, *166*(16), 1756–1762. https://doi.org/10.1001/archinte.166.16.1756

Kantasa, V. D., Kusumawardani, B., & Herniyati. (2016). Efek Stresor rasa sakit renjatan listrik terhadap limfosit dan makrofag pada gingiva tikus sprague dawley (The Effect

of electrical shock stressor on lymphocytes and macrophages in gingival tissue of sprague dawley rats). *Pustaka Kesehtan*, *4*(1), 48–54.

Kim, J., Yun, K. sang, Cho, A., Kim, D. H., Lee, Y. K., Choi, M. J., Kim, S. hyung, Kim, H., Yoon, J. W., & Park, H. C. (2022). High cortisol levels are associated with oxidative stress and mortality in maintenance hemodialysis patients. *BMC Nephrology*, *23*(1), 1–9. https://doi.org/10.1186/s12882-022-02722-w

Lestarianto, J. A. (2014). Hubungan antara internet addiction dan tingkat stres dengan kejadian insomnia pada mahasiswa keperawatan fakultas ilmu kesehatan universitas muhammadiyah. *Diss. Universitas Muhammadiyah Purwokerto*. http://repository.ump.ac.id/id/eprint/5503

Lichtenstein, G. R. (2015). The Importance of sleep. *Gastroenterology & Hepatology*, *11*(12), 790.

Mattice, C. D., Brooks, R., & Lee-Chiong, T. (2011). Fundamentals of sleep technology: Second edition. *Philadelphia: Lippincott Williams & Wilkins.*

Medic, G., Wille, M., & Hemels, M. E. H. (2017). Short- and long-term health consequences of sleep disruption. *Nature and Science of Sleep*, *9*, 151–161. https://doi.org/10.2147/NSS.S134864

Neto, J. C. R., Lira, F. S., Venancio, D. P., Cunha, C. A., Oyama, L. M., Pimentel, G. D., Tufik, S., Do Nascimento, C. M. O., Santos, R. V. T., & De Mello, M. T. (2010). Sleep deprivation affects inflammatory marker expression in adipose tissue. *Lipids in Health and Disease*, *9*(1), 125. https://doi.org/10.1186/1476-511X-9-125

Olayaki, L. A., Sulaiman, S. O., & Anoba, N. B. (2015). Vitamin C prevents sleep deprivation-induced elevation in cortisol and lipid peroxidation in the rat plasma. *Nigerian Journal of Physiological Sciences : Official Publication of the Physiological Society of Nigeria*, *30*(1–2), 5–9.

Patel, S. R., Zhu, X., Storfer-Isser, A., Mehra, R., Jenny, N. S., Tracy, R., & Redline, S. (2009). Sleep duration and biomarkers of inflammation. *Sleep*, *32*(2), 200–204. https://doi.org/10.1093/sleep/32.2.200

Patrick, Y., Lee, A., Raha, O., Pillai, K., Gupta, S., Sethi, S., Mukeshimana, F., Gerard, L., Moghal, M. U., Saleh, S. N., Smith, S. F., Morrell, M. J., & Moss, J. (2017). Effects of sleep deprivation on cognitive and physical performance in university students. *Sleep and Biological Rhythms*, *15*(3), 217–225. https://doi.org/10.1007/s41105-017-0099-5

Pejovic, S., Basta, M., Vgontzas, A. N., Kritikou, I., Shaffer, M. L., Tsaoussoglou, M., Stiffler, D., Stefanakis, Z., Bixler, E. O., & Chrousos, G. P. (2013). Effects of recovery sleep after one work week of mild sleep restriction on interleukin-6 and cortisol secretion and daytime sleepiness and performance. *American Journal of Physiology - Endocrinology and Metabolism*, *305*(7), 890–896. https://doi.org/10.1152/ajpendo.00301.2013

Praharaj, S. K., Gupta, R., & Gaur, N. (2018). Clinical practice guideline on management of sleep disorders in the elderly. *Indian Journal of Psychiatry*, *60*(7), S383–S396. https://doi.org/10.4103/0019-5545.224477

Putri, D. N. E., Nasrul, E., & Masri, M. (2017). Pengaruh Kurang Tidur terhadap Berat Badan pada Tikus Wistar Jantan. *Jurnal Kesehatan Andalas*, *6*(1), 78–82.

https://doi.org/10.25077/jka.v6i1.655

Rockstrom, M. D., Chen, L., Taishi, P., Nguyen, J. T., Gibbons, C. M., Veasey, S., & Krueger, J. M. (2018). Tumor necrosis factor alpha in sleep regulation. *Sleep Medicine Reviews*, *40*, 69–78. https://doi.org/10.1016/j.smrv.2017.10.005.Tumor

Ruiz, F. S., Andersen, M. L., Martins, R. C. S., Zager, A., Lopes, J. D., & Tufik, S. (2012). Immune alterations after selective rapid eye movement or total sleep deprivation in healthy male volunteers. *Innate Immunity*, *18*(1), 44–54. https://doi.org/10.1177/1753425910385962

Sá Gomes e Farias, A. V., de Lima Cavalcanti, M. P., de Passos Junior, M. A., & Vechio Koike, B. del. (2022). The association between sleep deprivation and arterial pressure variations: a systematic literature review. *Sleep Medicine: X*, *4*, 100042. https://doi.org/10.1016/j.sleepx.2022.100042

Saraiva, M., & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature Reviews Immunology*, *10*(3), 170–181. https://doi.org/10.1038/nri2711

Shearer, W. T., Reuben, J. M., Mullington, J. M., Price, N. J., Lee, B. N., O'brian Smith, E., Szuba, M. P., Van Dongen, H. P. A., & Dinges, D. F. (2001). Soluble TNF-α receptor 1 and IL-6 plasma levels in humans subjected to the sleep deprivation model of spaceflight. *Journal of Allergy and Clinical Immunology*, *107*(1), 165–170. https://doi.org/10.1067/mai.2001.112270

Simpson, N., & Dinges, D. F. (2007). Sleep and inflammation. *Nutrition Reviews*, *65*(SUPPL.3), S244–S252. https://doi.org/10.1111/j.1753-4887.2007.tb00371.x

Siregar, E. M. H. (2017). Korelasi kualitas tidur dengan kadar interleukin-6 serum pada pasien hemodialisis regular. In *Thesis Universitas Sumatera Utara*.

Sukendra, D. M. (2015). Efek olahraga ringan pada fungsi imunitas terhadap mikroba patogen: infeksi virus dengue. *Media Ilmu Keolahragaan Indonesia*, *5*(2), 57–65.

Van Dongen, H. P. A., Belenky, G., & Krueger, J. M. (2011). A Local, Bottom-up perspective on sleep deprivation and neurobehavioral performance. *Current Topics in Medicinal Chemistry*, *11*(19), 2414–2422. https://doi.org/doi: 10.2174/156802611797470286.

Vgontzas, A. N., Papanicolaou, D. A., Bixler, E. O., Hopper, K., Lotsikas, A., Lin, H. M., Kales, A., & Chrousos, G. P. (2000). Sleep apnea and daytime sleepiness and fatigue: Relation to visceral obesity, insulin resistance, and hypercytokinemia. *Journal of Clinical Endocrinology and Metabolism*, *85*(3), 1151–1158. https://doi.org/10.1210/jcem.85.3.6484

Welsh, P., Murray, H. M., Ford, I., Trompet, S., De Craen, A. J. M., Jukema, J. W., Stott, D. J., McInnes, I. B., Packard, C. J., Westendorp, R. G. J., & Sattar, N. (2011). Circulating interleukin-10 and risk of cardiovascular events: A prospective study in the elderly at risk. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(10), 2338–2344. https://doi.org/10.1161/ATVBAHA.111.231795

Wright Jr, K. P., Drake, A. L., Frey, D. J., Fleshner, M., Desouza, C. A., Gronfier, C., & Czeisler, C. A. (2015). Influence of sleep deprivation and circadian misalignment on cortisol, inflammatory markers, and cytokine balance. *Physiology & Behavior*, *47*, 24–34. https://doi.org/10.1016/j.bbi.2015.01.004.Influence

Xu, C., Wu, X., Hack, B. K., Bao, L., & Cunningham, P. N. (2015). TNF causes changes in glomerular endothelial permeability and morphology through a Rho and myosin light chain kinase-dependent mechanism. *Physiological Reports*, *3*(12), 1–16. https://doi.org/10.14814/phy2.12636

Zabidi, L., Supriatna, M., Departemen, M. M., Kesehatan, I., Fk-Undip, A., Rsup, /, Semarang, K., & Mexitalia, M. (2015). Kadar Kortisol Serum sebagai Indikator Prognosis Sepsis pada Anak (Serum Cortisol Level as Prognostic Indicator for Pediatric Sepsis. *Sari Pediatri*, *17*(2), 101–106.

LEVELS OF CORTISOL AND INFLAMMATORY CYTOKINES AFTER THE INDUCTION OF VARIOUS SLEEP DEPRIVATION STRESS MODELS IN MALE WISTAR RATS

KADAR KORTISOL DAN SITOKIN INFLAMASI PASCA INDUKSI BERBAGAI MODEL STRES *SLEEP DEPRIVATION* PADA TIKUS PUTIH JANTAN

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ABSTRACT

Sleep deprivation (SD) can modulate the production of various cytokines, including pro-inflammatory cytokines such as IL-6, TNF-α, and IFN-γ, and anti-inflammatory cytokines such as IL-10. Paradoxical sleep deprivation (PSD) increases the risk of inflammation but can be relieved by sleep recovery (SR). This study aimed to determine the differences in levels of cortisol and inflammatory cytokines (IL-6, IL-10, TNF-α, dan IFN-γ) in male Wistar rats (*Rattus norvegicus*) after induction of various sleep deprivation stress models. Twenty-five of male Wistar rats were randomly divided into five groups: control, PSD (20 hours of SD/day for five days), Total Sleep Deprivation or TSD (24 hours of SD/day for five days), PSD+SR (PSD followed by SR), and TSD+SR (TSD followed by SR). The plasma cortisol levels were measured with ELISA, and inflammatory cytokine levels were measured with immunoassay and calculated with fold change. Mean cortisol levels were significantly increased in treatment groups compared to the control group (p=0.029). Multivariate analysis showed no statistically significant difference in inflammatory cytokine levels of IL-6 (p=0.658), IL-10 (p=0.065), TNF-α (p=0.399), and IFN-γ (p=0.283) in all groups. In conclusion, various sleep deprivation stress models affect cortisol levels but not inflammatory cytokine levels of IL-6, IL-10, TNF-α, and IFN-γ among male Wistar rats.

Keywords: cortisol, inflammatory cytokines, sleep deprivation, sleep recovery

ABSTRAK

Sleep deprivation dapat memodulasi produksi berbagai sitokin dalam tubuh diantaranya yaitu sitokin pro-inflamasi seperti IL-6, TNF-α, IFN-γ, dan sitokin anti-inflamasi seperti IL-10. *Paradoxical sleep deprivation (PSD)* berhubungan dengan peningkatan risiko inflamasi, tetapi dapat diredakan dengan *sleep recovery (SR)*. Tujuan penelitian adalah mengetahui perbedaan kadar kortisol dan sitokin inflamasi (IL-6, IL-10, TNF-α, dan IFN-γ) pada tikus putih (*Rattus norvegicus*) jantan pasca induksi berbagai model stres *sleep deprivation*.

Sebanyak 25 ekor tikus putih jantan galur Wistar dibagi secara acak menjadi 5 kelompok: kontrol, PSD (20 jam SD/hari selama 5 hari), TSD (24 jam SD/hari selama 5 hari), PSD+SR (Induksi PSD dilanjutkan SR), dan TSD+SR (Induksi TSD dilanjutkan SR). Pengukuran kadar kortisol plasma dilakukan dengan ELISA, sedangkan pengukuran kadar sitokin inflamasi dilakukan dengan *immunoassay* yang dibandingkan kadarnya menggunakan *fold change*. Rerata kadar kortisol meningkat pada kelompok perlakuan dibandingkan dengan kelompok kontrol (p=0,029). Hasil analisis multivariat menunjukkan perbedaan yang tidak signifikan pada kadar sitokin inflamasi IL-6 (p=0,658), IL-10 (p=0,065), TNF-α (p=0,399), dan IFN-γ (p=0,283) pada seluruh kelompok. Kesimpulannya, berbagai model stress *sleep deprivation* mempengaruhi kadar kortisol tetapi tidak mempengaruhi kadar sitokin inflamasi IL-6, IL-10, TNF-α, dan IFN-γ pada tikus putih jantan galur Wistar.

Kata kunci: sitokin inflamasi, kortisol, *sleep deprivation, sleep recovery*

INTRODUCTION

Sleep plays a vital role in life by maintaining the optimal physiological function of the human body. It combines stimulation of afferent nerve delivery to the brain and activation of functional neurons in specific brain areas (Chokroverty & Ferini-Strambi, 2017). Good sleep is determined by the quality of sleep, how deep a person sleeps, and the quantity of sleep, amount of time as a person sleeps (Patrick et al., 2017). According to the National Sleep Foundation, a young adult (18-25 years) or adult (26-64 years) needs 7-9 hours of sleep daily, but not less than 6 hours or more than 11 hours for young adults or 10 hours for adults. Otherwise, an older adult (65 years and over) requires at least 7-8 hours of sleep daily, but not less than 5 hours or more than 9 hours (Lichtenstein, 2015). People who live in developed countries are chronically sleep-deprived because of their cultural and socioeconomic environments. Incidence of symptoms related to sleep deprivation has recently increased, suggesting its long-term detrimental health effects are more abundant than expected (Chokroverty & Ferini-Strambi, 2017).

A study by Hirshkowitz et al. (2015) showed that > 30% of men and women between the ages of 30-64 years sleep less than 6 hours per day, and 5-15% of the world population suffer from sleep disorders. Another study indicated that 40-70% of older adults have chronic sleep disorders that are more prominent in people with medical or psychiatric comorbidity (Praharaj et al., 2018). The National Sleep Foundation stated that 70% of Indonesian people experience sleep disturbances at least once a week, and 30 million people have sleeping difficulty every night (Lestarianto, 2014). A survey of the healthy lifestyle index by American International Assurance (AIA) in 2013 demonstrated that Indonesian people have an average sleep duration of 6.8 hours per day due to increased activity and

decreased sleep duration from its normal range, indicating many Indonesian people are suffered from sleep deprivation (Putri et al., 2017).

The relationship between sleep duration and the inflammation process has not been studied extensively, despite showing a similar correlation between sleep duration and mortality. People with long sleep duration (>8 hours/night) and short sleep duration (<7 hours/night) have a 30% and 12% greater risk of death, respectively, compared to people with moderate sleep duration (7-8 hours/night). Physiologically, sleep affects two major effector systems, the Hypothalamus-Pituitary-Adrenal (HPA) axis and the sympathetic nervous system, which simultaneously increases the release of pro-inflammatory cytokines and markers of systemic inflammation through β-adrenergic activation. The activity of sympathetic nervous system decreases when a person is sleeping, which may explain the relationship between sleep disorder, short sleep duration, and elevation of inflammatory markers (Chen et al., 2017). Patients that pathologically tend to fall asleep during daytime and easily get tired exhibit an increase in IL-6, a pro-inflammatory cytokine, in the circulatory system due to activation of the HPA Axis. People suffering from sleep deprivation produce more IL-6 during the day and less IL-6 at night, whereas good sleep reduces pro-inflammatory exposure to tissues. Furthermore, sleep deprivation upregulates pro-inflammatory cytokines through NF-kB activation, generating a pro-inflammatory state. Overexposure to the pro-inflammatory cytokines caused by sleep deprivation will increase tissue damage, decrease brain function, and increase the expression of IL-6 (Vgontzas et al., 2000).

Sleep deprivation correlates with the increase in TNF-α, indicating a pathological condition caused by sleep disorders (Chennaoui et al., 2011). Moreover, TNF-α is expressed in neurons and plays a role in brain neuroplasticity (Rockstrom et al., 2018). On the other hand, a clinical trial showed that IFN-γ was found in cases of fever, flu, and drowsiness, whereas IFN-γ can be somnogenic in the presence of TNF-α. Therefore, IFN-γ has a significant role in sleep regulation during viral infection (Van Dongen et al., 2011).

Studies suggest that cortisol levels as a physiological stress marker and systemic inflammatory marker may increase in sleep-deprived conditions. Sleep deprivation triggers the release of adrenocorticotropic hormone (ACTH) via the HPA axis, inducing the adrenal gland to release cortisol. Along with other stimulations, including norepinephrine and radical oxygen species (ROS), cortisol triggers inflammatory activation in the brain and peripheral immune cells by increasing the expression of pro-inflammatory cytokine genes such as IL-

1, IL-6, and TNF-α via transcriptional regulator of pro-inflammatory gene expression such as NF-κB. These pro-inflammatory cytokines then enter the systemic circulation inducing the increase of leukocytes, mainly neutrophils, CD4+ T-cells, B-cells, and monocyte leading to inflammatory reactions (Garbarino et al., 2021).

Sleep deprivation (SD) can be categorized into paradoxical sleep deprivation (PSD) and total sleep deprivation (TSD), and studied using the Modified Multiple Platform Method (MMPM). While both types can induce stress, total sleep deprivation causes poor decision-making control, triggers repetitive errors in working memory, and abolishes individual spontaneity in communication, making the subjects appear lazy, lethargic, and unmotivated (Gunn et al., 2017).

Previous studies showed that sleep deprivation affects cortisol and inflammatory cytokine levels in rats or humans by interfering with homeostatic sleep patterns and disrupting HPA axis activation (Chennaoui et al., 2011; Medic et al., 2017; Neto et al., 2010; Wright Jr et al., 2015). While sleep recovery may restore rat deteriorating conditions caused by sleep deprivation, no studies clearly define the effect of sleep recovery on pro-inflammatory and anti-inflammatory cytokine levels. This study directly compared cortisol and inflammatory cytokine level among rats treated with PSD, TSD, and sleep recovery after PSD and TSD treatment. Considering that many people have experienced sleep deprivation, this study is essential as initial research to determine the effect of sleep deprivation and sleep recovery on the levels of cortisol and inflammatory cytokines that impair various metabolic functions in mammals.

EXPERIMENTAL SECTION

Research Design

This study was an experimental study using a post-test only with control group design.

Subject

Wistar rats (*Rattus norvegicus)* with characteristics of male, 8-12 weeks old, and 200-300 grams of weight were obtained from the Laboratorium Penelitian dan Pengujian Terpadu (LPPT) 4 Universitas Gajah Mada. Sleep deprivation experiments were performed using the Modified Multiple Platform Method (MMPM) to prevent the rats from sleeping.

A total of 25 rats were randomly divided into five groups containing five rats in each group: control (no SD), PSD (20 hours of SD per day at 11.00 a.m. - 07.00 a.m. for 5 days and 4 hours rest at 07.00 a.m. - 11.00 a.m.), TSD (24 hours of SD per day at 11.00 a.m. - 11.00 a.m. for 5 days without rest), PSD+SR (PSD treatment for 5 days followed by sleep recovery (SR) for 5 days at 07.00 a.m. - 07.00 a.m.), and TSD+SR (TSD treatment for 5 days followed by sleep recovery for 5 days at 11.00 a.m. - 11.00 a.m.).

Research Procedure

Modified Multiple Platform Method (MMPM) tanks measuring 123 x 44 x 35 cm were used for the sleep deprivation stress model. The tanks were filled with water and equipped with 12 platforms of 6.5 cm in size with a distance of 10 cm between the platforms as a foothold for the rats that are instinctively avoiding water. Each tank contains five experimental rats equipped with a muscle atonia device, an instrument that gives an automatic shock effect every 10 minutes to keep the rats awake. The experiments were conducted in the Anatomy Laboratory of the Faculty of Medicine of Jenderal Soedirman University from August to September 2021.

Observation

Cortisol levels were measured by drawing 2 ml of rat blood for each group at 07.00-09.00 a.m. via retroorbital vein using a microhematocrit pipette into an EDTA tube, centrifuging for 15 minutes at 2,000-3,000 rpm, and storing in -20°C. A total of 50 ml of collected plasma was examined with the Enzyme-Linked Immunosorbent Assay (ELISA) method and read by the ELISA reader in 450 nm absorbance (Zabidi et al., 2015).

For inflammatory cytokines level measurement, rat blood plasma samples were collected from the retroorbital plexus as much as 55 ml. The levels of inflammatory cytokines of IL-6, IL-10, TNF-α, and IFN-γ were then measured using multiplex bead-based immunoassay from MILLIPLEX® MAP kit with Luminex technology in the Integrated Laboratory of Faculty of Medicine, Universitas Indonesia. Briefly, the frozen samples were thawed, stirred thoroughly with a vortex, and centrifuged to remove particulates before the immunoassay. The processed samples were then placed on the plate, added with conjugated beads from the MILLIPLEX® MAP kit, and incubated for 2 hours at 20-24°C or overnight at 4-8°C. After incubation, the beads were washed, incubated in biotinylated-detection antibodies, added with Streptavidin-PE (SAPE), and further incubated. The beads were then

washed, resuspended in an appropriate buffer, and placed on Luminex technology to measure cytokine levels. The plasma cytokine levels were analyzed based on the fold change value by comparing the treatment and the control group.

Statistical analysis

Normality analysis was performed by the Shapiro-Wilk test, and data homogeneity was analyzed by Levene's test. The multivariate analysis was then performed by the One-Way ANOVA and Post-hoc Least Significant Difference (LSD) to determine differences between groups. Kruskal-Wallis and Mann-Whitney tests were performed for non-parametric analysis.

All procedures were approved by The Medical Research Ethics Commission of The Faculty of Medicine of Jenderal Soedirman University on May 10, 2021 (reference number: 097/KEPK/V/2021).

RESULT AND DISCUSSION

Measurement and analysis results of mean cortisol levels among all groups are shown in **Figure 1**. Since the plasma cortisol levels were not normally distributed, non-parametric tests were used for further analysis.

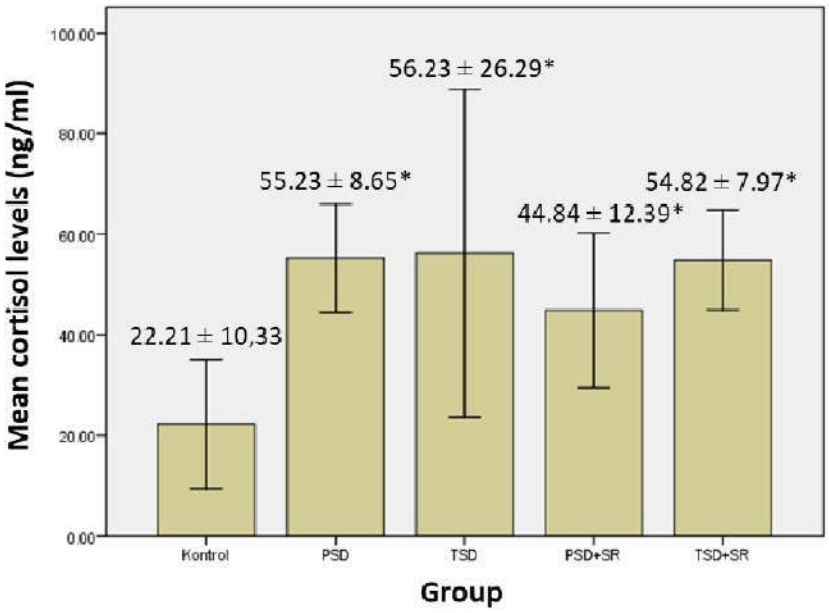

Figure 1. Mean cortisol levels in male Wistar rat (*Rattus norvegicus*). Control: no SD; PSD: 20 hours of SD with 4 hours rest for five days; TSD: 24 hours of SD for five days; PSD+SR: PSD followed by SR; TSD+SR: TSD followed by SR. SD: sleep deprivation, SR: sleep recovery, PSD: paradoxical sleep deprivation, TSD: total sleep deprivation. Error bars represent 95% confidence intervals. Statistical analysis was performed using Kruskal-

Wallis test (p = 0.029) and Mann-Whitney test for post-hoc analysis. *Post-hoc Mann-Whitney test compared to control, $p < 0.05$.

Kruskal-Wallis analysis showed substantial differences in cortisol levels between at least two groups for the assessed variables (p=0.029). Further post-hoc analysis with the Mann-Whitney test obtained significant results between every intervention group and the control ($p<0.05$). PSD for 20 hours/day increased cortisol levels in the afternoon and evening in rats by two times compared to control (Olayaki et al., 2015). Cortisol levels increased in the first six hours after PSD induction caused by HPA axis activation to maintain metabolism changes that were occurred during sleep deprivation (Galvão et al., 2009). Consistent with a study by Olayaki et al. (2015) demonstrating that TSD for five days increased cortisol levels compared to the control, this study pointed out that the TSD group had the highest mean cortisol levels among all groups (56.23±26.29 ng/ml).

The PSD group showed lower mean cortisol levels than the TSD group because PSD has a sleep period daily, thus the negative impact of sleep deprivation can be relieved. Two hours of an afternoon nap after one night of sleep deprivation decreased the secretion of cortisol hormone caused by inhibition of the HPA axis at the slow wave sleep (SWS) phase that reduces cortisol levels (Pejovic et al., 2013). The effects of sleep deprivation that impair HPA function cause an increase in evening cortisol levels as a result of an elevation in its secretory pulses amplitude, suggesting that the negative feedback of glucocorticoid mediated by hippocampal function may be affected by sleep-deprived condition (Hirotsu et al., 2015). Cortisol levels were higher in the subjects who stayed awake than in subjects who slept sufficiently, indicating that sleep deprivation affects the cortisol levels as a result of stress responses (Bassett et al., 2018). Cauter et al. (2008) emphasized that the initial consequence of partial sleep loss is a cortisol level increase in the evening. While under normal conditions, cortisol levels decrease rapidly and reach the minimum level just before sleeping time. Kantasa et al. (2016) stated that cortisol can suppress the immune system and induce an inflammatory pathway, making the body vulnerable to various diseases.

PSD + SR group exhibited lower cortisol levels than TSD + SR, and both PSD + SR and TSD + SR showed lower cortisol levels than PSD and TSD. A study by Mattice et al. (2011) showed that subjects induced by TSD for 24 hours and 48 hours followed by sleep recovery for 24 hours relieved their sleep deprivation effect for 72% and 42%, respectively. Sleep recovery for three days decreases cortisol levels and ameliorates the impact of sleep deprivation by restoring the HPA axis interaction (Pejovic et al., 2013). Sleep recovery also

decreases lipid peroxidase and free radical production by increasing glutathione and other enzymatic antioxidants (Hirotsu et al., 2015). Glutathione increases after sleep recovery for two days, indicated by the elevation of 6-PGD, an enzyme that acts on the pentose phosphate pathway in the carbohydrate metabolism that protects cells from oxidative stress in the form of NADPH, thus inhibiting stress oxidative that has a role in cortisol increase (Kim et al., 2022). Taken together, these results indicate that sleep recovery is able to decrease cortisol levels which increase after induction of PSD or TSD.

Results of data analysis showing the value of minimum, maximum, mean, median, and standard deviation for each group are shown in **Table 1.**

Table 1. Levels of inflammatory cytokine in male Wistar rat (pg/mL).

Group	N	Min.				Max.				Mean±SD			
		IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ
Control	5	311,14	15,14	2,89	736,59	1706,25	83,31	12,06	1375,77	974± 504	34,42±11,92	6,91±3,28	1038,08±272,26
PSD+SR	5	452,10	17,52	4,54	754,54	3103,86	93,76	23,89	1001,67	1281±1056	53,07±12,98	9,75±8,05	847,45±102,73
TSD+SR	5	311,14	57,89	6,54	718,80	2518,29	317,89	26,13	1001,67	1578±928	87,06±47,99	14,17±8,17	865,83±112,95
PSD	5	520,98	44,84	6,90	781,54	1858,94	74,98	12,24	1385,65	1192±544	59,47±5,44	9,39±2,62	1046,28±239,32
TSD	5	382,22	33,52	1,76	700,85	1365.64	178,02	14,17	1824,71	952±373	68,06±25,53	8,01±4,39	1167,39±432,52

Table 2. P-value of normality, homogeneity, and One-Way ANOVA test in the levels of inflammatory cytokines in male Wistar rat.

Group	Shapiro-Wilk (p-value)				Levene's (p-value)				One-Way Anova (p-value)			
	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ
Control	0,832	0,941	0,389	0,640								
PSD+SR	0,884	0,482	0,081	0,409								
TSD+SR	0,812	0,909	0,096	0,921	0,256	0,352	0,697	0,075	0,658	0,065	0,399	0,283
PSD	0,067	0,845	0,200	0,610								
TSD	0,622	0,500	0,615	0,750								

Normality analysis by the Shapiro-Wilk test and homogeneity analysis by the Levene's test showed a p-value >0.05 in all groups, indicating data of inflammatory cytokine levels were normally distributed and homogenous. Multivariate analysis using the One-Way ANOVA test demonstrated a p-value >0.05 in all groups, indicating no significant difference in inflammatory cytokine levels among the treatment groups, as shown in **Table 2**. The inflammatory cytokine levels were then compared among the treatment and control groups using fold change. The results demonstrated the highest increase in inflammatory cytokine levels was in the TSD treatment group (**Figure 2**). However, the multivariate analysis of the fold change of inflammatory cytokine levels using the One-Way ANOVA test did not show a significant difference ($p > 0.05$) in IL-6 ($p=0.658$), IL-10 ($p=0.085$), TNF-α ($p= 0.313$), and IFN-γ ($p=0.283$).

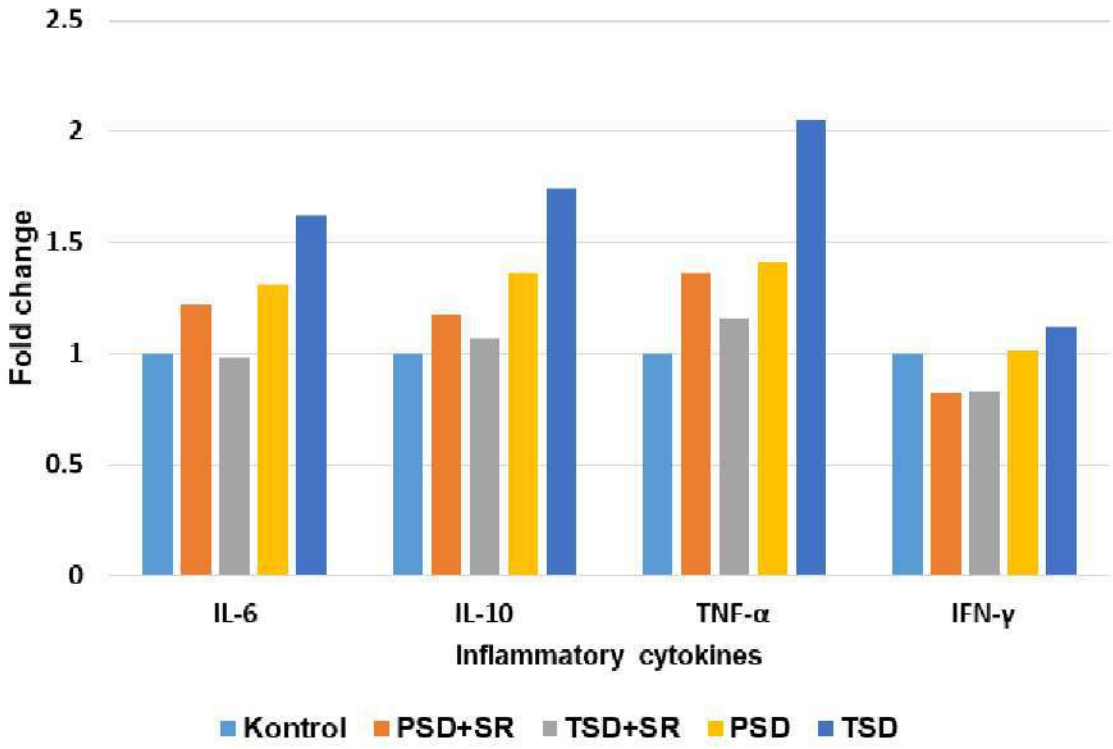

Figure 2. Mean of the fold change of inflammatory cytokine levels of IL-6, IL-10, TNF-α, and IFN-γ in male Wistar rat (*Rattus norvegicus*). Control group: no SD; PSD+SR: PSD followed by SR; TSD+SR: TSD followed by SR; PSD: 20 hours of SD with 4 hours rest for five days; TSD: 24 hours of SD for five days. SD: sleep deprivation, SR: sleep recovery, PSD: paradoxical sleep deprivation, TSD: total sleep deprivation.

Sleep deprivation triggers a stress response in animal models by increasing pro-inflammatory cytokines and activates the main stress axis in humans, the HPA axis. IL-6 can stimulate cortisol secretion directly in the adrenal gland or via activation of the hypothalamus

that induces the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. On the other hand, a slight elevation of IL-6 level in plasma induces the anti-inflammatory cytokines, IL-1ra and IL-10, along with c-reactive protein (CRP). Recent studies stated that apart from injury and infection, sleep deprivation triggers the pro-inflammatory response through the increase of pro-inflammatory cytokines secretion (Medic et al., 2017; Simpson & Dinges, 2007; Sukendra, 2015).

Activation of inflammatory responses caused by sleep deprivation can increase the levels of anti-inflammatory cytokines to prevent excessive inflammatory responses. IL-10, a potent anti-inflammatory cytokine, inhibits the activation of macrophages and dendritic cells, leading to the reduction of cytokine levels produced by T-helper1 (Th1) cells (Welsh et al., 2011). IL-10 also potently inhibits the production of IL-10 itself, IL-12, L-1α, IL-1β, IL-6, macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), leukemia inhibitory factor (LIF), and platelet-activating factor (PAF) from activated monocytes and macrophages (Saraiva & O'Garra, 2010).

This study showed that sleep deprivation did not significantly affect levels of IL-10 in each variable ($p = 0.065$). This result is consistent with the study of Neto et al. (2010), showing that sleep deprivation did not impair levels of anti-inflammatory cytokines, including IL-10. Otherwise, they also observed sleep deprivation increased IL-10 levels in different adipose tissue depots and decreased the TNF-α levels in the brain. Therefore, the profile of systemic anti-inflammatory cytokines after sleep deprivation remains controversial.

A study by Patel et al. (2009) also demonstrated that sleep deprivation did not affect levels of anti-inflammatory cytokines such as IL-1 and IL-10. They explained that the different responses of sleep deprivation on the levels of anti-inflammatory cytokines may depend on the differences in the effect of sleep on inflammatory components in each individual, the half-life of each inflammatory component, and the instrument performance for cytokine measurement. Intriguingly, anti-inflammatory cytokine levels increase in chronic sleep deprivation that lasts over weeks, months, or years.

Wright Jr et al. (2015) demonstrated that sleep deprivation significantly increased levels of IL-10 on day 26 to day 28 measured with ELISA. Sleep deprivation induces circadian rhythm impairment that, over weeks, will increase levels of anti-inflammatory cytokine (IL-10), the pro-inflammatory protein (TNF-α), and CRP. Otherwise, cortisol levels

increase during acute sleep deprivation, indicating that acute and chronic sleep deprivation provoke different responses of circadian rhythm impairment. Therefore, acute sleep deprivation is associated with physiological stress or metabolic response of increased cortisol levels, whereas chronic sleep deprivation is associated with physiological adaptation as a response to decreased cortisol levels followed by increased pro-inflammatory and anti-inflammatory cytokines.

No significant difference in TNF-α levels after induction of various sleep deprivation stress models in this study ($p = 0.366$) is consistent with the study by Ruiz et al. (2012), showing that total sleep deprivation for two days or paradoxical sleep deprivation for four days followed by sleep recovery for three days did not alter TNF-α levels. Other studies also demonstrated that ten days of sleep deprivation did not increase TNF-α or its receptors, regardless of sex characteristics (Shearer et al., 2001; Xu et al., 2015). In contrast, a study by Irwin et al. (2006) exhibited that sleep deprivation increased TNF-α production. Sleep deprivation probably induced TNF-α cellular expression through the activation of nuclear factor NF-kB, a key component in controlling the cellular expression of pro-inflammatory cytokines.

In agreement with the study by Crooks et al. (2019) that demonstrated no significant result between sleep quality and IL-6 levels, this study approved that there was no significant result between the levels of pro-inflammatory cytokines, IL-6, with various sleep deprivation stress models ($p = 0.658$). Conversely, a study by Siregar (2017) showed a significant result between sleep quality and IL-6 levels. Sleep deprivation may affect components of the immune system through modification of CD4+ cells, CD8+ cells, NK cells, and levels of pro-inflammatory cytokines such as TNF-α, INF-γ, and IL-6 (Ibarra-Coronado et al., 2015). Collectively, these results indicate that pure TSD and PSD or TSD and PSD followed by sleep recovery did not affect plasma cytokine levels of IL-6 and TNF-α.

Multivariate analysis among the treatment groups showed no significant difference between sleep deprivation and IFN-γ levels ($p=0.283$). This result is consistent with the study by Hirotsu et al. (2012), demonstrating that there was no significant difference in IFN-γ levels in psoriasis rats model induced by paradoxical sleep deprivation for 48 hours. IFN-γ production is generally unaffected by sleep deprivation, instead is increased at the end of sleep recovery. These insignificant results may be affected by sample processing that caused cytokine stability disruption, cytokine degradation by protease, or cytokine binding to its

soluble cellular receptors. These alterations may also explain the decrease in cytokine levels over time during sample storage (Hennø et al., 2017).

The main limitation of this study is that it only examined the level of inflammatory cytokine levels in plasma but not in the tissue resulting in an insignificant increase in inflammatory cytokine levels after sleep deprivation treatment. Induction of inflammatory cytokine release is likely more prominent in the brain or other tissues, which are initially released by immune and glial cells in the brain after activation of the HPA axis (Garbarino et al., 2021). Another limitation is that the stress induction protocol by sleep deprivation in this study was not enough to induce an increase in the cytokine level in plasma. Various sleep deprivation protocols may also cause different results in inflammatory cytokine levels among studies. Furthermore, variation in the circadian cycle may also affect the production of various cytokines, which confounds the levels of circulating cytokines in this study (Ruiz et al., 2012).

To date, studies concerning sleep deprivation and inflammatory cytokines remain elusive. Sleep deprivation indeed increases the main stress hormone, cortisol, which plays a role in various disease development, such as hypertension and cardiovascular disease (Sá Gomes e Farias et al., 2022). On the other hand, some studies indicated that people with sleep disorders showed an alteration in circulating levels of TNF-α and IL-6. However, other studies failed to demonstrate the change in TNF-α and IL-6 levels between normal and sleep-deprived subjects (Crooks et al., 2019; Irwin et al., 2006; Shearer et al., 2001; Siregar, 2017). Although this study showed insignificant results, sleep deprivation also has a potency to increase the pro-inflammatory cytokine expression that is associated with inflammatory reaction-related diseases such as cancer, neurodegeneration, and cardiovascular disease (Garbarino et al., 2021). Establishing how far sleep deprivation can affect cortisol and inflammatory cytokine levels and how much sleep recovery is required to alleviate the physiological disruption caused by sleep deprivation is essential to making recommendations for a healthy lifestyle. Furthermore, in this modern era, night shift job is increasing in various sectors, and the future development of this study can be used to make recommendations for working regulation to create work-life balance.

CONCLUSION

Stress induction by the various models of sleep deprivation modifies cortisol levels but not inflammatory cytokines levels of IL-6, IL-10, TNF-α, and IFN-γ in male Wistar rats (*Rattus norvegicus*). These results indicate cortisol levels can be used as a stress indicator induced by sleep deprivation, but inflammatory cytokines levels of IL-6, IL-10, TNF-α, and IFN-γ may not be used for this purpose. Furthermore, although not statistically different, sleep recovery restores the levels of cortisol and inflammatory cytokines after PSD or TSD treatment, suggesting sleep recovery able to bring back homeostasis conditions after stress. Further exploration of sleep deprivation and sleep recovery protocols is required to get valuable outcomes.

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REFERENCES

Bassett, S. M., Lupis, S. B., Gianferante, D., Rohleder, N., & Wolf, J. M. (2018). Sleep quality but not sleep quantity effects on cortisol responses to acute psychosocial stress. *Revista Del Colegio Americano de Cardiologia*, *72*(23), 2964–2979. https://doi.org/10.3109/10253890.2015.1087503.Sleep

Cauter, E. V., Spiegel, K., Tasali, E., & Leproult, R. (2008). Metabolic consequences of sleep and sleep loss. *Physiology & Behavior*, *176*(3), 139–148. https://doi.org/10.1016/S1389-9457(08)70013-3

Chen, L., Huang, J., Yang, L., Zeng, X. A., Zhang, Y., Wang, X., Chen, M., Li, X., Zhang, Y., & Zhang, M. (2017). Sleep deprivation accelerates the progression of alzheimer's disease by influencing Aβ-related metabolism. *Neuroscience Letters*, *650*, 146–152. https://doi.org/10.1016/j.neulet.2017.04.047

Chennaoui, M., Sauvet, F., Drogou, C., Van Beers, P., Langrume, C., Guillard, M., Gourby, B., Bourrilhon, C., Florence, G., & Gomez-Merino, D. (2011). Effect of one night of sleep loss on changes in tumor necrosis factor alpha (TNF-α) levels in healthy men. *Cytokine*, *56*(2), 318–324. https://doi.org/10.1016/j.cyto.2011.06.002

Chokroverty, S., & Ferini-Strambi, L. (2017). Oxford textbook of sleep disorders. In *Oxford*

University Press.

Crooks, E., Hansen, D. A., Satterfield, B. C., Layton, M. E., & Van Dongen, H. P. A. (2019). Cardiac autonomic activity during sleep deprivation with and without caffeine administration. *Physiology & Behavior*, *210*, 112643. https://doi.org/10.1016/j.physbeh.2019.112643.Cardiac

Galvão, M. de O. L., Sinigaglia-Coimbra, R., Kawakami, S. E., Tufik, S., & Suchecki, D. (2009). Paradoxical sleep deprivation activates hypothalamic nuclei that regulate food intake and stress response. *Psychoneuroendocrinology*, *34*(8), 1176–1183. https://doi.org/10.1016/j.psyneuen.2009.03.003

Garbarino, S., Lanteri, P., Bragazzi, N. L., Magnavita, N., & Scoditti, E. (2021). Role of sleep deprivation in immune-related disease risk and outcomes. *Communications Biology*, *4*(1). https://doi.org/10.1038/s42003-021-02825-4

Gunn, B. G., Cox, C. D., Chen, Y., Frotscher, M., Gall, C. M., Baram, T. Z., & Lynch, G. (2017). The endogenous stress hormone CRH modulates excitatory transmission and network physiology in hippocampus. *Cerebral Cortex*, *27*(8), 4182–4198. https://doi.org/10.1093/cercor/bhx103

Hennø, L. T., Storjord, E., Christiansen, D., Bergseth, G., Ludviksen, J. K., Fure, H., Barene, S., Waage-Nielsen, E., Mollnes, T. E., & Brekke, O. L. (2017). Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines – Consequences for defining reference values in healthy humans. *Cytokine*, *97*(May), 86–95. https://doi.org/10.1016/j.cyto.2017.05.014

Hirotsu, C., Rydlewski, M., Araújo, M. S., Tufik, S., & Andersen, M. L. (2012). Sleep Loss and Cytokines Levels in an Experimental Model of Psoriasis. *PLoS ONE*, *7*(11), 1–9. https://doi.org/10.1371/journal.pone.0051183

Hirotsu, C., Tufik, S., & Andersen, M. L. (2015). Interactions between sleep, stress, and metabolism: From physiological to pathological conditions. *Sleep Science*, *8*(3), 143–152. https://doi.org/10.1016/j.slsci.2015.09.002

Hirshkowitz, M., Whiton, K., Albert, S. M., Alessi, C., Bruni, O., DonCarlos, L., Hazen, N., Herman, J., Katz, E. S., Kheirandish-Gozal, L., Neubauer, D. N., O'Donnell, A. E., Ohayon, M., Peever, J., Rawding, R., Sachdeva, R. C., Setters, B., Vitiello, M. V., Ware, J. C., & Adams Hillard, P. J. (2015). National sleep foundation's sleep time duration recommendations: Methodology and results summary. *Sleep Health*, *1*(1), 40–43. https://doi.org/10.1016/j.sleh.2014.12.010

Ibarra-Coronado, E. G., Pantaleón-Martínez, A. M., Velazquéz-Moctezuma, J., Prospéro-García, O., Méndez-Díaz, M., Pérez-Tapia, M., Pavón, L., & Morales-Montor, J. (2015). The Bidirectional Relationship between Sleep and Immunity against Infections. *Journal of Immunology Research*, *2015*, 678164. https://doi.org/10.1155/2015/678164

Irwin, M. R., Wang, M., Campomayor, C. O., Collado-Hidalgo, A., & Cole, S. (2006). Sleep deprivation and activation of morning levels of cellular and genomic markers of inflammation. *Archives of Internal Medicine*, *166*(16), 1756–1762. https://doi.org/10.1001/archinte.166.16.1756

Kantasa, V. D., Kusumawardani, B., & Herniyati. (2016). Efek Stresor Rasa Sakit Renjatan Listrik terhadap Limfosit dan Makrofag pada Gingiva Tikus Sprague Dawley (The

Effect of Electrical Shock Stressor on Lymphocytes and Macrophages in Gingival Tissue of Sprague Dawley Rats). *Pustaka Kesehtan*, *4*(1), 48–54.

Kim, J., Yun, K. sang, Cho, A., Kim, D. H., Lee, Y. K., Choi, M. J., Kim, S. hyung, Kim, H., Yoon, J. W., & Park, H. C. (2022). High cortisol levels are associated with oxidative stress and mortality in maintenance hemodialysis patients. *BMC Nephrology*, *23*(1), 1–9. https://doi.org/10.1186/s12882-022-02722-w

Lestarianto, J. A. (2014). Hubungan antara internet addiction dan tingkat stres dengan kejadian insomnia pada mahasiswa keperawatan fakultas ilmu kesehatan universitas muhammadiyah. *Diss. Universitas Muhammadiyah Purwokerto*. http://repository.ump.ac.id/id/eprint/5503

Lichtenstein, G. R. (2015). The Importance of Sleep. *Gastroenterology & Hepatology*, *11*(12), 790.

Mattice, C. D., Brooks, R., & Lee-Chiong, T. (2011). Fundamentals of Sleep Technology: Second edition. *Philadelphia: Lippincott Williams & Wilkins.*

Medic, G., Wille, M., & Hemels, M. E. H. (2017). Short- and long-term health consequences of sleep disruption. *Nature and Science of Sleep*, *9*, 151–161. https://doi.org/10.2147/NSS.S134864

Neto, J. C. R., Lira, F. S., Venancio, D. P., Cunha, C. A., Oyama, L. M., Pimentel, G. D., Tufik, S., Do Nascimento, C. M. O., Santos, R. V. T., & De Mello, M. T. (2010). Sleep deprivation affects inflammatory marker expression in adipose tissue. *Lipids in Health and Disease*, *9*(1), 125. https://doi.org/10.1186/1476-511X-9-125

Olayaki, L. A., Sulaiman, S. O., & Anoba, N. B. (2015). Vitamin C Prevents Sleep Deprivation-induced Elevation in Cortisol and Lipid Peroxidation in the Rat Plasma. *Nigerian Journal of Physiological Sciences : Official Publication of the Physiological Society of Nigeria*, *30*(1–2), 5–9.

Patel, S. R., Zhu, X., Storfer-Isser, A., Mehra, R., Jenny, N. S., Tracy, R., & Redline, S. (2009). Sleep duration and biomarkers of inflammation. *Sleep*, *32*(2), 200–204. https://doi.org/10.1093/sleep/32.2.200

Patrick, Y., Lee, A., Raha, O., Pillai, K., Gupta, S., Sethi, S., Mukeshimana, F., Gerard, L., Moghal, M. U., Saleh, S. N., Smith, S. F., Morrell, M. J., & Moss, J. (2017). Effects of sleep deprivation on cognitive and physical performance in university students. *Sleep and Biological Rhythms*, *15*(3), 217–225. https://doi.org/10.1007/s41105-017-0099-5

Pejovic, S., Basta, M., Vgontzas, A. N., Kritikou, I., Shaffer, M. L., Tsaoussoglou, M., Stiffler, D., Stefanakis, Z., Bixler, E. O., & Chrousos, G. P. (2013). Effects of recovery sleep after one work week of mild sleep restriction on interleukin-6 and cortisol secretion and daytime sleepiness and performance. *American Journal of Physiology - Endocrinology and Metabolism*, *305*(7), 890–896. https://doi.org/10.1152/ajpendo.00301.2013

Praharaj, S. K., Gupta, R., & Gaur, N. (2018). Clinical practice guideline on management of sleep disorders in the elderly. *Indian Journal of Psychiatry*, *60*(7), S383–S396. https://doi.org/10.4103/0019-5545.224477

Putri, D. N. E., Nasrul, E., & Masri, M. (2017). Pengaruh Kurang Tidur terhadap Berat Badan pada Tikus Wistar Jantan. *Jurnal Kesehatan Andalas*, *6*(1), 78–82.

https://doi.org/10.25077/jka.v6i1.655

Rockstrom, M. D., Chen, L., Taishi, P., Nguyen, J. T., Gibbons, C. M., Veasey, S., & Krueger, J. M. (2018). Tumor necrosis factor alpha in sleep regulation. *Sleep Medicine Reviews*, *40*, 69–78. https://doi.org/10.1016/j.smrv.2017.10.005.Tumor

Ruiz, F. S., Andersen, M. L., Martins, R. C. S., Zager, A., Lopes, J. D., & Tufik, S. (2012). Immune alterations after selective rapid eye movement or total sleep deprivation in healthy male volunteers. *Innate Immunity*, *18*(1), 44–54. https://doi.org/10.1177/1753425910385962

Sá Gomes e Farias, A. V., de Lima Cavalcanti, M. P., de Passos Junior, M. A., & Vechio Koike, B. del. (2022). The association between sleep deprivation and arterial pressure variations: a systematic literature review. *Sleep Medicine: X*, *4*, 100042. https://doi.org/10.1016/j.sleepx.2022.100042

Saraiva, M., & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature Reviews Immunology*, *10*(3), 170–181. https://doi.org/10.1038/nri2711

Shearer, W. T., Reuben, J. M., Mullington, J. M., Price, N. J., Lee, B. N., O'brian Smith, E., Szuba, M. P., Van Dongen, H. P. A., & Dinges, D. F. (2001). Soluble TNF-α receptor 1 and IL-6 plasma levels in humans subjected to the sleep deprivation model of spaceflight. *Journal of Allergy and Clinical Immunology*, *107*(1), 165–170. https://doi.org/10.1067/mai.2001.112270

Simpson, N., & Dinges, D. F. (2007). Sleep and Inflammation. *Nutrition Reviews*, *65*(SUPPL.3), S244–S252. https://doi.org/10.1111/j.1753-4887.2007.tb00371.x

Siregar, E. M. H. (2017). Korelasi Kualitas Tidur dengan Kadar Interleukin-6 Serum pada Pasien Hemodialisis Regular. In *Thesis Universitas Sumatera Utara*.

Sukendra, D. M. (2015). Efek Olahraga Ringan Pada Fungsi Imunitas Terhadap Mikroba Patogen: Infeksi Virus Dengue. *Media Ilmu Keolahragaan Indonesia*, *5*(2), 57–65.

Van Dongen, H. P. A., Belenky, G., & Krueger, J. M. (2011). A Local, Bottom-Up Perspective on Sleep Deprivation and Neurobehavioral Performance. *Current Topics in Medicinal Chemistry*, *11*(19), 2414–2422. https://doi.org/doi: 10.2174/156802611797470286.

Vgontzas, A. N., Papanicolaou, D. A., Bixler, E. O., Hopper, K., Lotsikas, A., Lin, H. M., Kales, A., & Chrousos, G. P. (2000). Sleep apnea and daytime sleepiness and fatigue: Relation to visceral obesity, insulin resistance, and hypercytokinemia. *Journal of Clinical Endocrinology and Metabolism*, *85*(3), 1151–1158. https://doi.org/10.1210/jcem.85.3.6484

Welsh, P., Murray, H. M., Ford, I., Trompet, S., De Craen, A. J. M., Jukema, J. W., Stott, D. J., McInnes, I. B., Packard, C. J., Westendorp, R. G. J., & Sattar, N. (2011). Circulating interleukin-10 and risk of cardiovascular events: A prospective study in the elderly at risk. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(10), 2338–2344. https://doi.org/10.1161/ATVBAHA.111.231795

Wright Jr, K. P., Drake, A. L., Frey, D. J., Fleshner, M., Desouza, C. A., Gronfier, C., & Czeisler, C. A. (2015). Influence of sleep deprivation and circadian misalignment on cortisol, inflammatory markers, and cytokine balance. *Physiology & Behavior*, *47*, 24–34. https://doi.org/10.1016/j.bbi.2015.01.004.Influence

Xu, C., Wu, X., Hack, B. K., Bao, L., & Cunningham, P. N. (2015). TNF causes changes in glomerular endothelial permeability and morphology through a Rho and myosin light chain kinase-dependent mechanism. *Physiological Reports*, *3*(12), 1–16. https://doi.org/10.14814/phy2.12636

Zabidi, L., Supriatna, M., Departemen, M. M., Kesehatan, I., Fk-Undip, A., Rsup, /, Semarang, K., & Mexitalia, M. (2015). Kadar Kortisol Serum sebagai Indikator Prognosis Sepsis pada Anak Serum Cortisol Level as Prognostic Indicator for Pediatric Sepsis. *Sari Pediatri*, *17*(2), 101–106.

LEVELS OF CORTISOL AND INFLAMMATORY CYTOKINES AFTER THE INDUCTION OF VARIOUS SLEEP DEPRIVATION STRESS MODELS IN MALE WISTAR RATS

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ABSTRACT

Sleep deprivation (SD) can modulate the production of various cytokines, including pro-inflammatory cytokines such as IL-6, TNF-α, and IFN-γ, and anti-inflammatory cytokines such as IL-10. Paradoxical sleep deprivation (PSD) increases the risk of inflammation but can be relieved by sleep recovery (SR). This study aimed to determine the differences in levels of cortisol and inflammatory cytokines (IL-6, IL-10, TNF-α, dan IFN-γ) in male Wistar rats (*Rattus norvegicus*) after induction of various sleep deprivation stress models. Twenty-five of male Wistar rats were randomly divided into five groups: control, PSD (20 hours of SD/day for five days), Total Sleep Deprivation or TSD (24 hours of SD/day for five days), PSD+SR (PSD followed by SR), and TSD+SR (TSD followed by SR). The plasma cortisol levels were measured with ELISA, and inflammatory cytokine levels were measured with immunoassay and calculated with fold change. Mean cortisol levels were significantly increased in treatment groups compared to the control group (p=0.029). Multivariate analysis showed no statistically significant difference in inflammatory cytokine levels of IL-6 (p=0.658), IL-10 (p=0.065), TNF-α (p=0.399), and IFN-γ (p=0.283) in all groups. In conclusion, various sleep deprivation stress models affect cortisol levels but not inflammatory cytokine levels of IL-6, IL-10, TNF-α, and IFN-γ among male Wistar rats.

Keywords: cortisol, inflammatory cytokines, sleep deprivation, sleep recovery

INTRODUCTION

Sleep plays a vital role in life by maintaining the optimal physiological function of the human body. It combines stimulation of afferent nerve delivery to the brain and activation of functional neurons in specific brain areas (Chokroverty et al., 2017). Good sleep is determined by the quality of sleep, how deep a person sleeps, and the quantity of sleep, amount of time as a person sleeps (Patrick et al., 2017). According to the National Sleep Foundation, a young adult (18-25 years) or adult (26-64 years) needs 7-9 hours of sleep daily, but not less than 6 hours or more than 11 hours for young adults or 10 hours for

adults. Otherwise, an older adult (65 years and over) requires at least 7-8 hours of sleep daily, but not less than 5 hours or more than 9 hours (Lichtenstein, 2015). People who live in developed countries are chronically sleep-deprived because of their cultural and socioeconomic environments. Incidence of symptoms related to sleep deprivation has recently increased, suggesting its long-term detrimental health effects are more abundant than expected (Chokroverty & Ferini-Strambi, 2017).

A study by Hirshkowitz et al. (2015) showed that > 30% of men and women between the ages of 30-64 years sleep less than 6 hours per day, and 5-15% of the world population suffer from sleep disorders. Another study indicated that 40-70% of older adults have chronic sleep disorders that are more prominent in people with medical or psychiatric comorbidity (Praharaj et al., 2018). The National Sleep Foundation stated that 70% of Indonesian people experience sleep disturbances at least once a week, and 30 million people have sleeping difficulty every night (Lestarianto, 2014). A survey of the healthy lifestyle index by American International Assurance (AIA) in 2013 demonstrated that Indonesian people have an average sleep duration of 6.8 hours per day due to increased activity and decreased sleep duration from its normal range, indicating many Indonesian people are suffered from sleep deprivation (Putri et al., 2017).

The relationship between sleep duration and the inflammation process has not been studied extensively, despite showing a similar correlation between sleep duration and mortality. People with long sleep duration (>8 hours/night) and short sleep duration (<7 hours/night) have a 30% and 12% greater risk of death, respectively, compared to people with moderate sleep duration (7-8 hours/night). Physiologically, sleep affects two major effector systems, the Hypothalamus-Pituitary-Adrenal (HPA) axis and the sympathetic nervous system, which simultaneously increases the release of pro-inflammatory cytokines and markers of systemic inflammation through β-adrenergic activation. The activity of sympathetic nervous system decreases when a person is sleeping, which may explain the relationship between sleep disorder, short sleep duration, and elevation of inflammatory markers (Chen et al., 2017). Patients that pathologically tend to fall asleep during daytime and easily get tired exhibit an increase in IL-6, a pro-inflammatory cytokine, in the circulatory system due to activation of the HPA Axis. People suffering from sleep deprivation produce more IL-6 during the day and less IL-6 at night, whereas good sleep reduces pro-inflammatory exposure to tissues. Furthermore, sleep deprivation upregulates pro-inflammatory cytokines through NF-kB activation, generating a pro-inflammatory state.

Overexposure to the pro-inflammatory cytokines caused by sleep deprivation will increase tissue damage, decrease brain function, and increase the expression of IL-6 (Vgontzas et al., 2000).

Sleep deprivation correlates with the increase in TNF-α, indicating a pathological condition caused by sleep disorders (Chennaoui et al., 2011). Moreover, TNF-α is expressed in neurons and plays a role in brain neuroplasticity (Rockstrom et al., 2018). On the other hand, a clinical trial showed that IFN-γ was found in cases of fever, flu, and drowsiness, whereas IFN-γ can be somnogenic in the presence of TNF-α. Therefore, IFN-γ has a significant role in sleep regulation during viral infection (Van Dongen et al., 2011).

Studies suggest that cortisol levels as a physiological stress marker and systemic inflammatory marker may increase in sleep-deprived conditions. Sleep deprivation triggers the release of adrenocorticotropic hormone (ACTH) via the HPA axis, inducing the adrenal gland to release cortisol. Along with other stimulations, including norepinephrine and radical oxygen species (ROS), cortisol triggers inflammatory activation in the brain and peripheral immune cells by increasing the expression of pro-inflammatory cytokine genes such as IL-1, IL-6, and TNF-α via transcriptional regulator of pro-inflammatory gene expression such as NF-κB. These pro-inflammatory cytokines then enter the systemic circulation inducing the increase of leukocytes, mainly neutrophils, CD4+ T-cells, B-cells, and monocyte leading to inflammatory reactions (Garbarino et al., 2021).

Sleep deprivation (SD) can be categorized into paradoxical sleep deprivation (PSD) and total sleep deprivation (TSD), and studied using the Modified Multiple Platform Method (MMPM). While both types can induce stress, total sleep deprivation causes poor decision-making control, triggers repetitive errors in working memory, and abolishes individual spontaneity in communication, making the subjects appear lazy, lethargic, and unmotivated (Gunn et al., 2017).

Previous studies showed that sleep deprivation affects cortisol and inflammatory cytokine levels in rats or humans by interfering with homeostatic sleep patterns and disrupting HPA axis activation (Chennaoui et al., 2011; Medic et al., 2017; Neto et al., 2010; Wright et al., 2015). While sleep recovery may restore rat deteriorating conditions caused by sleep deprivation, no studies clearly define the effect of sleep recovery on pro-inflammatory and anti-inflammatory cytokine levels. This study directly compared cortisol and inflammatory cytokine level among rats treated with PSD, TSD, and sleep recovery after PSD and TSD treatment. Considering that many people have experienced sleep deprivation,

this study is essential as initial research to determine the effect of sleep deprivation and sleep recovery on the levels of cortisol and inflammatory cytokines that impair various metabolic functions in mammals.

EXPERIMENTAL SECTION

Research Design

This study was an experimental study using a post-test only with control group design.

Subject

Wistar rats (*Rattus norvegicus)* with characteristics of male, 8-12 weeks old, and 200-300 grams of weight were obtained from the Laboratorium Penelitian dan Pengujian Terpadu (LPPT) 4 Universitas Gajah Mada. Sleep deprivation experiments were performed using the Modified Multiple Platform Method (MMPM) to prevent the rats from sleeping. A total of 25 rats were randomly divided into five groups containing five rats in each group: control (no SD), PSD (20 hours of SD per day at 11.00 a.m. - 07.00 a.m. for 5 days and 4 hours rest at 07.00 a.m. - 11.00 a.m.), TSD (24 hours of SD per day at 11.00 a.m. - 11.00 a.m. for 5 days without rest), PSD+SR (PSD treatment for 5 days followed by sleep recovery (SR) for 5 days at 07.00 a.m. - 07.00 a.m.), and TSD+SR (TSD treatment for 5 days followed by sleep recovery for 5 days at 11.00 a.m. - 11.00 a.m.).

Research Procedure

Modified Multiple Platform Method (MMPM) tanks measuring 123 x 44 x 35 cm were used for the sleep deprivation stress model. The tanks were filled with water and equipped with 12 platforms of 6.5 cm in size with a distance of 10 cm between the platforms as a foothold for the rats that are instinctively avoiding water. Each tank contains five experimental rats equipped with a muscle atonia device, an instrument that gives an automatic shock effect every 10 minutes to keep the rats awake. The experiments were conducted in the Anatomy Laboratory of the Faculty of Medicine of Jenderal Soedirman University from August to September 2021.

Observation

Cortisol levels were measured by drawing 2 ml of rat blood for each group at 07.00-09.00 a.m. via retroorbital vein using a microhematocrit pipette into an EDTA tube,

centrifuging for 15 minutes at 2,000-3,000 rpm, and storing in -20 ℃. A total of 50 mL of collected plasma was examined with the Enzyme-Linked Immunosorbent Assay (ELISA) method and read by the ELISA reader in 450 nm absorbance (Zabidi et al., 2015).

For inflammatory cytokines level measurement, rat blood plasma samples were collected from the retroorbital plexus as much as 55 mL. The levels of inflammatory cytokines of IL-6, IL-10, TNF-α, and IFN-γ were then measured using multiplex bead-based immunoassay from MILLIPLEX® MAP kit with Luminex technology in the Integrated Laboratory of Faculty of Medicine, Universitas Indonesia. Briefly, the frozen samples were thawed, stirred thoroughly with a vortex, and centrifuged to remove particulates before the immunoassay. The processed samples were then placed on the plate, added with conjugated beads from the MILLIPLEX® MAP kit, and incubated for 2 hours at 20-24 ˚C or overnight at 4-8 ˚C. After incubation, the beads were washed, incubated in biotinylated-detection antibodies, added with Streptavidin-PE (SAPE), and further incubated. The beads were then washed, resuspended in an appropriate buffer, and placed on Luminex technology to measure cytokine levels. The plasma cytokine levels were analyzed based on the fold change value by comparing the treatment and the control group.

Statistical analysis

Normality analysis was performed by the Shapiro-Wilk test, and data homogeneity was analyzed by Levene's test. The multivariate analysis was then performed by the One-Way ANOVA and Post-hoc Least Significant Difference (LSD) to determine differences between groups. Kruskal-Wallis and Mann-Whitney tests were performed for non-parametric analysis.

All procedures were approved by The Medical Research Ethics Commission of The Faculty of Medicine of Jenderal Soedirman University on May 10, 2021 (reference number: 097/KEPK/V/2021).

RESULT AND DISCUSSION

Measurement and analysis results of mean cortisol levels among all groups are shown in **Figure 1**. Since the plasma cortisol levels were not normally distributed, non-parametric tests were used for further analysis.

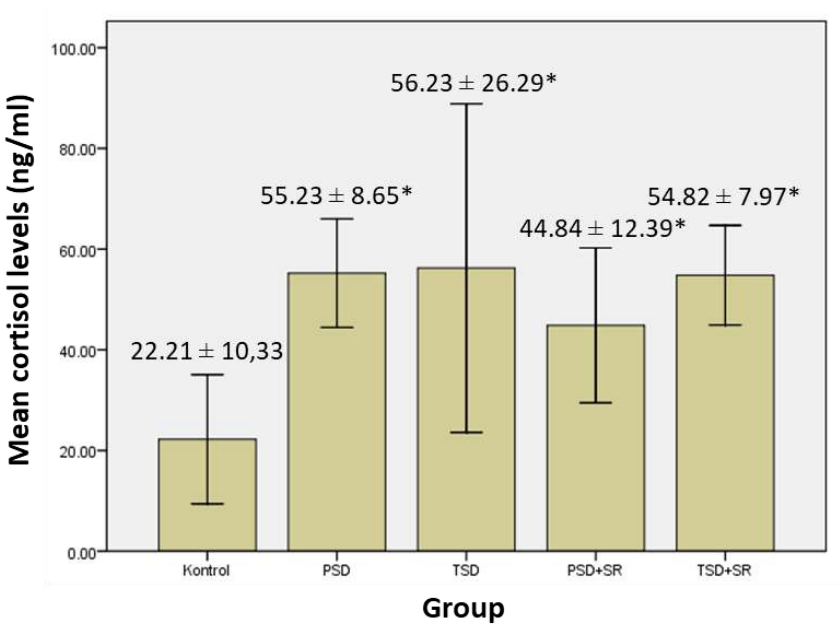

Figure 1. Mean cortisol levels in male Wistar rat (*Rattus norvegicus*). Control: no SD; PSD: 20 hours of SD with 4 hours rest for five days; TSD: 24 hours of SD for five days; PSD+SR: PSD followed by SR; TSD+SR: TSD followed by SR. SD: sleep deprivation, SR: sleep recovery, PSD: paradoxical sleep deprivation, TSD: total sleep deprivation. Error bars represent 95% confidence intervals. Statistical analysis was performed using Kruskal-Wallis test ($p = 0.029$) and Mann-Whitney test for post-hoc analysis. *Post-hoc Mann-Whitney test compared to control, $p < 0.05$.

Kruskal-Wallis analysis showed substantial differences in cortisol levels between at least two groups for the assessed variables ($p=0.029$). Further post-hoc analysis with the Mann-Whitney test obtained significant results between every intervention group and the control ($p<0.05$). PSD for 20 hours/day increased cortisol levels in the afternoon and evening in rats by two times compared to control (Olayaki et al., 2015). Cortisol levels increased in the first six hours after PSD induction caused by HPA axis activation to maintain metabolism changes that were occurred during sleep deprivation (Galvão et al., 2009). Consistent with a study by Olayaki et al. (2015) demonstrating that TSD for five days increased cortisol levels compared to the control, this study pointed out that the TSD group had the highest mean cortisol levels among all groups (56.23±26.29 ng/mL).

The PSD group showed lower mean cortisol levels than the TSD group because PSD has a sleep period daily, thus the negative impact of sleep deprivation can be relieved. Two hours of an afternoon nap after one night of sleep deprivation decreased the secretion of cortisol hormone caused by inhibition of the HPA axis at the slow wave sleep (SWS) phase that reduces cortisol levels (Pejovic et al., 2013). The effects of sleep deprivation that impair HPA function cause an increase in evening cortisol levels as a result of an elevation in its

secretory pulses amplitude, suggesting that the negative feedback of glucocorticoid mediated by hippocampal function may be affected by sleep-deprived condition (Hirotsu et al., 2015). Cortisol levels were higher in the subjects who stayed awake than in subjects who slept sufficiently, indicating that sleep deprivation affects the cortisol levels as a result of stress responses (Bassett et al., 2018). Cauter et al. (2008) emphasized that the initial consequence of partial sleep loss is a cortisol level increase in the evening. While under normal conditions, cortisol levels decrease rapidly and reach the minimum level just before sleeping time. Kantasa et al. (2016) stated that cortisol can suppress the immune system and induce an inflammatory pathway, making the body vulnerable to various diseases.

PSD + SR group exhibited lower cortisol levels than TSD + SR, and both PSD + SR and TSD + SR showed lower cortisol levels than PSD and TSD. A study by Mattice et al. (2011) showed that subjects induced by TSD for 24 hours and 48 hours followed by sleep recovery for 24 hours relieved their sleep deprivation effect for 72% and 42%, respectively. Sleep recovery for three days decreases cortisol levels and ameliorates the impact of sleep deprivation by restoring the HPA axis interaction (Pejovic et al., 2013). Sleep recovery also decreases lipid peroxidase and free radical production by increasing glutathione and other enzymatic antioxidants (Hirotsu et al., 2015). Glutathione increases after sleep recovery for two days, indicated by the elevation of 6-PGD, an enzyme that acts on the pentose phosphate pathway in the carbohydrate metabolism that protects cells from oxidative stress in the form of NADPH, thus inhibiting stress oxidative that has a role in cortisol increase (Kim et al., 2022). Taken together, these results indicate that sleep recovery is able to decrease cortisol levels which increase after induction of PSD or TSD.

Results of data analysis showing the value of minimum, maximum, mean, median, and standard deviation for each group are shown in **Table 1.**

Table 1. Levels of inflammatory cytokine in male Wistar rat (pg/mL).

Group	N	Min.				Max.				Mean±SD			
		IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ
Control	5	311.14	15.14	2.89	736.59	1706.25	83.31	12.06	1375.77	974± 504	34.42±11.92	6.91±3.28	1038.08±272.26
PSD+SR	5	452.10	17.52	4.54	754.54	3103.86	93.76	23.89	1001.67	1281±1056	53.07±12.98	9.75±8.05	847.45±102.73
TSD+SR	5	311.14	57.89	6.54	718.80	2518.29	317.89	26.13	1001.67	1578±928	87.06±47.99	14.17±8.17	865.83±112.95
PSD	5	520.98	44.84	6.90	781.54	1858.94	74.98	12.24	1385.65	1192±544	59.47±5.44	9.39±2.62	1046.28±239.32
TSD	5	382.22	33.52	1.76	700.85	1365.64	178.02	14.17	1824.71	952±373	68.06±25.53	8.01±4.39	1167.39±432.52

Table 2. P-value of normality, homogeneity, and One-Way ANOVA test in the levels of inflammatory cytokines in male Wistar rat.

Group	Shapiro-Wilk (p-value)				Levene's (p-value)				One-Way Anova (p-value)			
	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ	IL-6	IL-10	TNF-α	IFN-γ
Control	0.832	0.941	0.389	0.640								
PSD+SR	0.884	0.482	0.081	0.409								
TSD+SR	0.812	0.909	0.096	0.921	0.256	0.352	0.697	0.075	0.658	0.065	0.399	0.283
PSD	0.067	0.845	0.200	0.610								
TSD	0.622	0.500	0.615	0.750								

Normality analysis by the Shapiro-Wilk test and homogeneity analysis by the Levene's test showed a p-value >0.05 in all groups, indicating data of inflammatory cytokine levels were normally distributed and homogenous. Multivariate analysis using the One-Way ANOVA test demonstrated a p-value >0.05 in all groups, indicating no significant difference in inflammatory cytokine levels among the treatment groups, as shown in **Table 2**. The inflammatory cytokine levels were then compared among the treatment and control groups using fold change. The results demonstrated the highest increase in inflammatory cytokine levels was in the TSD treatment group (**Figure 2**). However, the multivariate analysis of the fold change of inflammatory cytokine levels using the One-Way ANOVA test did not show a significant difference ($p > 0.05$) in IL-6 ($p=0.658$), IL-10 ($p=0.085$), TNF-α ($p= 0.313$), and IFN-γ ($p=0.283$).

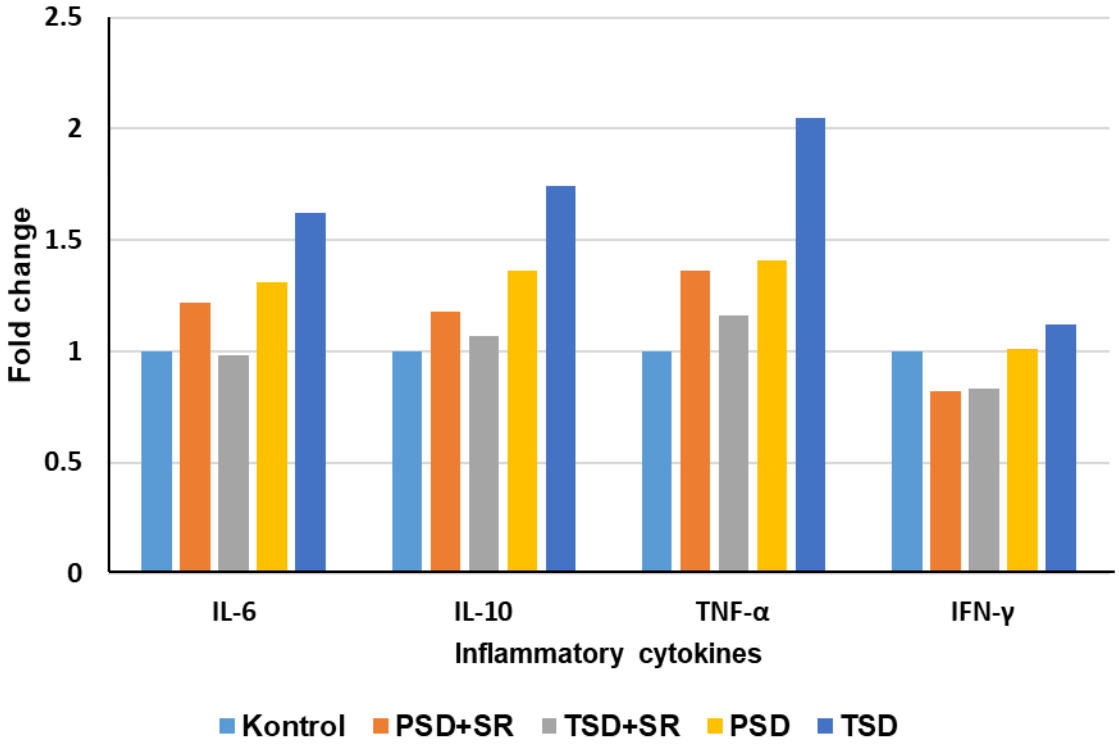

Figure 2. Mean of the fold change of inflammatory cytokine levels of IL-6, IL-10, TNF-α, and IFN-γ in male Wistar rat (*Rattus norvegicus*). Control group: no SD; PSD+SR: PSD followed by SR; TSD+SR: TSD followed by SR; PSD: 20 hours of SD with 4 hours rest for five days; TSD: 24 hours of SD for five days. SD: sleep deprivation, SR: sleep recovery, PSD: paradoxical sleep deprivation, TSD: total sleep deprivation.

Sleep deprivation triggers a stress response in animal models by increasing pro-inflammatory cytokines and activates the main stress axis in humans, the HPA axis. IL-6 can

stimulate cortisol secretion directly in the adrenal gland or via activation of the hypothalamus that induces the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. On the other hand, a slight elevation of IL-6 level in plasma induces the anti-inflammatory cytokines, IL-1ra and IL-10, along with c-reactive protein (CRP). Recent studies stated that apart from injury and infection, sleep deprivation triggers the pro-inflammatory response through the increase of pro-inflammatory cytokines secretion (Medic et al., 2017; Simpson & Dinges, 2007; Sukendra, 2015).

Activation of inflammatory responses caused by sleep deprivation can increase the levels of anti-inflammatory cytokines to prevent excessive inflammatory responses. IL-10, a potent anti-inflammatory cytokine, inhibits the activation of macrophages and dendritic cells, leading to the reduction of cytokine levels produced by T-helper1 (Th1) cells (Welsh et al., 2011). IL-10 also potently inhibits the production of IL-10 itself, IL-12, L-1α, IL-1β, IL-6, macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), leukemia inhibitory factor (LIF), and platelet-activating factor (PAF) from activated monocytes and macrophages (Saraiva & O'Garra, 2010).

This study showed that sleep deprivation did not significantly affect levels of IL-10 in each variable ($p = 0.065$). This result is consistent with the study of Neto et al. (2010), showing that sleep deprivation did not impair levels of anti-inflammatory cytokines, including IL-10. Otherwise, they also observed sleep deprivation increased IL-10 levels in different adipose tissue depots and decreased the TNF-α levels in the brain. Therefore, the profile of systemic anti-inflammatory cytokines after sleep deprivation remains controversial.

A study by Patel et al. (2009) also demonstrated that sleep deprivation did not affect levels of anti-inflammatory cytokines such as IL-1 and IL-10. They explained that the different responses of sleep deprivation on the levels of anti-inflammatory cytokines may depend on the differences in the effect of sleep on inflammatory components in each individual, the half-life of each inflammatory component, and the instrument performance for cytokine measurement. Intriguingly, anti-inflammatory cytokine levels increase in chronic sleep deprivation that lasts over weeks, months, or years.

Wright et al. (2015) demonstrated that sleep deprivation significantly increased levels of IL-10 on day 26 to day 28 measured with ELISA. Sleep deprivation induces circadian rhythm impairment that, over weeks, will increase levels of anti-inflammatory cytokine (IL-

10), the pro-inflammatory protein (TNF-α), and CRP. Otherwise, cortisol levels increase during acute sleep deprivation, indicating that acute and chronic sleep deprivation provoke different responses of circadian rhythm impairment. Therefore, acute sleep deprivation is associated with physiological stress or metabolic response of increased cortisol levels, whereas chronic sleep deprivation is associated with physiological adaptation as a response to decreased cortisol levels followed by increased pro-inflammatory and anti-inflammatory cytokines.

No significant difference in TNF-α levels after induction of various sleep deprivation stress models in this study ($p = 0.366$) is consistent with the study by Ruiz et al. (2012), showing that total sleep deprivation for two days or paradoxical sleep deprivation for four days followed by sleep recovery for three days did not alter TNF-α levels. Other studies also demonstrated that ten days of sleep deprivation did not increase TNF-α or its receptors, regardless of sex characteristics (Shearer et al., 2001; Xu et al., 2015). In contrast, a study by Irwin et al. (2006) exhibited that sleep deprivation increased TNF-α production. Sleep deprivation probably induced TNF-α cellular expression through the activation of nuclear factor NF-kB, a key component in controlling the cellular expression of pro-inflammatory cytokines.

In agreement with the study by Crooks et al. (2019) that demonstrated no significant result between sleep quality and IL-6 levels, this study approved that there was no significant result between the levels of pro-inflammatory cytokines, IL-6, with various sleep deprivation stress models ($p = 0.658$). Conversely, a study by Siregar (2017) showed a significant result between sleep quality and IL-6 levels. Sleep deprivation may affect components of the immune system through modification of CD4+ cells, CD8+ cells, NK cells, and levels of pro-inflammatory cytokines such as TNF-α, INF-γ, and IL-6 (Ibarra-Coronado et al., 2015). Collectively, these results indicate that pure TSD and PSD or TSD and PSD followed by sleep recovery did not affect plasma cytokine levels of IL-6 and TNF-α.

Multivariate analysis among the treatment groups showed no significant difference between sleep deprivation and IFN-γ levels ($p=0.283$). This result is consistent with the study by Hirotsu et al. (2012), demonstrating that there was no significant difference in IFN-γ levels in psoriasis rats model induced by paradoxical sleep deprivation for 48 hours. IFN-γ production is generally unaffected by sleep deprivation, instead is increased at the end of sleep recovery. These insignificant results may be affected by sample processing that caused cytokine stability disruption, cytokine degradation by protease, or cytokine binding to its

soluble cellular receptors. These alterations may also explain the decrease in cytokine levels over time during sample storage (Hennø et al., 2017).

The main limitation of this study is that it only examined the level of inflammatory cytokine levels in plasma but not in the tissue resulting in an insignificant increase in inflammatory cytokine levels after sleep deprivation treatment. Induction of inflammatory cytokine release is likely more prominent in the brain or other tissues, which are initially released by immune and glial cells in the brain after activation of the HPA axis (Garbarino et al., 2021). Another limitation is that the stress induction protocol by sleep deprivation in this study was not enough to induce an increase in the cytokine level in plasma. Various sleep deprivation protocols may also cause different results in inflammatory cytokine levels among studies. Furthermore, variation in the circadian cycle may also affect the production of various cytokines, which confounds the levels of circulating cytokines in this study (Ruiz et al., 2012).

To date, studies concerning sleep deprivation and inflammatory cytokines remain elusive. Sleep deprivation indeed increases the main stress hormone, cortisol, which plays a role in various disease development, such as hypertension and cardiovascular disease (Sá Gomes e Farias et al., 2022). On the other hand, some studies indicated that people with sleep disorders showed an alteration in circulating levels of TNF-α and IL-6. However, other studies failed to demonstrate the change in TNF-α and IL-6 levels between normal and sleep-deprived subjects (Crooks et al., 2019; Irwin et al., 2006; Shearer et al., 2001; Siregar, 2017). Although this study showed insignificant results, sleep deprivation also has a potency to increase the pro-inflammatory cytokine expression that is associated with inflammatory reaction-related diseases such as cancer, neurodegeneration, and cardiovascular disease (Garbarino et al., 2021). Establishing how far sleep deprivation can affect cortisol and inflammatory cytokine levels and how much sleep recovery is required to alleviate the physiological disruption caused by sleep deprivation is essential to making recommendations for a healthy lifestyle. Furthermore, in this modern era, night shift job is increasing in various sectors, and the future development of this study can be used to make recommendations for working regulation to create work-life balance.

CONCLUSION

Stress induction by the various models of sleep deprivation modifies cortisol levels but not inflammatory cytokines levels of IL-6, IL-10, TNF-α, and IFN-γ in male Wistar rats (*Rattus norvegicus*). These results indicate cortisol levels can be used as a stress indicator induced by sleep deprivation, but inflammatory cytokines levels of IL-6, IL-10, TNF-α, and IFN-γ may not be used for this purpose. Furthermore, although not statistically different, sleep recovery restores the levels of cortisol and inflammatory cytokines after PSD or TSD treatment, suggesting sleep recovery able to bring back homeostasis conditions after stress. Further exploration of sleep deprivation and sleep recovery protocols is required to get valuable outcomes.

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REFERENCES

Bassett, S. M., Lupis, S. B., Gianferante, D., Rohleder, N., & Wolf, J. M. (2018). Sleep quality but not sleep quantity effects on cortisol responses to acute psychosocial stress. *Revista Del Colegio Americano de Cardiologia*, *72*(23), 2964–2979. https://doi.org/10.3109/10253890.2015.1087503.Sleep

Cauter, E. V., Spiegel, K., Tasali, E., & Leproult, R. (2008). Metabolic consequences of sleep and sleep loss. *Physiology & Behavior*, *176*(3), 139–148. https://doi.org/10.1016/S1389-9457(08)70013-3

Chen, L., Huang, J., Yang, L., Zeng, X. A., Zhang, Y., Wang, X., Chen, M., Li, X., Zhang, Y., & Zhang, M. (2017). Sleep deprivation accelerates the progression of alzheimer's disease by influencing Aβ-related metabolism. *Neuroscience Letters*, *650*, 146–152. https://doi.org/10.1016/j.neulet.2017.04.047

Chennaoui, M., Sauvet, F., Drogou, C., Van Beers, P., Langrume, C., Guillard, M., Gourby, B., Bourrilhon, C., Florence, G., & Gomez-Merino, D. (2011). Effect of one night of sleep loss on changes in tumor necrosis factor alpha (TNF-α) levels in healthy men. *Cytokine*, *56*(2), 318–324. https://doi.org/10.1016/j.cyto.2011.06.002

Chokroverty, S., & Ferini-Strambi, L. (2017). Oxford textbook of sleep disorders. In *Oxford*

University Press.

Crooks, E., Hansen, D. A., Satterfield, B. C., Layton, M. E., & Van Dongen, H. P. A. (2019). Cardiac autonomic activity during sleep deprivation with and without caffeine administration. *Physiology & Behavior*, *210*, 112643. https://doi.org/10.1016/j.physbeh.2019.112643.Cardiac

Galvão, M. de O. L., Sinigaglia-Coimbra, R., Kawakami, S. E., Tufik, S., & Suchecki, D. (2009). Paradoxical sleep deprivation activates hypothalamic nuclei that regulate food intake and stress response. *Psychoneuroendocrinology*, *34*(8), 1176–1183. https://doi.org/10.1016/j.psyneuen.2009.03.003

Garbarino, S., Lanteri, P., Bragazzi, N. L., Magnavita, N., & Scoditti, E. (2021). Role of sleep deprivation in immune-related disease risk and outcomes. *Communications Biology*, *4*(1). https://doi.org/10.1038/s42003-021-02825-4

Gunn, B. G., Cox, C. D., Chen, Y., Frotscher, M., Gall, C. M., Baram, T. Z., & Lynch, G. (2017). The endogenous stress hormone CRH modulates excitatory transmission and network physiology in hippocampus. *Cerebral Cortex*, *27*(8), 4182–4198. https://doi.org/10.1093/cercor/bhx103

Hennø, L. T., Storjord, E., Christiansen, D., Bergseth, G., Ludviksen, J. K., Fure, H., Barene, S., Waage-Nielsen, E., Mollnes, T. E., & Brekke, O. L. (2017). Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines – Consequences for defining reference values in healthy humans. *Cytokine*, *97*(May), 86–95. https://doi.org/10.1016/j.cyto.2017.05.014

Hirotsu, C., Rydlewski, M., Araújo, M. S., Tufik, S., & Andersen, M. L. (2012). Sleep loss and cytokines levels in an experimental model of psoriasis. *PLoS ONE*, *7*(11), 1–9. https://doi.org/10.1371/journal.pone.0051183

Hirotsu, C., Tufik, S., & Andersen, M. L. (2015). Interactions between sleep, stress, and metabolism: From physiological to pathological conditions. *Sleep Science*, *8*(3), 143–152. https://doi.org/10.1016/j.slsci.2015.09.002

Hirshkowitz, M., Whiton, K., Albert, S. M., Alessi, C., Bruni, O., DonCarlos, L., Hazen, N., Herman, J., Katz, E. S., Kheirandish-Gozal, L., Neubauer, D. N., O'Donnell, A. E., Ohayon, M., Peever, J., Rawding, R., Sachdeva, R. C., Setters, B., Vitiello, M. V., Ware, J. C., & Adams Hillard, P. J. (2015). National sleep foundation's sleep time duration recommendations: Methodology and results summary. *Sleep Health*, *1*(1), 40–43. https://doi.org/10.1016/j.sleh.2014.12.010

Ibarra-Coronado, E. G., Pantaleón-Martínez, A. M., Velazquéz-Moctezuma, J., Prospéro-García, O., Méndez-Díaz, M., Pérez-Tapia, M., Pavón, L., & Morales-Montor, J. (2015). The Bidirectional relationship between sleep and immunity against infections. *Journal of Immunology Research*, *2015*, 678164. https://doi.org/10.1155/2015/678164

Irwin, M. R., Wang, M., Campomayor, C. O., Collado-Hidalgo, A., & Cole, S. (2006). Sleep deprivation and activation of morning levels of cellular and genomic markers of inflammation. *Archives of Internal Medicine*, *166*(16), 1756–1762. https://doi.org/10.1001/archinte.166.16.1756

Kantasa, V. D., Kusumawardani, B., & Herniyati. (2016). Efek Stresor rasa sakit renjatan listrik terhadap limfosit dan makrofag pada gingiva tikus sprague dawley (The Effect

of electrical shock stressor on lymphocytes and macrophages in gingival tissue of sprague dawley rats). *Pustaka Kesehtan*, *4*(1), 48–54.

Kim, J., Yun, K. sang, Cho, A., Kim, D. H., Lee, Y. K., Choi, M. J., Kim, S. hyung, Kim, H., Yoon, J. W., & Park, H. C. (2022). High cortisol levels are associated with oxidative stress and mortality in maintenance hemodialysis patients. *BMC Nephrology*, *23*(1), 1–9. https://doi.org/10.1186/s12882-022-02722-w

Lestarianto, J. A. (2014). Hubungan antara internet addiction dan tingkat stres dengan kejadian insomnia pada mahasiswa keperawatan Fakultas Ilmu Kesehatan Universitas Nuhammadiyah (correlation between internet addiction and stress levels with the incidence of insomnia in nursing students at the Faculty of Health Sciences of University of Muhammadiyah). *Diss. Universitas Muhammadiyah Purwokerto*. http://repository.ump.ac.id/id/eprint/5503

Lichtenstein, G. R. (2015). The Importance of sleep. *Gastroenterology & Hepatology*, *11*(12), 790.

Mattice, C. D., Brooks, R., & Lee-Chiong, T. (2011). Fundamentals of sleep technology: Second edition. *Philadelphia: Lippincott Williams & Wilkins.*

Medic, G., Wille, M., & Hemels, M. E. H. (2017). Short- and long-term health consequences of sleep disruption. *Nature and Science of Sleep*, *9*, 151–161. https://doi.org/10.2147/NSS.S134864

Neto, J. C. R., Lira, F. S., Venancio, D. P., Cunha, C. A., Oyama, L. M., Pimentel, G. D., Tufik, S., Do Nascimento, C. M. O., Santos, R. V. T., & De Mello, M. T. (2010). Sleep deprivation affects inflammatory marker expression in adipose tissue. *Lipids in Health and Disease*, *9*(1), 125. https://doi.org/10.1186/1476-511X-9-125

Olayaki, L. A., Sulaiman, S. O., & Anoba, N. B. (2015). Vitamin C prevents sleep deprivation-induced elevation in cortisol and lipid peroxidation in the rat plasma. *Nigerian Journal of Physiological Sciences : Official Publication of the Physiological Society of Nigeria*, *30*(1–2), 5–9.

Patel, S. R., Zhu, X., Storfer-Isser, A., Mehra, R., Jenny, N. S., Tracy, R., & Redline, S. (2009). Sleep duration and biomarkers of inflammation. *Sleep*, *32*(2), 200–204. https://doi.org/10.1093/sleep/32.2.200

Patrick, Y., Lee, A., Raha, O., Pillai, K., Gupta, S., Sethi, S., Mukeshimana, F., Gerard, L., Moghal, M. U., Saleh, S. N., Smith, S. F., Morrell, M. J., & Moss, J. (2017). Effects of sleep deprivation on cognitive and physical performance in university students. *Sleep and Biological Rhythms*, *15*(3), 217–225. https://doi.org/10.1007/s41105-017-0099-5

Pejovic, S., Basta, M., Vgontzas, A. N., Kritikou, I., Shaffer, M. L., Tsaoussoglou, M., Stiffler, D., Stefanakis, Z., Bixler, E. O., & Chrousos, G. P. (2013). Effects of recovery sleep after one work week of mild sleep restriction on interleukin-6 and cortisol secretion and daytime sleepiness and performance. *American Journal of Physiology - Endocrinology and Metabolism*, *305*(7), 890–896. https://doi.org/10.1152/ajpendo.00301.2013

Praharaj, S. K., Gupta, R., & Gaur, N. (2018). Clinical practice guideline on management of sleep disorders in the elderly. *Indian Journal of Psychiatry*, *60*(7), S383–S396. https://doi.org/10.4103/0019-5545.224477

Putri, D. N. E., Nasrul, E., & Masri, M. (2017). Pengaruh kurang tidur terhadap berat badan pada tikus Wistar jantan (effect of sleep deprivation on body weight in male Wistar rats). *Jurnal Kesehatan Andalas*, *6*(1), 78–82. https://doi.org/10.25077/jka.v6i1.655

Rockstrom, M. D., Chen, L., Taishi, P., Nguyen, J. T., Gibbons, C. M., Veasey, S., & Krueger, J. M. (2018). Tumor necrosis factor alpha in sleep regulation. *Sleep Medicine Reviews*, *40*, 69–78. https://doi.org/10.1016/j.smrv.2017.10.005.Tumor

Ruiz, F. S., Andersen, M. L., Martins, R. C. S., Zager, A., Lopes, J. D., & Tufik, S. (2012). Immune alterations after selective rapid eye movement or total sleep deprivation in healthy male volunteers. *Innate Immunity*, *18*(1), 44–54. https://doi.org/10.1177/1753425910385962

Sá Gomes e Farias, A. V., de Lima Cavalcanti, M. P., de Passos Junior, M. A., & Vechio Koike, B. del. (2022). The association between sleep deprivation and arterial pressure variations: a systematic literature review. *Sleep Medicine: X*, *4*, 100042. https://doi.org/10.1016/j.sleepx.2022.100042

Saraiva, M., & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature Reviews Immunology*, *10*(3), 170–181. https://doi.org/10.1038/nri2711

Shearer, W. T., Reuben, J. M., Mullington, J. M., Price, N. J., Lee, B. N., O'brian Smith, E., Szuba, M. P., Van Dongen, H. P. A., & Dinges, D. F. (2001). Soluble TNF-α receptor 1 and IL-6 plasma levels in humans subjected to the sleep deprivation model of spaceflight. *Journal of Allergy and Clinical Immunology*, *107*(1), 165–170. https://doi.org/10.1067/mai.2001.112270

Simpson, N., & Dinges, D. F. (2007). Sleep and inflammation. *Nutrition Reviews*, *65*(SUPPL.3), S244–S252. https://doi.org/10.1111/j.1753-4887.2007.tb00371.x

Siregar, E. M. H. (2017). Korelasi kualitas tidur dengan kadar interleukin-6 serum pada pasien hemodialisis regular (correlation of sleep quality with interleukin-6 serum levels in regular hemodialysis patients). In *Thesis Universitas Sumatera Utara*.

Sukendra, D. M. (2015). Efek olahraga ringan pada fungsi imunitas terhadap mikroba patogen: infeksi virus dengue (effects of light exercise on immune function against microbial pathogens: dengue virus infection). *Media Ilmu Keolahragaan Indonesia*, *5*(2), 57–65.

Van Dongen, H. P. A., Belenky, G., & Krueger, J. M. (2011). A Local, Bottom-up perspective on sleep deprivation and neurobehavioral performance. *Current Topics in Medicinal Chemistry*, *11*(19), 2414–2422. https://doi.org/doi: 10.2174/156802611797470286.

Vgontzas, A. N., Papanicolaou, D. A., Bixler, E. O., Hopper, K., Lotsikas, A., Lin, H. M., Kales, A., & Chrousos, G. P. (2000). Sleep apnea and daytime sleepiness and fatigue: Relation to visceral obesity, insulin resistance, and hypercytokinemia. *Journal of Clinical Endocrinology and Metabolism*, *85*(3), 1151–1158. https://doi.org/10.1210/jcem.85.3.6484

Welsh, P., Murray, H. M., Ford, I., Trompet, S., De Craen, A. J. M., Jukema, J. W., Stott, D. J., McInnes, I. B., Packard, C. J., Westendorp, R. G. J., & Sattar, N. (2011). Circulating interleukin-10 and risk of cardiovascular events: A prospective study in the elderly at risk. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(10), 2338–2344.

https://doi.org/10.1161/ATVBAHA.111.231795

Wright Jr, K. P., Drake, A. L., Frey, D. J., Fleshner, M., Desouza, C. A., Gronfier, C., & Czeisler, C. A. (2015). Influence of sleep deprivation and circadian misalignment on cortisol, inflammatory markers, and cytokine balance. *Physiology & Behavior*, *47*, 24–34. https://doi.org/10.1016/j.bbi.2015.01.004.Influence

Xu, C., Wu, X., Hack, B. K., Bao, L., & Cunningham, P. N. (2015). TNF causes changes in glomerular endothelial permeability and morphology through a Rho and myosin light chain kinase-dependent mechanism. *Physiological Reports*, *3*(12), 1–16. https://doi.org/10.14814/phy2.12636

Zabidi, L., Supriatna, M., Departemen, M. M., Kesehatan, I., Fk-Undip, A., Rsup, /, Semarang, K., & Mexitalia, M. (2015). Kadar kortisol serum sebagai indikator prognosis sepsis pada anak (serum cortisol level as prognostic indicator for pediatric sepsis). *Sari Pediatri*, *17*(2), 101–106.