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# The Physiological Interaction of Sleep Deprivation and Zoledronate on Distal Femur Trabecular Thickness of Ovariectomized Rats

## Comments

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## Research Article

# The Physiological Interaction of Sleep Deprivation and Zoledronate on Distal Femur Trabecular Thickness of Ovariectomized Rats

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### Abstract

Osteoporosis, a disease resulting in an increased risk of fracture due to compromised bone, affects 1 in 3 postmenopausal women. Discontinuities in the microarchitecture of bone, such as trabeculae, are seen in postmenopausal osteoporosis. This study aimed to evaluate how sleep deprivation affects the distal femur trabecular thickness of estrogen-deficient rats treated with Zoledronate. 29 ovariectomized Wistar female rats were separated into 4 groups. The control group (C) was housed in standard housing with a 12-hour light/dark cycle and was given an intravenous injection of 0.45 mL of 0.9% saline. The Zoledronate group (Z) were also housed in standard conditions but given an intravenous injection of 50 ug/kg of 10% Zoledronate. The Sleep Deprived group (SD) were given an intravenous saline injection, but were housed in chambers that did not permit sleep for 18 hours, then moved to standard chambers that permitted 6 hours of sleep daily. The Sleep-Deprived Zoledronate group (SDZ) was housed the same as the SD group, but was given an intravenous injection of Zoledronate. After 5 weeks, tibiae and femora were harvested and stored at -80°C until high-resolution micro-CT was done. SDZ had improved distal femur trabecular thickness compared to C (75.5 microns and 67 microns, respectively;  $p=0.0001$ ). Multi-factor ANOVA revealed a significant interaction between Zoledronate and sleep deprivation ( $p=0.0078$ ). More research is needed to determine how this interaction impacts executive women who often suffer from sleep deprivation and demanding professions.

**Keywords:** Bone; Executive women; Osteoporosis; Postmenopausal zoledronate; Sleep deprivation

### Introduction

Osteoporosis is a skeletal disorder that compromises the strength of bone, leading to increased risk for fracture [1]. Osteoporosis can be detrimental to physical and mental health, yet it is not a popular topic despite being identified as one of the most significant diseases affecting human health. Women are more prone to osteoporosis than men. One in three women over the age of 50 will experience a fracture due to osteoporosis [2]. The mechanism of how osteoporosis affects bone is understood, but there are many factors, including menopause, that may influence the progression of the disease. Bone is a metabolically active tissue with osteoblasts creating inorganic substance and osteoclasts breaking down the inorganic substance when minerals, such as calcium, are needed. The inorganic substance makes up 60 percent of bone and gives it its strength and rigidity [3]. Typically, osteoblast and

osteoclast activity is about equal, but osteoporosis is characterized by overactive osteoclastic activity, decreased osteoblastic activity, or a significant decrease in the activity of both [4].

Bones consist of a dense portion known as cortical bone and an internal network of filaments known as trabecular bone [2]. The progressive architectural deterioration of trabecular bone can be seen by the disconnection of trabeculae due to loss of bone mineral content. Depletion of calcium in the trabeculae is one of the abnormalities in bone that leads to fracture in osteoporosis [2]. Trabecular bone is 3-4 times more metabolically active than cortical bone making it a good indicator of disease as it responds quicker than cortical bone [5]. This study focuses on trabecular thickness because recent research suggests that the quality of bone may be a better indicator of bone health [2]. While elderly men and women are at a greater risk of developing osteoporosis, postmenopausal women have the highest age-adjusted risk of fracture [1]. It is understood that estrogens play a vital role in bone protection by silencing osteoclasts [6]. When a woman goes through menopause

and estrogen is no longer regularly produced, osteoclast activity increases, and bone mineral density and bone strength decrease [1,6]. There is no cure for postmenopausal osteoporosis, but research is beginning to look at different medications and lifestyle changes that could influence the disease by preventing bone resorption.

As the population ages and retires later in life, concern for health implications due to inadequate sleep are viable [7-9]. In 1910 average sleep duration was approximately 9 hours, but currently, the average sleep duration among adults is 7.5 hours [8]. With more women working now than ever before, concern for sleep duration and sleep disturbances have become more responsible for the health maintenance area of research [9,10]. While data has not indefinitely suggested the relationship between sleep and osteoporosis, research suggests that lack of sleep leads to decreased bone mineral density and strength [11,12], particularly for postmenopausal women [10]. Another area of study for postmenopausal osteoporosis is the pharmaceutical role of bisphosphonates [5]. Bisphosphonates are used to slow the progression of bone resorption by binding to hydroxyapatite crystals, which inhibits the dissolution of the calcium phosphate [13,14]. This binding mechanism to hydroxyapatite crystals of bone leads to slower recruitment of osteoclasts and eventually apoptosis [13]. There are many different kinds of bisphosphonates, but Zoledronate is considered to be one of the most potent [13]. Zoledronate is a nitrogen-based bisphosphonate which allows for stronger binding to bone [14]. Zoledronate has been shown to improve the trabeculae in rats, and is generally well-tolerated in clinical trials by humans, making it a promising choice as a treatment for postmenopausal osteoporosis [13,15]. An annual intravenous infusion of Zoledronate in clinical practice has been preferred by patients compared to other bisphosphonates [16]. While it is well understood how Zoledronate affects osteoclasts, there also has been some research on how Zoledronate affects osteoblasts. One study found that, for postmenopausal osteoporosis, smaller doses such as 0.5 mg/kg body weight is ideal because it inhibits osteoclasts without interfering in osteoblast activity [17]. Lifestyle changes and pharmaceuticals have been studied well individually in postmenopausal osteoporosis, but there is limited research that predicated the implications of both.

## Materials and Methods

Wistar female rats (n=29), who had a mean weight of 341g and were 8-months old, were ovariectomized by Charles River Laboratories, Boston, MA, prior to the reception. This was done to simulate an estrogen-deficient environment comparable to menopause in humans. There are different strategies that manipulate the hormonal milieu that simulate a post-menopausal state in the animals. However, we felt that to best mimic the cessation of ovarian function, the surgical removal of the ovaries was the best and least complicating hormonally of the procedures.

The rats had a 1-week adjustment period where they were placed in their individual housing cages and were provided with Purina rat chow and water ad libitum before the experimental period began. A 12-hour light/dark cycle was kept during this week where lights came on at 7:00 and turned off at 19:00. After the week, the rats were then randomly assigned into different groups (Table 1), including a control group (C, n=5), a sleep-deprived group (SD, n=8), a group treated with Zoledronate (Z, n=8), and a sleep-deprived group who also received Zoledronate for treatment (SDZ, n=8). The University Institutional Review Board (IRB) approved the protocol and methodology for this study. All animal procedures, including housing, food provision, anesthesia, surgeries, and sacrifice, were in accordance with the 2015 IACUC guidelines.

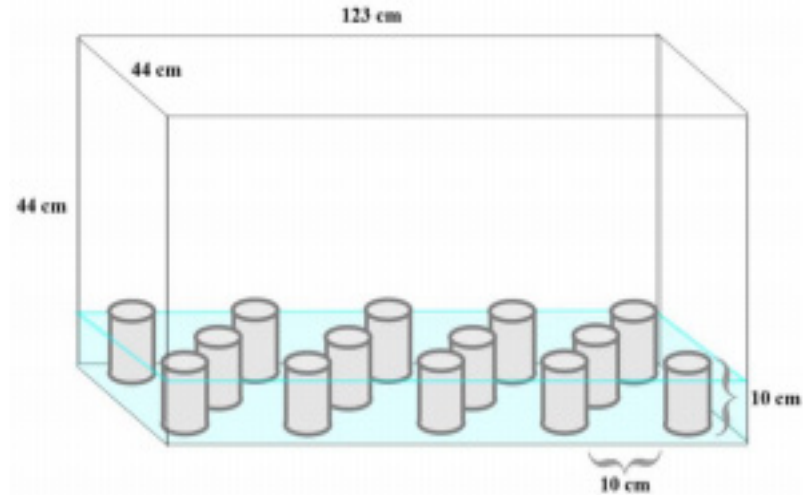
Group	Number of Rats	Injection Given	Sleep Deprived
C	5	0.45 mL 0.9% saline	No
SD	8	0.45 mL 0.9% saline	Yes
Z	8	50 ug/kg Zoledronate	No
SDZ	8	50 ug/kg Zoledronate	Yes

C= Control; Z= Zoledronate; SD= Sleep-deprived; SDZ= Sleep-Deprived Zoledronate

**Table 1:** Group classifications.

The groups that received Zoledronate, including the Z and the SDZ groups, were given an intravenous injection through the tail vein of 50 ug/kg of body weight. Only one injection was needed for the five-week study due to the potency of Zoledronate and its ability to bind to the hydroxyapatite crystals of bone. The C and SD groups were given a one-time intravenous injection of 0.45mL of 0.9% saline to experience the same stress of injection as the Z and SDZ had. After the injection, the 5-week experiment was started. During the five-week period, sleep deprivation was simulated by the Modified Multiple Platform Method (MMPM) [18]. We modified rectangular acrylic fish tanks (144cmx44cmx44cm); one for the SD group and one for the SDZ group, that were fitted with 14 circular platforms that had a diameter of 6.5cm (Figure 1). The platforms were 10cm away from each other. Each tank was filled with room-temperature water until the water reached 1cm below the top of the platforms (Figure 1). Purina rat chow and drinking water were available in the tanks at all times. The food and fresh water were placed in accessible containers positioned at the top of the tanks and away from the standing water and platforms. These tanks allowed the rats to move around, but if they fell asleep, they would fall into the water and be woken up. Therefore, these rats were unable to maintain a normal sleep cycle. The SD and SDZ rats participated in 1 of 3 different sleep cycles each day (Table 2). The sleep cycle was assigned using a random number generator. Each day at 14:00, the rats were moved into the MMPM tanks.

Then between 07:00 and 09:00, they were moved to a tank with wood chip bedding, food and water, and that allowed sleep in the darkness. This provided the rats with 5-7 hours of sleep per day, depending on the cycle permitted (Table 3). The C and Z groups were housed under standard conditions and permitted 12 hours of light/dark.



**Figure 1:** Modified multiple platform tank.

Sleep Cycle	Start Time	End Time	Time Awake (hrs)	Time Allowed for Sleep (hrs)
SC1	14:00	9:00	19	5
SC2	14:00	8:00	18	6
SC3	14:00	7:00	17	7

A random number generator would select one of the three sleep cycles listed above, daily. The rats would be placed in the MMP tank during the wake time, then they would be moved to a standard chamber for sleeping.

**Table 2:** Sleep cycles.

Day	Time Awake (hr.)	Time Asleep (hr.)	Sleep Cycle
1	17	7	3
2	18	6	2
3	19	5	1
4	17	7	3
5	18	6	2
6	19	5	1
7	18	6	2
8	17	7	3
9	19	5	1
10	17	7	3
11	17	7	3

12	17	7	3
13	19	5	1
14	17	7	3
15	17	7	3
16	17	7	3
17	17	7	3
18	19	5	1
19	19	5	1
20	19	5	1
21	18	6	2
22	19	5	1
23	18	6	2
24	19	5	1
25	18	6	2
26	19	5	1
27	19	5	1
28	17	7	3
29	19	5	1
30	18	6	2
31	18	6	2
32	18	6	2
33	18	6	2
34	17	7	3
35	17	7	3

Amount of sleep permitted as indicated by the sleep cycle. Sleep cycle was determined by a random number generator. The amount of wake time was spent in the MMP tank.

**Table 3:** Daily Sleep/Wake Times.

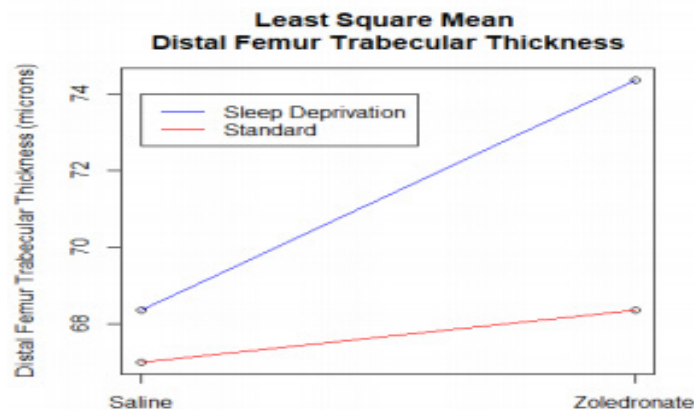
At the end of 5 weeks, blood and tissue samples were collected and frozen prior to death. The rats were sedated with 0.001 mL/g of ketamine/atropine/xylazine cocktail. Following sedation, all animals were decapitated and exsanguinated. Blood was obtained and centrifuged at 14,000 RPM for 5 minutes at 5°C and frozen at -80°C for studies not reported in this paper. Femora and tibiae were harvested, wrapped in saline-soaked gauze, and frozen at -80°C. The right tibiae and femora of each rat were shipped to Novartis Institute for Biomedical Research. Ex vivo DEXA scans ex vivo high-resolution micro-Computed Tomography (micro-CT) and peripheral Quantitative computed tomography (pQCT) of the distal and midshaft femur (Figure 2). The scans and data were then sent to Chapman University for analysis. This report is limited to the femora data, which was statistically analyzed with the use of multiple comparisons tests and ANOVA in RStudio.



**Figure 2:** Depiction of Location of micro-CT and pQCT of the Distal Femur Ex vivo location of the micro-CT and pQCT of the rat’s distal femur.

**Results**

Data from the micro-CT suggested an increase in distal femur trabecular thickness between the C and SDZ groups (67, 75.5 microns, respectively;  $p=0.0001$ ) (Table 4). Additionally, sleep deprivation seemed to improve distal femur trabecular thickness between the Z and SDZ groups (68.375, 75.5 microns, respectively,  $p=0.00007$ ). Because the sleep deprivation seemed to improve distal femur trabecular thickness, a multi-factor ANOVA was conducted and revealed a significant interaction between the treatment and the amount of sleep the rats received ( $p=0.0078$ ) (Figure 3).



**Figure 3:** Interaction plot between Treatment and Housing.

Estimates from an ANOVA model with treatments as main effect and an interaction term as covariates ( $p=0.0078$ ,  $R^2=0.612$ ).

Group	Average Distal Femur Trabecular Thickness (microns)
Control (C)	67 ± 0.721
Zoledronate (Z)	68.375 ± 0.240
Sleep-Deprived (SD)	68.375 ± 0.188
Sleep-Deprived + Zoledronate (SDZ)	75.5*** ± 0.400

The average distal femur trabecular thickness of each group including standard error. A significant difference was determined with ANOVA between the Control and Sleep-Deprived Zoledronate groups.  
\*\*\* $p=0.0001$

**Table 4:** Average Distal Femur Trabecular Thickness of Each Group.

**Discussion**

It was expected that sleep deprivation would cause thinner trabeculae in the distal femora of ovariectomized rats and that treatment with Zoledronate would improve this. However, data suggested that the sleep-deprived groups had a greater distal femur trabecular thickness. Because the SDZ group had a much greater increase in distal femur trabecular thickness than the Z group, an interaction plot was created using ANOVA. The plot revealed a possible physiological interaction between the amount of sleep and the use of Zoledronate. Further studies are needed to determine the mechanism behind the physiological interaction, but results could have a profound impact on the use of Zoledronate in modern society where many people are getting less sleep because of work Demands. While the animals in this study were provided lab chow ad libitum, future studies should include the evaluation of the amount of food daily ingested. It is widely observed that food intake may be modified by the stress of decreased sleep [19]. This could be a factor in the metabolism of bone tissue. Another consideration that should be addressed is the sample size of this study. Though there was significance between the C and SDZ groups, the small sample size of 32 rats may not have been robust enough to provide more illumination into the effects of this protocol. Other studies that have tested Zoledronate in ovariectomized rats have had at least 80 rats [20-22]. The duration of this study was carefully considered, with it being estimated that the relative age for humans during a postmenopausal period over 1 year is equal to 11.8 days for rats [23]. Given this information, it was determined that a 5-week trial for the rats would be approximately a 3-year trial for postmenopausal women, which is a common timeline for bisphosphonate clinical trials.

While the MMPM has been a commonly used technique for sleep deprivation in animal studies, it may have stimulated osteoblast activity in this study. One study that looked at the effects of exercise on ovariectomized rats found that the rats who were



forced to run each day had significantly greater bone mass especially in the trabecular bone [24]. In humans, this phenomenon (Wolff's Law) causes improved bone quality and increased bone mineral content when the bone experiences greater mechanical stress over time [25]. In future studies, consideration should be taken of the type of bisphosphonate used and the timing of the injection. While Zoledronate is one of the most potent bisphosphonates, there have been recent studies that suggest diluting it with propanol or parathyroid hormone has been more effective than Zoledronate by itself [26,27].

## Conclusion

Executive women, who often suffer from sleep deprivation, are a growing subclass of postmenopausal patients suffering from osteoporosis. This study attempted to evaluate Zoledronate as an intervention to reduce bone loss. While these data suggested a significance in the SDZ group compared to the C group, a trial of longer duration and greater sample size may be more illuminating. Rats held in the sleep deprivation tanks may have been using muscles which may have stimulated osteoblasts. Overall, Zoledronate may have a protective effect in sleep-deprived ovariectomized rats and suggests promise as a therapeutic intervention for postmenopausal executive women with osteoporosis.

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## Authorship Confirmation Statement

Authors of this work are in compliance with the definition of Author, as defined by the International Committee of Medical Journal Editors. This manuscript is submitted with the understanding that they have neither been published, nor are under consideration for publication elsewhere.

## Authors Disclosure Statements

**Competing Interests:** There are no competing interests with respect to this study.

**Personal Financial Interests:** There are no financial interests associated with this study or with any of the authors.

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**Other Competing Interests:** There are no competing interests, which compromise the integrity or objectivity of this study.

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