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Comparison of Effects of Sleep Fragmentation on Pro-Inflammatory Cytokine IL-1β of Male and Female Adult Mice

Manzar Rzayeva

COMPARISON OF EFFECTS OF SLEEP FRAGMENTATION ON PRO-INFLAMMATORY CYTOKINE IL-1β OF MALE AND FEMALE ADULT MICE

A Capstone Experience/Thesis Project Presented in Partial Fulfillment

of the Requirements for the Degree Bachelor of Science

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By

Manzar Rzayeva

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CE/T Committee:

Dr. Noah Ashley, Chair

Dr. Ajay Srivastava

Dr. Martha Day

ABSTRACT

Sleep plays an essential role throughout the body by affecting the physiological function and regulation of many systems. One of these systems that receives effects as a result of the adequacy of sleep is the immune system. Previous studies have demonstrated effects of sleep fragmentation upon the immune system; however, sexual differences of these effects have not been studied in depth. To analyze these variances amongst the genders, male and female adult mice were subjected to acute sleep fragmentation (SF) for 24 hours in an automated SF cage that includes a bar sweeping across the cage every two minutes. Meanwhile, the control group of male and female adult mice were left undisturbed. Following SF, the brain, spleen, liver, and white adipose tissues of both SF and control groups of mice were collected and stored in RNA*later* solution. Total RNA of each of the tissues belonging to the mice were isolated. Additionally, RNA was reverse transcribed into cDNA. Then, gene expression of proinflammatory cytokine IL-1β was assessed using RTPCR with Taqman primer/probes. Results show that female mice exhibited more elevation of IL-1β gene expression in spleen from acute sleep fragmentation compared with males. However, sleep fragmentation did not increase IL-1β gene expression in liver or white adipose tissue for either sex. Taken together, these data provide more evidence of a sexual difference in immune response with a focus upon obstructive sleep apnea.

I dedicate this to my parents, who took a courageous leap in the pursuit of providing better education for their children. I also dedicate this to the fourteen-year-old me, whom, without her confidence, dedication, and determination, I would not have come this far.

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VITA

EDUCATION

Western Kentucky University, Bowling Green, KY — May 2022

B.S. in Chemistry and Biology – Mahurin Honors College Graduate

Honors CE/T: *Comparison of Effects of Sleep Fragmentation on Pro-Inflammatory Cytokine IL-1β of Male and Female Adult Mice*

Greenwood High School, Bowling Green, KY — May 2018

PROFESSIONAL EXPERIENCE

Chemistry Department Teacher Assistant	January 2019 - Present
Biology Department Learning Assistant, Intern	January 2022 - Present
Med Center Health Patient Coordinator, Intern	January 2022 - Present
Starbucks Barista & Shift Supervisor	May 2019 - January 2022

AWARDS & HONORS

Magna Cum Laude, WKU, May 2022

Honors College Graduate, WKU, May 2022

Nell Skean Lab Assistant Award, WKU, March 2022

Outstanding Biotechnology Award, WKU, March 2022

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INTRODUCTION

Sleep plays an essential role throughout the body by affecting the physiological function and regulation of many systems. Inadequate duration and quality of sleep is directly correlated with deficits in cognitive functions and psychological well-being and can impair immune functioning. These effects can culminate in individuals being prone to cardiovascular diseases (Worley, 2018). It is inevitable that sleep plays a fundamental role with many functions of the body, and more specifically the immune system which is designed to assist with protecting the body from various diseases and infections. As much as sleep itself has many effects and causes within the human body, the adequacy of duration and quality of sleep also plays a vital role. Inadequate duration and/or quality of sleep has strong associations with many medical conditions, including increased mortality & morbidity (Hanson, 2022).

Despite the significance of sleep, studies have found that there are up to 70 million people in the US and 45 million people in Europe who have chronic sleep disorders that impact daily functioning and health (Medic, 2017). With there being many lifestyle factors that correlate with sleep disruption, such as being a college student, consuming excessive amounts of caffeine, and many more, there has also been a variety of sleep disorders which lead to disruptions in sleep quantity and/or quality (Medic, 2017). Regardless of sleep disorders being common across many populations, there are many instances of misdiagnoses of sleep orders. One of the consequences for this is the

manifestation of effects of sleep loss and disorders being identified or misinterpreted as other clinical, physiological, and psychological conditions (Bonsignore, 2019).

The consequences of these misdiagnosis have also been seen within gender differences too. A study completed on sex differences in obstructive sleep apnea (OSA), which is a type of sleep disorder that causes disturbances in breathing, have revealed that males are more likely to be diagnosed with sleep apnea compared to females with a ratio of 3:1 to 5:1 in general populations, and from 8:1 to 10:1 in selected populations (Lin, 2008). These gender-based differences in addition to failure of some healthcare providers to recognize the clinical presentation of OSA have caused and could lead to further underdiagnosis or misdiagnosis of many sleep disorders in females (Cubala, 2010). Subsequently, these misdiagnoses have also been observed with inappropriate treatment of sleep disorders between males and females. For example, only recently has the Food and Drug Administration reduced recommended dose of Zolpidem for women by half where up until then this drug was administered at the same dosage for both men and women (FDA, 2013). This change in dosing of Zolpidem, which is a sedative-hypnotic benzodiazepine receptor agonist (BZRA) prescribed for insomnia treatment, was the result of observed gender differences in metabolism (Cubala, 2010).

As much as there are differences of how male and female immune systems respond to sleep fragmentation, there are also similarities in it as well. It is known that sleep and immune system are bidirectionally linked where immune system activation mediates sleep; meanwhile, sleep affects the innate and adaptive responses of defense system of human body. In a time of distress, such as the body not receiving adequate amount of sleep, the immune system is able to activate and release sleep regulatory

substances as a defense mechanism to assist with regulation of homeostasis (Besedovsky, 2019). These sleep regulatory substances are also referred to as pro-inflammatory cytokines which are released by the immune system in response to stress being applied to the body and causing disturbances of normal regulatory functions. For this study, the emphasis was on analyzing the response of interleukin-1-beta (IL-1β) as a pro-inflammatory cytokine in immune system which mediated physiological and pathological responses to stress and sterile inflammatory stimuli (Song, 2018). Within periphery tissues, IL-1β has been found to promote inflammation, development and maturation of immune cells, immune function, and sleep regulation (Jewett, 2012).

The aim of the study was to analyze the expression of IL-1β as a pro-inflammatory cytokine in peripheral tissues of spleen, liver, and white adipose tissue between male and female adult mice that have experienced sleep fragmentation. The effects of sleep fragmentation on the immune system of mice in general have been studied ceaselessly. However, whether inflammatory responses to sleep loss are dependent upon gender have been poorly studied. Therefore, by taking a deeper look and assessing the response of male and female mice to sleep fragmentation, the goal of the study was to bring awareness into the medical field and showcase the importance of adequate sleep and its effect on the immune system and many related functions of the body. With consideration of many misdiagnoses or mistreatments of sleep fragmentation between males and females, the aim of the study was also to raise awareness for potential gender differences and allow more gender-dependent treatments and diagnosis to be completed instead of generalized approaches. It is hypothesized both female and male adult mice show increased expression of IL-1β from acute sleep fragmentation (SF)

compared to the control (C) group (no SF). In addition, I also predicted that females would show increased IL-1β cytokine compared to males.

MATERIALS AND METHODS

Animals

Adult (>17 weeks of age) C57BL/6J mice were used for this study (18 male and 20 female). Out of the collection, 9 males and 10 females were Sleep Fragmented (SF) while the rest were designated to be in the Control (C) group. Both groups were maintained in a colony room at the Western Kentucky University in 12-hour light (lights on at 8:00 am) and 12 hours dark (lights off at 8:00 pm) while separated based on their genders. Each cage of mice was provided with corncob bedding, and food, and water ad libitum. Due to the research involving animals, following CITI program courses have been completed: Working with IACUC, Working with Mice in Research Settings, and Reducing Pain and Distress in Lab. All research was approved by the WKU IACUC committee (protocol #19-14).

Figure 1. C57BL/6J Adult Mice

Sleep Fragmentation

Male and female adult mice were assigned to control (N=10) or sleep-fragmented (N=10). Both groups of mice were separated based on their genders and whether they were SF or C groups. These mice were placed into Model 80390 automated SF chambers.

Replication of sleep fragmentation was done through these cages that involved a horizontal bar sweeping across the bottom of the cage every two minutes. The SF groups experienced the bar movement every two minutes with no harm done to the animal. Meanwhile, the C group were left undisturbed (no bar movement). Both groups of mice were housed in cages in the colony room for a 24 h period.

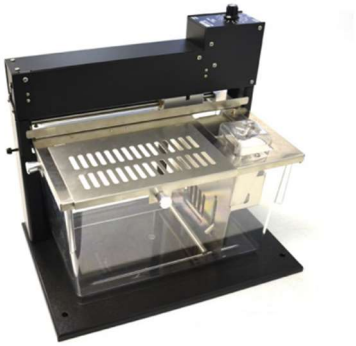

Figure 2. Model 80390 Sleep Fragmentation Chamber

Tissue Collection

After spending 24 hours in SF or C conditions, mice were then euthanized with carbon dioxide overdose. For each set of collection, tissues of C (N=5) and SF (N=5) at a time were collected. For this study, peripheral tissues of spleen, liver, and WAT of mice were collected and stored in RNA*later* (Qiagen) at 4˚C.

RNA Extraction and Reverse Transcription

Using RNeasy kit (Qiagen), the RNA was isolated from each set of peripheral tissues, spleen, liver, and WAT. Then, concentration of RNA for each set of tissues was measured using Nanodrop 2000 Spectrophotometer (Thermo Scientific). Once

concentrations were acquired, then RNA was reverse transcribed to cDNA by utilizing a High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific).

Real-Time PCR

Following the acquiring of cDNA that was reverse transcribed from RNA of the peripheral tissues, using ABI 7300-RT PCR system, the IL-1β cytokine expression was measured for each set of tissues where samples were duplicated and normalized to control gene of 18S. All of the samples were run in duplicate. The amplification protocol provided by Applied Biosystems 7300 machine was utilized and followed where, each plate was amplified at 50°C for two minutes, 95°C for ten minutes, and 95°C for fifteen seconds with forty cycles and 60°C for one minute. The protocol was followed as it was, with the exception of the volumes of plate being reduced to 20 μL from 50 μL.

Data Analysis

Acquired data from ABI 7300-RT PCR system which included fold change in mRNA levels were then calculated as relative mRNA expression levels, $2^{-\Delta\Delta Ct}$. Each Ct value was normalized to the lowest Ct value of control sample per tissue and gender type. The data was then graphed using Excel and results were analyzed using a two-way ANOVA with sex and sleep treatment as main factors.

RESULTS

Spleen

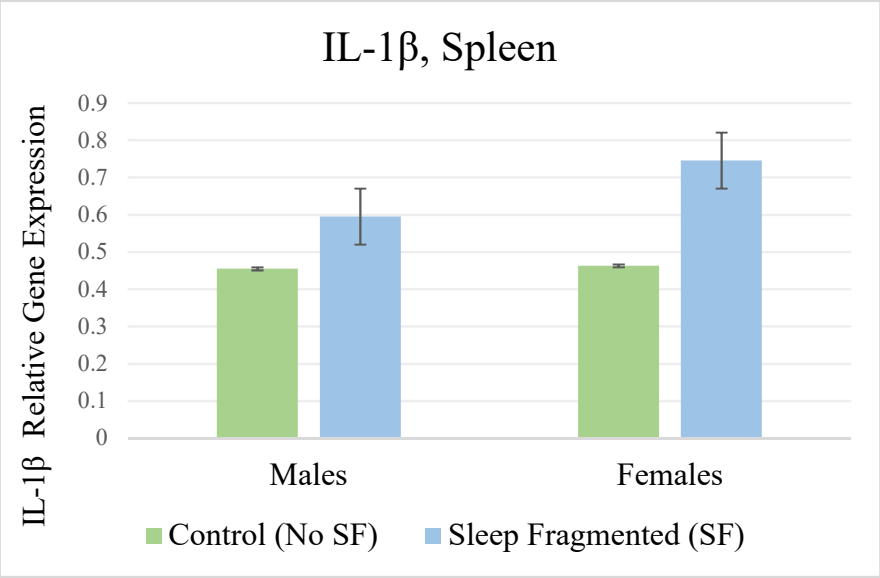

Figure 3. IL-1β Relative Gene Expression in Spleen

Expression of IL-1β between spleen of Control and Sleep Fragmented male and female was analyzed. SF significantly increased IL-1β gene expression in males and females (p=0.04, two-way ANOVA). After sleep fragmentation, compared to males, females showed a larger increase in IL-1β gene expression compared with controls (p= 0.03).

Liver

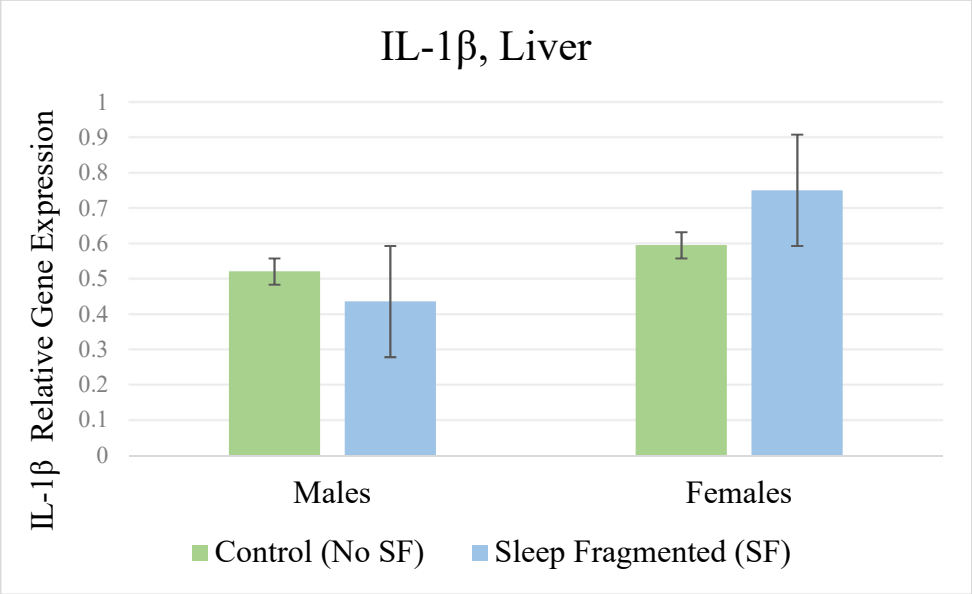

Figure 4. IL-1β Relative Gene Expression in Liver

Expression of IL-1β between liver tissues of Control and Sleep Fragmented Male and Female was analyzed. There was no significant effect of sleep fragmentation on liver IL-1β in male or female mice (p=.71) However, females had higher IL-1β expression compared with males (p=0.01).

WAT

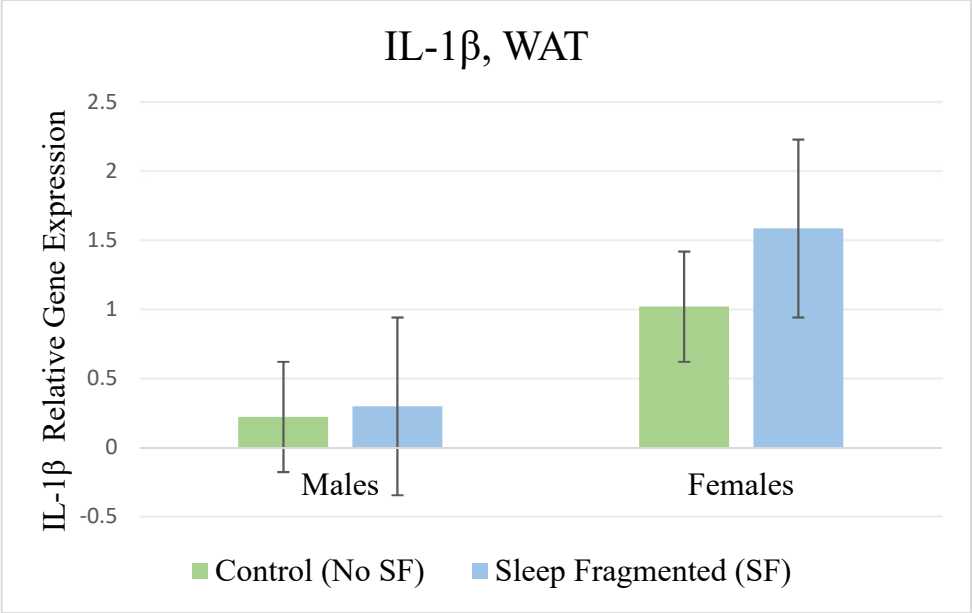

Figure 5. IL-1β Relative Gene Expression in WAT

Lastly, IL-1β expression of white adipose tissue of control and sleep fragmented male and female mice was analyzed. There were no significant increases in IL-1β expression from SF in males or females ($p = 0.54$). On average, females show increased IL-1β gene expression compared to males ($p = 0.04$).

DISCUSSION

It was hypothesized that both female and male adult mice would show increased expression of IL-1β from acute sleep fragmentation (SF) compared to the control (C) group (no SF). Subsequently, it was also predicted that the females would show increased IL-1β cytokine compared to males.

As hypothesized, both female and male adult mice signified showcased increase of IL-1β cytokine expression in SF group compared to the C group. Additionally, females displayed higher IL-1β expression compared with the males for spleen, liver, and WAT peripheral tissues. From previous studies conducted regarding role of adequate sleep on immune system, it was evidently concluded that following sleep fragmentation cytokine gene expression is altered and therefore, there is an impact on the development of inflammation (Dumaine and Ashley, 2015). Results of our studies also indicated the evidence of proinflammatory cytokines, for example IL-1β, displaying increase in expression when induced with inadequate and poor quality of sleep compared to the undisturbed C group of adult mice.

Furthermore, when compared to males, the female adult mice indicated higher IL-1β expression compared to males, most prominently after sleep fragmentation. These findings regarding the existing gender differences in response of immune system to alteration of sleep patterns and quality, further supports the aim of the study which is to raise awareness for potential gender differences with allowance of gender-dependent

treatments and diagnosis regarding sleep related diseases. All peripheral tissues of the female adult mice indicated higher expression of IL-1β compared to the male adult mice.

The evident difference of cytokine expression was mainly observed on the spleen of the mice. Results indicated there was significant increase of IL-1β gene expression in spleen of males and females (p=0.04). Furthermore, there following sleep fragmentation IL-1β gene expression indicated much larger increase in females compared to males and control groups (p=0.03). This increase of expression can be attributed to role of spleen tissue in the immune system, which is its ability to fight against infections within the body (UPMC, 2022). Sleep fragmentation causes immune system to be more susceptible to various infections which results with release of pro-inflammatory cytokines as a defense mechanism from the immune system (Asif, 2017). This explains the increase of the IL-1β gene expression in SF compared to C groups of males and females.

Subsequently, the expression of IL-1β in liver tissues of males and females, showcased no significant effect of sleep fragmentation (p=0.71). Although the liver plays an essential role within immune system, similar to spleen, the resulted expression of IL-1β could be allocated to the role of IL-1 superfamily of cytokines within liver where they could either serve as protective or proinflammatory cytokines (Barbier, 2019). In spite of the fact that there have not been many studies concluded on IL-1β serving as protective cytokines, instead of pro-inflammatory cytokines, the results of this study could potentially allow further studies to be done on IL-1 superfamily cytokines and their functions in liver tissues.

Lastly, the expression of IL-1β was analyzed in WAT of mice. Although there were no significant increases in IL-1β expression when induced with sleep fragmentation

in males or females (p=0.54), females show increased IL-1β gene expression compared to males, overall. This could be resulted due to the WAT of adult mice were collected from lower abdominal area, closer to the reproductive system. The increase in pro-inflammatory cytokine IL-1β of females, especially in the mice which were sleep fragmented, could be attributed to its proximity to reproductive system and its related hormones, and additional inflammatory cytokines. For this study, the reproductive systems or analysis of estrous cycles of female adult mice were not taken into consideration, which would have potentially helped to better analyze the greater expression of IL-1β cytokines in WAT of females compared to males.

The future research is needed to establish as continuation of this study and analyze pro-inflammatory cytokine Tumor Necrosis Factor-alpha (TMF- α) in addition to IL-1β. Although both cytokines have similar roles of being required for innate immune response, activation of macrophages and neutrophils, in the case of many pro-inflammatory response when one cytokine is activated it causes subsequent cytokines also to be activated (Ott, 2007). Therefore, a continuous study could be conducted to see if females continue to indicate increase of expression of pro-inflammatory cytokines in peripheral tissues (spleen, liver, and WAT) when induced with sleep fragmentation. Thereafter, the analysis of expression of TMF- α and IL-1β would allow to better analyze the gender related differences within response of immune system to acute sleep fragmentation.

Based on the acquired results for WAT of female adult mice, another future study is needed to establish the role of reproductive system and hormones levels of females in regard to their response for expression of pro-inflammatory cytokines when deprived

from sleep. In a future study, by considering estrous cycle and hormone levels of female, there could be more prominent data results and analysis of effects of sleep fragmentation on immune system of males and females.

Overall, the results from the study strongly and evidently indicated that females have overall higher increase of IL-1β pro-inflammatory cytokines compared to males, especially when analyzed the spleen tissue of both sleep fragmented and control groups of males and females. These overall findings combined with possibilities for future studies, would potentially and hopefully allow to raise further awareness towards gender differences exhibited in response of immune system to sleep fragmentation and allow more gender-dependent treatments and diagnosis to be completed instead of generalized approaches. The effects of adequacy and quality of the sleep on the immune system is inevitable, however, the existing gender differences amongst response of immune system to the sleep also should be left unnoticeable.

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