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Effect of Acute Sleep Fragmentation Upon Inflammatory Response of Brown and White Adipose Tissue in Male Mice

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EFFECT OF ACUTE SLEEP FRAGMENTATION UPON INFLAMMATORY RESPONSE OF BROWN AND WHITE ADIPOSE TISSUE IN MALE MICE

A Capstone Experience/Thesis Project Presented in Partial Fulfillment

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By

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ABSTRACT

Sleep is an important process required for vertebrates, including humans, to function. When sleep is disrupted, it leads to deleterious effects such as inflammatory responses throughout the body. Past studies have shown that acute (24 h) sleep fragmentation (SF) leads to an inflammatory response in white adipose tissue. However, whether brown adipose tissue responds in a similar fashion is unknown. Male adult (>8 weeks of age) C57BL/6j mice were subjected to SF for 24 h using a cage outfitted with a bar that moves horizontally across the cage every 2 min to periodically awaken mice (N =10). Controls were housed in a similar cage but experienced no bar movement (N=10). After SF, inguinal and epididymal white adipose tissue, as well as brown adipose tissue, were collected. Next, RNA was extracted from samples, reverse transcribed into cDNA, and then pro-inflammatory gene expression (IL-1ß and TNF-α) was assessed using real-time PCR. For both cytokines, there was differential expression in the different types of adipose tissue. Specifically, pro-inflammatory gene expression was elevated in white, but not brown, adipose tissue among SF mice. The difference in function of brown versus white adipose could serve as an explanation as why they respond differently to a stressor, such as sleep loss.

I dedicate this thesis to my parents, Jamos and Beverly Wriedt, who have supported me through every endeavor and are a great inspiration to me.

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INTRODUCTION

While not understood completely from a functional standpoint, sleep is imperative for survival. Sleep impacts cognitive ability and memory, hormone secretion, glucose metabolism, immune function, core body temperature, and renal function (Dumaine and Ashley, 2015). There is evidence suggesting that the quality and quantity of sleep have neurocognitive impacts, which can affect the morbidity and mortality of individuals (Simpson and Dinges, 2007). Additionally, a reduction in sleep can lead to severe pathologies such as cardiovascular disease and obesity (Dumaine and Ashley, 2015). One specific type of sleep loss affecting humans is obstructive sleep apnea (OSA). OSA is characterized by pharyngeal collapse, which leads to intermittent hypoxia and periodic arousals that lead to sleep fragmentation (SF). OSA is common within the United States affecting 14% of men and 5% of women (Peppard *et al*. 2013), and 60% of OSA patients are obese (Peppard *et al*. 2000). OSA also is a risk factor for cardiovascular diseases such as coronary artery disease, systemic arterial hypertension, and heart failure (Ryan, 2016). With such severe implications of sleep loss, it is important to understand how sleep loss functionally impacts the body and the immune system.

Sleep loss has a direct effect on the sympathetic nervous system (SNS). When the SNS is stimulated, it can activate the immune response, culminating in the release of cytokines. Cytokines are small peptides, which have pro-inflammatory or anti-inflammatory effects on the body. Cytokines lead to the activation of macrophages, which are involved in the upregulation of inflammatory responses (Zhang and An, 2007).

Measuring cytokine gene expression allows for the assessment of the immune response due to sleep loss.

Sleep loss increases pro-inflammatory cytokine gene expression, IL-1ß and TNF-α, in various tissues throughout the body such as white adipose tissue, heart tissue, and hypothalamus (Dumaine and Ashley, 2015). This can lead to further inflammation throughout the body's tissues, which, if persistent, results in chronic ailments. Adipose tissue is of particular interest due to its functional importance in regulation of obesity and metabolism of an individual. In adipose tissue, macrophages have two main phenotypes M1 and M2, which are pro-inflammatory and anti-inflammatory, respectively. M1 macrophages produce cytokines, such as TNF- α and IL-1ß, contributing to inflammation observed in adipose tissue leading to the development of chronic issues such as insulin resistance (Chylikova *et al*. 2018). The infiltration of these macrophages is the main trigger for inflammation in individuals with obesity (Weisberg *et al*. 2003). The M2 macrophages are polarized in obese individuals, which is associated with greater populations of M1 macrophages and, in turn, more cytokine expression.

Adipose tissue is categorized into two major categories, white and brown adipose tissue (WAT and BAT, respectively). WAT and BAT have antagonistic functions. While WAT stores excess energy through triglycerides, BAT dissipates energy through thermogenesis. A substantial storage of BAT in adult humans is associated with lower body weights; however, as age increases BAT declines and body weight increases. BAT is of clinical importance due to its potential connection to obesity and associated metabolic disorders (Saely *et al*. 2012). Macrophages have been shown to infiltrate WAT in both mice (Weisberg *et al*. 2003) and humans (Xu *et al*. 2003). This infiltration, in

combination with polarization, of macrophages contributes to the inflammatory response observed in obese individuals. In comparison, low (Roberts-Toler *et al*. 2015) or no (Fitzgibbons *et al*. 2011) macrophage infiltration in BAT has been observed in mice fed an energy-rich diet, which is associated with diet-induced inflammation.

The aim of this study was to investigate the effects of acute sleep fragmentation (24 h) upon proinflammatory responses in male mice, specifically focusing upon effects found in different types of adipose tissue. Previous studies (Dumaine and Ashley, 2015; Poroyko et al. 2016) had evaluated the effect of sleep fragmentation upon white adipose tissue: inguinal and epididymal; however, brown adipose tissue (BAT) had not been evaluated. I predicted that there would be differential expression in pro-inflammatory gene expression between white and brown adipose tissue in mice receiving acute SF. Specifically, I hypothesized that white adipose tissue would experience an increase in cytokine gene expression of IL-1ß and TNF-α from acute SF, whereas brown adipose tissue would not be affected.

MATERIALS AND METHODS

Animals

Male C57BL/6j mice were housed in a colony room (12:12-h light-dark cycle, lights on at 0800; 21°C ± 1°C) at Western Kentucky University. After weaning at 21 days of age, mice were placed in polypropene cages with same-sex littermates. Within each cage, mice were provided with corncob bedding, food (rodent RM4 pellets), and water *ad libitum*. This study was conducted under the approval of the Institutional Animal Care and Use Committee at Western Kentucky University (#19-14), and procedures followed the National Institutes of Health's "Guide for the Use and Care of Laboratory Animals" and international ethical standards.

Sleep Fragmentation

Male adult mice (≥ 8 wk of age) were selected for experimentation and randomly assigned to control (N = 10) or sleep-fragmented (N = 10) groups. Before starting experiments, mice were briefly exposed to isoflurane vapors, weighed, and ear tagged for identification. Mice were placed into a Model 80390 automated sleep fragmentation (SF) chamber (Fig. 1) already supplied with a thin layer of corncob bedding (up to 5 mice per session). Food and water were provided *ad libitum*. Mice were given at least three days to acclimate to their new environment. If mice were subjected to SF, then additional methods were followed: at 0800 the computerized swipe bar of the sleep fragmentation chamber was turned on. Mice experienced the 2 min bar sweep for the next 24 h period, which approximates the rate of arousals of a human exhibiting severe sleep apnea.

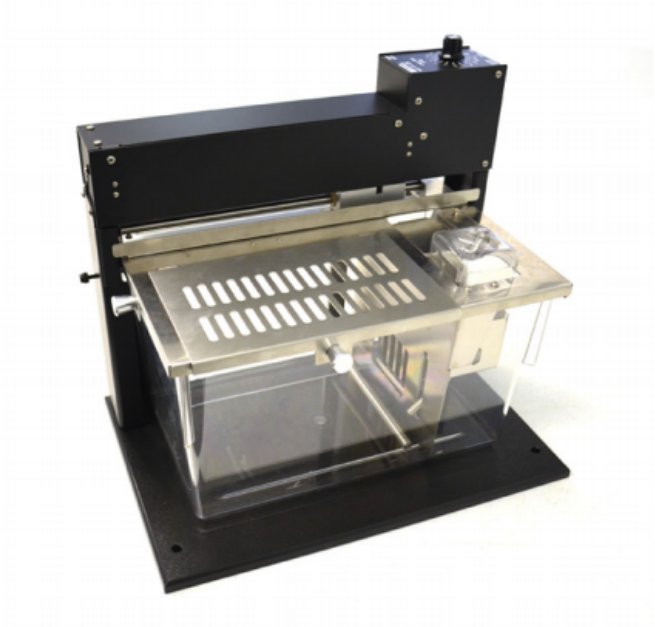

Figure 1. Model 80390 Sleep Fragmentation Chamber

Tissue Collection

After mice experienced control or SF for 24 h, mice were euthanized using carbon dioxide and secondarily cervical dislocation. EWAT, IWAT, and BAT were collected from each mouse and stored in RNAlater (Qiagen) at 4˚C.

RNA Extraction and Reverse Transcription

RNA was extracted from EWAT, IWAT, and BAT using a RNeasy mini kit (Qiagen). RNA concentration was measured using a Nanodrop 2000 Spectrophotometer (Thermo Scientific).

RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific). The reactions were run according to the manufacturer's specification using a thermal cycler.

RT-PCR

Real-Time PCR was conducted using an Applied Biosystems 7300 machine. Taqman Gene Expression RT-PCR Master Mix was used in conjunction with the following primer/probes:IL-1ß, TNF-α. Both probes, which were labeled with fluorescent marker 5-FAM at the 5' end and quencher MGB at the 3'end, were used for genes of interest along with 18S (VIC-labelled probe) as the endogenous control according to the manufacturer's instructions. The amplification conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The reaction was carried out according to the manufacturer's protocol and the relative expression of IL-1ß and TNF-α were measured. Samples were run in duplicates and the fold change in mRNA levels was calculated as the relative mRNA expression levels, $2^{-\Delta\Delta Ct}$. Briefly, the cycle threshold (Ct) obtained by fluorescence exceeding background levels was used to calculate ΔCt(Ct[target gene] - Ct[18s]). Each Ct value was normalized against the lowest Ct value of a control sample. The negative value of this powered to 2 ($2^{-\Delta\Delta Ct}$) was plotted.

Statistical Analysis

Unpaired, two-tailed Student's t-tests were used to evaluate the statistical significance of differences between groups. Logarithmic transformation was necessary in some cases to satisfy homogeneity of variances. When data failed normality or homogeneity of variances, non-parametric Mann-Whitney U tests were employed. P-values < 0.05 were considered statistically significant.

RESULTS

Epididymal White Adipose Tissue

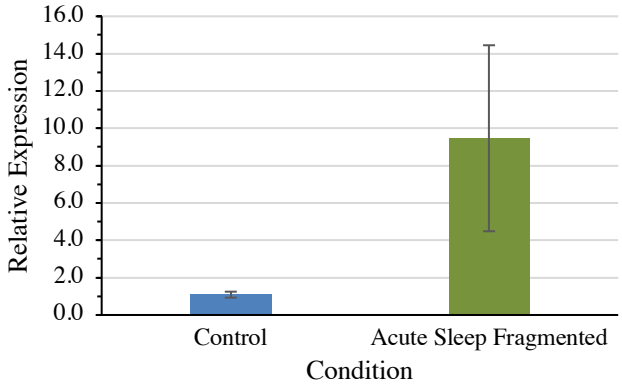

Figure 2: Epididymal White Adipose Tissue IL-1ß Expression

Acute SF significantly increased IL-1ß gene expression in EWAT compared to controls (Student's t-test, $P = 0.0038$, $t = 3.3154$, df = 18; Fig. 2).

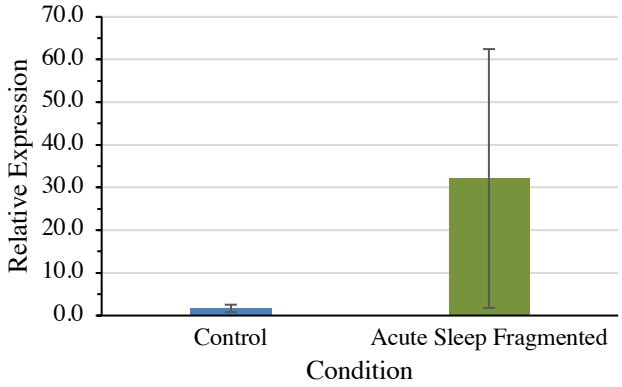

Figure 3: Epididymal White Adipose Tissue TNF-α Expression

Acute SF significantly increased TNF-α gene expression in EWAT compared to controls (Mann-Whitney U Test; $P = 0.0455$, $U = 23$; Fig. 3).

Inguinal White Adipose Tissue

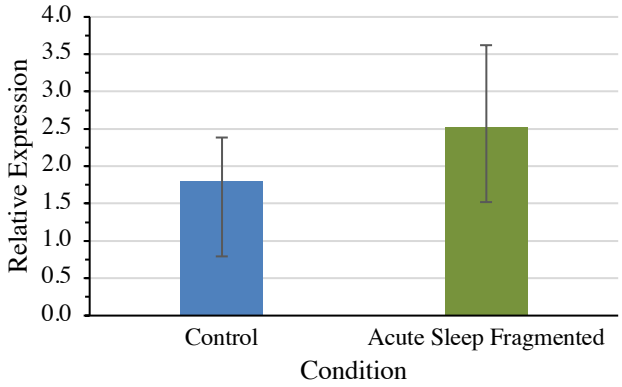

Figure 4: Inguinal White Adipose Tissue IL-1ß Expression

There was no significant effect of acute SF upon IWAT IL-1ß expression compared with controls (Student's t-test, $P = .5681$; Fig. 4).

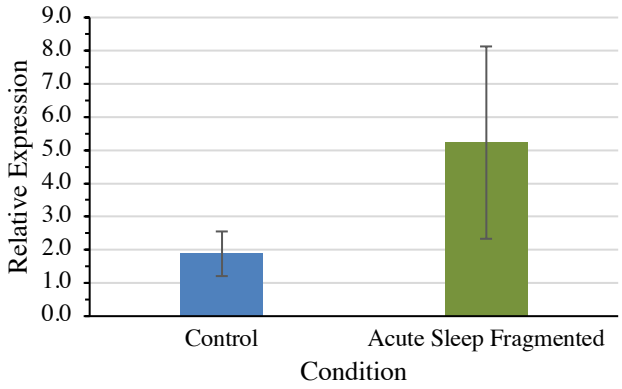

Figure 5: Inguinal White Adipose Tissue TNF-α Expression

There was no significant effect of acute SF upon IWAT TNF-α expression (Mann Whitney U test, $P = .4743$; Fig. 5).

Brown Adipose Tissue

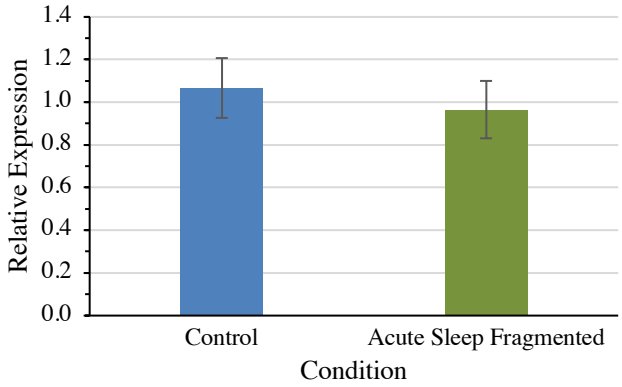

Figure 6: Brown Adipose Tissue IL-1ß Expression

There was no significant effect of acute SF upon BAT IL-1ß expression compared with controls (Student's t-test, $P = .6083$; Fig. 6).

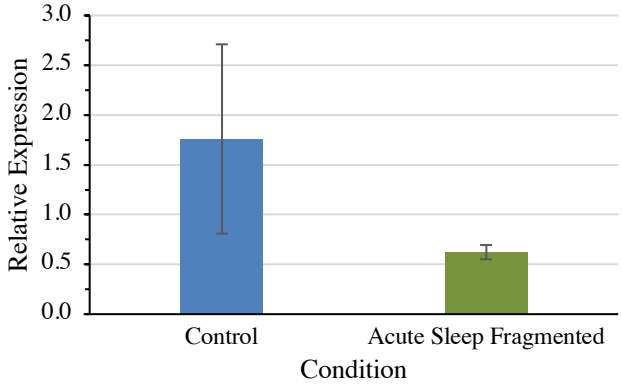

Figure 7: Brown Adipose Tissue TNF-α Expression

There was no significant effect of acute SF upon BAT TNF-α expression compared with controls (Student's t-test, $P = .0928$; Fig. 7).

DISCUSSION

It was hypothesized that there would be differential expression of the pro-inflammatory response in white adipose tissue versus brown adipose tissue. Specifically, white adipose (EWAT and IWAT) would display an increase in IL-1ß and TNF-α pro-inflammatory cytokine gene expression after SF, and brown adipose tissue (BAT) would not show the same increase in expression.

As hypothesized, EWAT showed a significant upregulation in expression of both cytokines. On the other hand, IWAT did not show a significant increased expression in either of the cytokines. One potential reason to explain this difference is that EWAT harbors significantly more immune cells than IWAT (Nolsalski and Guzik, 2017). An increased immune cell population allows for a significant increase in cytokine expression in EWAT, but not IWAT. Previous studies describe an increase in TNF-α levels in peripheral tissues (Ramesh *et al*. 2012; Zhang *et al*. 2014); however, these studies were chronic SF studies. This indicates that the duration of sleep fragmentation has an impact on the inflammatory response of these tissues such as EWAT and IWAT. This could explain why TNF-α expression in IWAT was not significantly different from controls.

The inflammatory response observed in adipose tissue can be attributed to the interaction between enlarged adipocytes and the macrophages that infiltrate the tissue (Tanaka, 2020). There are two types of adipose macrophages, M1 and M2, and the M1 macrophages have been associated with inflammation in this tissue. More specifically, M1 macrophages form crown like structures (CLS) around dying adipocytes that allows

for adipocyte-macrophage cross talk (Cinti *et al*. 2005). Furthermore, M1 produces pro-inflammatory cytokines, such as TNF-α, which leads to inflammation of the tissue. Additionally, the ratio of M1 to M2 macrophages also has been shown to increase (Lumeng *et al*. 2007). What regulates the inflammation? It has been associated with Macrophage-inducible C-type lectin (Mincle), which is a type II transmembrane Ca^{2+} - dependent lectin that is induced in macrophages by lipopolysaccharides (Matsumoto *et al*. 1999). The function of Mincle is to induce the production of pro-inflammatory cytokines and chemokines (Yamasaki *et al*. 2009). Also, Mincle has been shown to be expressed significantly higher in M1 macrophages in individuals with obese adipose tissue (Tanaka, 2014).

BAT did not show a significant increase in cytokine gene expression of IL-1ß or TNF-α. This indicates that acute SF does not lead to an inflammatory response within this tissue. The lack of response could be attributed to lower infiltration of pro-inflammatory immune cells to the tissue. When mice have been fed a high-fat diet, a lower infiltration of pro-inflammatory immune cells occurred in BAT compared to WAT (Fitzgibbons *et al*. 2011); however, if mice were fed a high-fat diet for an extended period of time, then there was a significant increase in TNF-α expression (Roberts-Toler *et al*. 2015). It would be of interest in the future to research if chronic SF leads to an increase in cytokine gene expression in BAT. Additionally, resident macrophages are mostly of the M2 subtype in the BAT of lean individuals (Thomas and Apoyian, 2017). This could also explain why the inflammatory response from acute SF was not as pronounced as EWAT since M2 macrophages are not associated with the pro-inflammatory response as M1 macrophages are. Also, this lack of inflammation could be accredited to the importance of BAT's

functions in metabolism. BAT is involved in the process of insulin sensitivity. When pro-inflammatory cells infiltrate BAT, this insulin sensitivity is impaired in both mice (Ferré *et al*. 1986) and humans (Orava *et al*. 2013). Additionally, the thermogenic function of BAT is also impaired due to the infiltration of pro-inflammatory immune cells, which can lead to lipotoxicity (Villarroya *et al*. 2018). If BAT was easily infiltrated by pro-inflammatory immune cells, then this would have adverse effects on the health of the individual.

It is important to take into consideration that this study was performed with only male mice. Female mice may be able to withstand more stress than their male counterparts. It would be of interest to perform further studies that compare male and female responses to acute SF and examine cytokine gene expression in both white and brown adipose tissue. Moreover, evaluating the effects of chronic SF upon these tissues would be important to understanding the immune and inflammatory response. Another area of interest in future studies could be analyzing the different effects of acute and chronic SF on mice that are lean and obese.

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