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# Inflammatory Response to Sleep Fragmentation in Skeletal, Cardiac, and Smooth Muscle Tissues in Female Mice

Patton Allen

# INFLAMMATORY RESPONSE TO SLEEP FRAGMENTATION IN SKELETAL, CARDIAC, AND SMOOTH MUSCLE TISSUES IN FEMALE MICE

A Capstone Experience/Thesis Project Presented in Partial Fulfillment

of the Requirements for the Degree Bachelor of Science

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By

Patton H. Allen

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CE/T Committee:

Dr. Noah T. Ashley

Dr. Simran Banga

Dr. Dennis Wilson

## ABSTRACT

Sleep is a critical process that the body undergoes. When sleep is interrupted, so that an individual is awakened for some period before going back into sleep, the sleep can be described as fragmented. Studies in the past have shown that sleep fragmentation (SF) promotes an inflammatory environment, especially in the brain and peripheral tissue. However, studies have not been conducted to observe inflammatory responses in muscle. To examine this, C57BL/6J female mice were subjected to either a control group (no SF) or a SF group which involved using an automated SF chamber to disrupt sleep every 2 min over a 24-h period. Afterwards, mice were euthanized, and tissues were collected from different types of muscle (skeletal (pectoralis and gastrocnemius), smooth (uterus), and cardiac muscle). Total RNA was then extracted by a fibrous tissue extraction method using RNeasy kits. The total RNA was then reverse transcribed into cDNA. Pro-inflammatory cytokine gene expression (tumor necrosis factor-alpha) was measured using RTPCR. We predicted that an inflammatory response would occur in cardiac and skeletal muscle, but not smooth muscle. Data collection and analysis later showed that there was an inflammatory response observed in cardiac tissue based on a significant difference of TNF-α expression between fragmented and control groups, but there were no significant differences observed in other tissues. Results of this study will increase our understanding of how sleep loss can affect multiple tissues and organ systems.

I dedicate this thesis to my parents, Mark and Lena Allen, my sisters Kerra Ogden and Kelsey Allen, my niece and nephew Layla and Brayden Ogden, Annabelle Cooper, and my role model William “Spike” Funk, who have loved and supported me in all walks of life. Without them, I would not be where I am now.

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# VITA

*EDUCATION*

Western Kentucky University, Bowling Green, KY — May 2022
B.S. in Biology (Pre-Med), Minor in Chemistry
Mahurin Honors College Graduate
Honors CE/T: *Inflammatory Response to Sleep Fragmentation in Skeletal, Cardiac, and Smooth Muscle Tissues in Female Mice*

Gatton Academy of Mathematics and Science, Bowling Green, KY — May 2018

Butler County High School, Morgantown, KY — May 2018
Valedictorian

*PROFESSIONAL EXPERIENCE*

WKU Anatomy and Physiology Lab — Aug. 2020 – May 2021
Teaching Assistant

*AWARDS & HONORS*

Western Kentucky University Gatton Academy 4 Year Scholarship, 2018 to 2022

Western Kentucky University Academy Merit 4 Year Scholarship, 2018 to 2022

Morgantown Bank Leadership Scholarship, 2018-2019

Rural Health Scholars 2021 Selectee

UK/WKU Medical Early Assurance Selectee – Gatton Academy - 2018

*PRESENTATIONS*

(March 2022) *Inflammatory Response to Sleep Fragmentation in Skeletal, Cardiac, and Smooth Muscle Tissues in Female Mice.* Oral Presentation at the WKU Student Research Conference. Bowling Green, KY.

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# INTRODUCTION

## *Obstructive Sleep Apnea*

Sleep is a critical process that the body undergoes. It is a time where the body recuperates and recovers from the stress of the day so that one may wake up and do it all over again the next. When thinking about sleep and its importance, understanding normal sleep patterns and sleep cycles is pertinent. Normal sleep can be defined by a set of four different stages. The first stage of sleep, N1, is the time in which the body is beginning to relax and is easily awakened; this stage typically lasts about 1-5 minutes and bodily functions start to slow down in preparation for the next stage.[7] As the body enters the next stage, N2, temperature and heart rate slow and muscles relax. An individual who has entered this stage will show distinct changes in brain activity and compared to the N1 stage, is harder to awaken. Once past N2, the body enters N3. [7] N3 Sleep can be identified by delta waves in the brain and is a time when the body may restore itself and grow. Considering the restorative properties of the N3 stage, it is one of the most important. The final stage of sleep is REM, or rapid eye movement sleep. Normal amounts of REM sleep are thought to allow for normal cognitive function and without it, cognition may decline.[7]

Contrary to normal sleep, one may observe sleep patterns that are irregular, such as obstructive sleep apnea (OSA). An individual suffering from OSA may have an entire or partial collapse of their airway during the night. This airway collapse will cause the individual to partially awaken during the night. Recurrent episodes of airway collapse and awakening can be described as fragmented, as this happens repeatedly throughout the

night.[4] An individual who is experiencing fragmented sleep may never make it past either the N1 or N2 phases that constitute normal sleep. Considering the great need for the latter stages, N3 and REM, this individual may experience complications to cardiovascular, cognitive, and metabolic functions due to sleep fragmentation (SF) or OSA.[4,7] According to a journal published by the International Journal of Molecular Sciences some of the complications associated with OSA include "chronic kidney disease, erectile dysfunction, ocular conditions, Alzheimer disease, nocturia, and even cancer."[7] For these reasons, gaining a better understanding of obstructive sleep apnea is particularly important.

*Inflammatory Response to Sleep Fragmentation*

With an understanding of the importance of normal sleep and OSA, one may question why OSA occurs and in what ways this condition may affect the body. When normal sleep patterns are disturbed due to OSA or when individuals experience any sort of sleep deprivation, the body will experience increased levels of inflammation. Inflammation is the body's response to damage or shifts from homeostasis.[1] Inflammation due to acute sleep fragmentation has been shown to be associated with increased levels of pro-inflammatory gene expression, such as TNF- α and IL-6, in heart tissue, white adipose tissue, brown adipose tissue, and the hypothalamus.[2,6] Since past research has shown differential expression in these tissues, the question was raised as to whether differential expression may be present in different types of muscle tissues.

The aim of this study would be to investigate the proinflammatory response to acute sleep fragmentation within cardiac, skeletal, and smooth muscle types in female mice. To observe skeletal muscle, I examined two skeletal muscle tissues, gastrocnemius

and pectoralis muscle. To assess cardiac muscle, we examined heart. To assess smooth muscle, we examined uterus tissue. The only tissue that I examined during this study that has been examined before is the heart which has shown different results in differential expression of TNF – α depending on the study.[2,6] This study will give more evidence as to whether differential expression of TNF- α will occur in response to SF. It is hypothesized that cardiac and skeletal muscle will produce differential expression of TNF- α in response to sleep fragmentation in the female mice model, but smooth muscle would not produce differential expression of TNF- α.

# MATERIALS AND METHODS

## *Animals*

This project adhered to the National Institute of Health's "Guide for the Use and Care of Laboratory Animals" as well as guidelines set by the Institutional Animal Care and Use Committee at Western Kentucky University. The study's subjects were female C57BL/6j mice, which were housed in a light and temperature controlled (12:12-h light-dark cycle, lights on at 0800; 21°C ± 1°C) colony room. At 21 weeks old, the mice were weaned from their mother; following this separation they were housed in polypropene cages and provided with water, food (rodent RM4 pellets), and corncob bedding. Additionally, the mice were placed with other pups from the same biological sex. All interactions with the mice in this experiment followed international ethical standards.

## *Sleep Fragmentation*

Experimentation was performed on adult female mice (>8 weeks of age), which were separated into two groups: a control (N=10) and a sleep fragmented (N=10) group. Mice were then transferred to a Model 80390 automated sleep fragmentation chamber with a layer of corncob bedding. A maximum of 5 mice per session were placed in the chamber and were provided with food and water as desired. These mice were given three days to become accustomed to their new environment before further experimentation. Additional procedures were observed for mice exposed to the sleep fragmentation machine, such as the automated swipe bar of the sleep fragmentation chamber being turned on at 0800. Furthermore, the experimental group was awakened every 2 minutes

over the 24 hours period by the sleep fragmentation machine bar. This process would simulate an individual experiencing severe sleep apnea.

*Tissue Collection*

Carbon dioxide and cervical dislocation were used in conjunction to ensure euthanasia of all mice. Gastrocnemius, heart, uterus, and pectoralis tissue was collected from each mouse and stored in RNA*later* (Qiagen) at 4˚C.

*RNA-Isolation*

RNA was extracted from each of the tissues (aforementioned) using a Fibrous Tissue RNeasy mini kit (Qiagen). The collected RNA was then measured using a Nanodrop 2000 Spectrophotometer (Thermo Scientific).

*cDNA Synthesis*

A High-Capacity cDNA Reverse Transcription Kit (Thermo Scientifc) was used to transcribe the RNA into cDNA. A thermal cycler was then used to run the reactions as described by the manufacturer.

*RTPCR*

RT-PCR was performed by loading a 96-well PCR plate with cDNA, 18s (endogenous control) and TNF-α (gene of interest) probes, and Taqman Gene Expression Master Mix (Thermofisher Scientific). The plate was loaded into the Applied Biosystems 7300 machine for amplification. The amplification protocol for each plate was as follows: 50°C for two minutes, 95°C for ten minutes, and forty cycles at 95°C for fifteen seconds and 60°C for one minute. The manufacturer's protocol was followed except that reaction volumes were reduced from the manufacturer protocol of 50 μL to 20 μL, and each sample was run in duplicate. A comparative $C_t$ analysis was performed. $C_t$ values were

averaged for each sample, resulting in a value termed the $\Delta C_t$. $C_t$ values for each TNF-$\alpha$ sample against the lowest $C_t$ value of the respective control sample were then normalized, resulting in a value termed the $\Delta\Delta C_t$. The negative value of this powered to 2 ($2^{-\Delta\Delta Ct}$) was plotted.

*Statistical Analysis*

Statistical analysis was performed using a two-tailed Student T-Test.

RESULTS

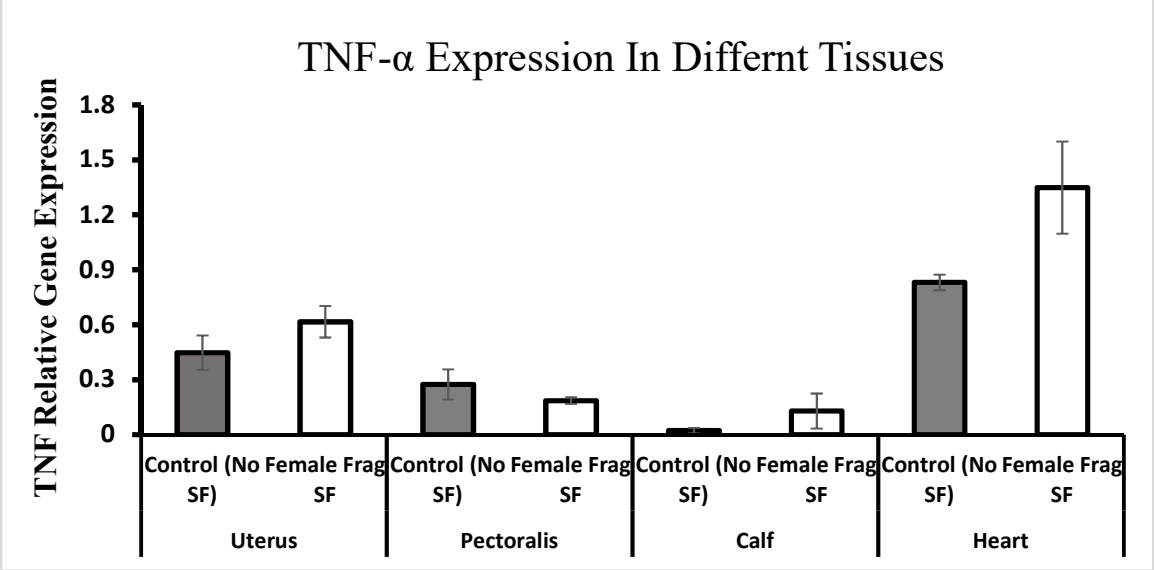


Figure 1: TNF-α Expression in Different Tissues

There was no significant difference in the differential expression of TNF-α in the uterus, pectoralis, or calf. There was a significant difference in the TNF - α expression in the heart.

| | Uterus | Pectoralis | Calf | Heart |
|---|---|---|---|---|
| P-Value | 0.0690 | 0.1419 | 0.1437 | 0.0067 |

Table 1: P-Values for Different Tissues

The table above shows the p-values associated with each of the four tested tissues. These P-Values were obtained using a Two-Tailed Student T-Test.

# DISCUSSION

It was hypothesized that cardiac and skeletal muscle would produce differential expression of TNF-α in response to sleep fragmentation in the female mice model, but smooth muscle would not produce differential expression of TNF-α. My results supported the hypothesis that differential expression would occur in the heart, but all other tissues did not show differential expression.

In the heart, acute sleep fragmentation did have a significant effect on TNF-α expression. Compared to the control group, there was an upregulation of TNF-α in the heart tissue. This result was interesting as past studies have shown that TNF-α expression was downregulated in response to acute sleep fragmentation. However, the same study showed that in response to chronic sleep fragmentation, there was an upregulation of TNF-α.[6] Furthermore, another study which evaluated high frequency sleep fragmentation versus low frequency sleep fragmentation showed no significant difference in the expression of TNF-α in either the two groups compared to control groups.[2] The results of this study along with the study that showed upregulation of TNF-α in response to chronic sleep could explain why individuals with OSA display cardiovascular issues that are thought to be a result of chronic inflammation in cardiovascular tissues.[2,6] However, the acute SF group that went along with the chronic SF study and the other study that showed no significant difference in the expression of TNF- α in response to different kinds of TNF- α suggest that more other interactions could be involved.[2,6]

In the pectoralis, calf and uterus, acute sleep fragmentation did not have a significant effect on the expression of TNF- α. The original hypothesis postulated that there would be a difference in the expression of TNF- α in the skeletal muscle tissues. The reasoning behind this hypothesis was that the mice in the SF group would have to move much more than the control group due to the swipe bar that would make them move around. However, this did not seem to be the case. Although not enough to suggest a significant difference, the uterus was the closest of any to having a P value which would have indicated differential expression.

In conclusion, this study has given the scientific community more insight into the effects of SF on inflammatory responses in the body and may help us better understand OSA. This study used female mice and a future study similar to this study might examine male mice instead as males and females have different proportions of muscle mass which could result in a difference in the expression of pro-inflammatory cytokine.[3] Another change to the study could include accessing sleep fragmentation after the mice do some sort of exercise routine, such as running on an exercise wheel, as changes in inflammation result from muscle hypertrophy in response to exercise in muscle tissue.[5]

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