Effects of 6 Months of Voluntary Alcohol Self-Administration on Intracortical Bone remodeling in Young Adult Male Cynomolgus Monkeys

by
Amida F. Kuah

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biochemistry and Biophysics
(Honors Scholar)

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AN ABSTRACT TO THE THESIS OF

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Title: Effects of 6 Months of Voluntary Alcohol Self-Administration on Intracortical Bone Remodeling in Young Adult Male Cynomolgus Monkeys.

Approved by:

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Urszula T. Iwaniec

Heavy chronic alcohol consumption has been linked to a myriad of detrimental skeletal effects. Many studies have been conducted to examine the effects of alcohol abuse upon cancellous bone. However, little is known about its effect upon cortical bone. The skeleton is comprised of approximately 80% cortical bone and thus alcohol induced alterations may play a role in increased fracture risk. A 6 month voluntary self-administration of ethanol study was conducted on cynomolgus monkeys to determine the effects of heavy alcohol consumption on bone mass, microarchitecture, and intracortical bone remodeling. Animals consumed an average of 1.83g per kg body weight of ethanol (4%v/v) per day, approximately equivalent to 10 standard drinks a day for a 75kg person. Examinations of the 3rd lumbar vertebra, distal femur, and distal tibia were conducted using DXA, μCT, and histological techniques. There were no differences in the bone mass or bone microarchitecture between alcohol consuming animals and the control group. Alcohol consuming animals showed a trend towards lower intracortical bone resorption and formation. The data suggests that 6 months of heavy alcohol

consumption does not affect bone mass or microarchitecture but may depress intracortical bone remodeling.

Key Words: Alcohol abuse, Cynomolgus model, Cortical bone remodeling, Haversian remodeling, Histomorphometry, Microcomputed tomography

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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EFFECTS OF 6 MONTHS OF VOLUNTARY ALCOHOL SELF-ADMINISTRATION ON INTRACORITCAL BONE REMODELING IN MALE CYNOMOLGUS MONKEYS

INTRODUCTION

Human alcohol consumption can be dated back to the Neolithic period. Today, approximately two thirds of Americans report drinking in the past year. The U.S Dietary Guidelines for Americans recommend that daily alcohol consumption should be limited to around 1 drink for women and 2 drinks for men. In the United States, a standard alcohol drink is defined as 14g (17.7ml) of pure ethanol. This is approximately equivalent to consuming 12 fluid ounces of beer, 8 fluid ounces of malt liquor, 5 fluid ounces of wine, or a 1.5 fluid ounce shot of 80-proof distilled spirits (30).

Roughly, 1 in 6 adults report having engaged in 4 binge drinking episodes per month, consuming an average of 8 drinks per binging episode (7). About 16.9 million (3.8%) Americans have engaged in heavy drinking in the past 30 days and roughly 4.3 million meet the criteria of alcohol dependence (8).

A common finding of epidemiological studies investigating the relationship between alcohol consumption and general health is that there is a J-shaped association between the amounts of alcohol consumed and measured health outcomes (37). These findings have suggested that consuming small-to-moderate levels of alcohol promotes superior health outcomes than either abstinence or heavy drinking. Studies investigating the relationship between

bone mineral density (BMD) and alcohol consumption have also reported that a J-shaped relationship between BMD and ethanol consumption (21).

Bone is a multifunctional organ system and plays a role in mechanical support and protection, mineral homeostasis, and hematopoiesis. Bone's protective functions serve to prevent injury to vital organs. The marrow cavity within bone serves as the site for hematopoiesis. Bone also functions as the body's major reservoir for calcium storage. Calcium is both deposited and released from bone to contribute to the regulation of blood mineral levels (31). Bone is categorized into two main components: cortical bone and cancellous bone (Figure 1). Cortical bone is the primary component of the shafts of long bones. Cortical bone is dense and biomechanically takes on the main role of weight bearing. Cancellous bone is found mainly in the epiphysis and metaphysis of the long bones, the vertebrae and ribs. Cancellous bone is more porous and functions structurally to redirect loading stressors upon the stronger cortical shell. Architecturally, cancellous bone is composed of rods and plates called trabeculae that run through at various angles and act as struts for additional support for the outer cortical structure without greatly increasing the weight of the bone (31).

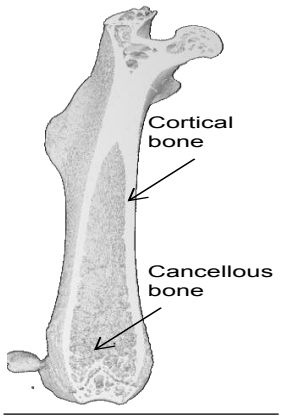

Figure 1. MicroCT image of a femur showing cortical and cancellous bone.

Throughout life, bone is constantly being remodeled in response to factors such as microdamage caused by wear and tear induced through everyday activities. Bone turnover depends on the coordinated actions of osteoclasts and osteoblasts. A long-term imbalance in the rate of formation and the rate of resorption will act to alter the quality and the structural integrity of bone. Osteoclasts are the primary cells that are involved in bone resorption. Osteoblasts are the primary cells responsible for bone formation. At the bone surface, preosteoclasts differentiate into mature osteoclasts that resorb mineralized bone. Following bone resorption, osteoblasts secrete osteoid which mineralizes to form bone. Under filling or overfilling of the resorption cavity results in a change in bone volume (31).

Bone mineral density is a biological predictor of fracture risk in the general population (1-3). Controlling for BMD, alcohol abusers exhibit higher rates of fracture than the general population. This suggests that heavy drinking can reduce the structural integrity of the skeleton in a manner that does not necessarily alter BMD. This implies that binge drinking acts to decrease bone quality as well as quantity (10-20).

Experimentally, heavy alcohol consumption has been shown to lower BMD and reduce histological indices of cancellous bone remodeling at the axial and appendicular skeleton in animals (22, 23, 25). In addition, experimental findings suggest that chronic alcoholism is associated with lower compressive and tensile breaking strength, along with altered microarchitecture in both cancellous and cortical bone (24, 27). There is substantial evidence that

suggests that alcohol abuse strongly reduces cancellous bone remodeling and more broadly, disrupts global bone remodeling (22, 23, 24). However, not much is known about how alcohol abuse affects intracortical bone remodeling.

The skeleton is comprised of approximately 80% cortical bone and due to its importance in structural support, alcohol-induced modifications in the remodeling of cortical bone may play a crucial role in which alcohol acts to reduce the quality of cortical bone (28). Intracortical bone remodeling is the primary process through which cortical bone is remodeled in humans. Although most studies evaluating the impact of alcohol on the skeleton using animal models have been performed in rodents, small rodents do not exhibit intracortical bone remodeling under normal circumstances. In contrast, intracortical bone remodeling is present in non-human primates. In a previous study, rhesus macaques given free access to alcohol for 12 months had significantly lower intracortical bone formation and bone resorption compared to the control group. In the study described here, a different species (cynomolgus monkeys) was used to model chronic alcohol abuse over a 6 month time period. Data collected from this study allows us to determine how heavy alcohol consumption impacts bone in a shorter time period. Cynomolgus monkeys undergo intracortical bone remodeling that closely resembles bone remodeling in humans (29). Cortical bone is the primary component of the diaphysis of long and short bones of the appendicular skeleton. Intracortical bone remodeling requires formation of secondary osteons or Haversian systems where existing bone is resorbed and replaced with new bone. The purpose of this investigation was to test the

hypothesis that 6 months of chronic alcohol consumption acts to decrease intracortical bone remodeling in young adult cynomolgus monkeys.

METHODS

Animals

Eleven late adolescent male *M. fascicularis* monkeys were used in the experiment. The animals were born, housed, and sacrificed at Oregon National Primate Research Center (Beaverton, OR). Each monkey was housed in an individual quadrant cage with temperature (20-22ºC), humidity (65%), and light/dark cycles controlled during the entire duration of study.

Experimental Design

The monkeys were divided into two treatment groups, control (n=3) and ethanol (n=8). In the induction phase of the study, monkeys from both groups were trained to self-administer food, water, and in the alcohol group, ethanol. Monkeys in the ethanol treatment group were then habituated to consume ethanol by increasing dietary intake of ethanol over 4 consecutive 30 day periods, in total, the induction phase of the study lasted 120 days. The induction procedure of the experimental design is summarized in Figure 2. Following, the monkeys in the alcohol group were given continuous access to ethanol (4%v/v) and water for the 6 months of study. Under the same conditions, control monkeys were given a maltose-dextrin solution that was isocaloric to the alcohol solution consumed by the ethanol treatment group. Individual drinking data was recorded daily.

To determine active bone mineralization sites and rates of bone formation, the fluorochrome tetracycline hydrochloride (20mg/kg) was orally administered at 17 and 3 days prior to sacrifice. At time of sacrifice, lumbar vertebrae 1-5, distal

femora, tibiae and fibulae from each monkey were harvested and stored in 70% ethanol at 4ºC until analysis.

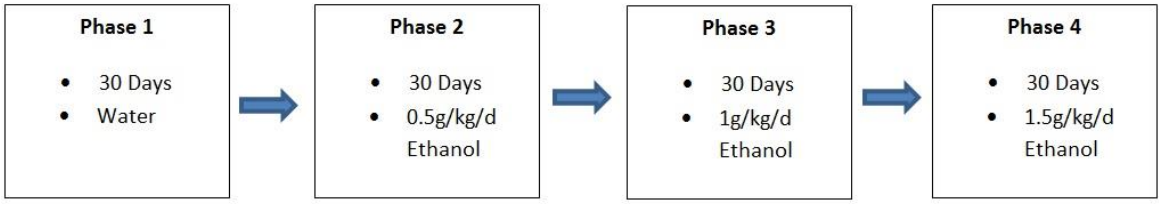

Figure 2. 120 day step-wise alcohol induction protocol. Throughout the first period animals were fed water. During the second period, animals in the alcohol group were fed 0.5g/kg/d ethanol. During the third and fourth period, monkeys were fed 1.0 and 1.5 g/kg/d ethanol, respectively. This step-wise conditioning of alcohol induction was conducted to prevent ethanol taste aversion.

Blood Alcohol Concentrations

Blood samples were collected from the monkeys in the alcohol group every 2-5 days through the saphenous vein. Blood samples were sealed in airtight vials containing 0.02 mL of 10% isopropyl alcohol, and stored at -4ºC before being analyzed using gas chromatography (Hewlett-Packard 5890 Series II, Avondale, PA).

Dual-energy X-ray Absorptiometry (DXA)

Bone mineral content (BMC, g), bone area (cm^2), and BMD (g/cm^2) in the lumbar vertebrae (LV1-4) and tibiae/fibulae were analyzed using DXA (Hologic Discovery A, Waltham, MA, USA) and Hologic APEX System Software, Version 3.1.1. Quality control checks were performed against the Anthropomorphic Spine Phantom and Small Animal Step Phantom, provided by the manufacturer. The

coefficient of variation evaluation test-retest reliability for DXA in our laboratory is 1.0% for BMC, bone area, and BMD. Tibial/fibular scans were conducted in the in the cranial-caudal view and vertebral scans were conducted in the ventral-dorsal view. All samples were scanned using the small animal regional high-resolution option.

Micro Computed Tomography (μCT) Analysis

μCT was used as a method for the nondestructive 3D analysis of cancellous and cortical bone architecture. Distal femurs and third lumbar vertebrae were scanned using a Scanco μCT40 scanner (Scanco Medical AG, Basserdof, Switzerland) at 36 x 36 x 36 micron voxel size, and the distal third of the tibias was scanned at 30 x 30 x 30 micron voxel size. Cancellous bone was measured at an empirically determined threshold of 175 (0-1000) and cortical bone was measured at a threshold of 245 (0-1000). The volumes analyzed in the distal femur (cancellous bone in the metaphysis and epiphysis), lumbar vertebra (cancellous bone), and tibia (cortical bone in distal tibial diaphysis) are depicted in Figure 3A-C. μCT cancellous measurements included bone volume/tissue volume, connectivity density, structural model index, trabecular number, trabecular thickness, and trabecular separation. Cortical measurements included cross-sectional volume, cortical bone volume, marrow volume, cortical thickness, and moments of inertia (I_{max}, I_{min}, and $I_{polar)}$.

Histomorphometry

Tibia length was measured from the proximal tip of the intercondylar eminence to the distal tip of the medial malleolus. Cross-sectional samples of the tibial diaphysis were cut at 1/3 of the length from the distal end of the tibia using an IsoMet® Low Speed Saw (Buehler, Lake Bluff, IL, USA). The resulting thick sections were then ground down to ~25 μm through the use of a 220 grit aluminum oxide powder upon a roughened glass surface, mounted on glass slides and coverslipped (32).

An Olympus BH2 Microscope equipped with an Olympus DP71 digital camera and attached to a computer system with OsteoMeasure software was used to collect all histomorphometric data. Histological samples were assessed for bone area (mm^2) and the number of incomplete (Figure 4A) and complete osteons per bone area ($\#/mm^2$). Incomplete osteons were recognized as having a large Haversian canal. Fluorochrome-based measurement of bone formation in the cortical bone consisted of counting the number of single (Figure 4B) or double labeled osteon (Figure 4C) per bone area. Details of procedure have been described elsewhere (32.)

Statistical Analysis

A two tailed t-test was used for statistical analysis. Data were analyzed using Microsoft Excel Version 2010 (Redmond, WA). Data are shown as means ± SE.

RESULTS

Age, weight, alcohol consumption, and blood alcohol concentration of the control and ethanol treated monkeys are shown in Table 1. There were no differences in age or body weight between the control and alcohol treatment groups. Monkeys in the alcohol treatment group drank an average of 1.83g of ethanol per kg body weight per day over the course of 6 months (Table 1). The control group did not consume any alcohol due to experimental design.

Alcohol Consumption Pattern

Total ethanol consumption is shown in Table 2. The monkeys engaged in patterns of heavy binging episodes, followed by abstention, then repeated by further binging (Figure 5). This drinking behavior caused a sporadic rise and drop in blood alcohol concentration that reflects the pattern in human alcohol abusers (38).

Lumbar Vertebra

Total Bone (DXA)

The effects of chronic alcohol consumption on bone area, BMC, and BMD in the lumbar vertebrae (LV1-4) were analyzed using DXA (Table 3). There were no significant differences seen in bone area, BMC and BMD in the lumbar vertebrae

Cancellous Bone (μCT)

The effects of heavy alcohol consumption on cancellous bone volume and architecture in lumbar vertebra are shown in Table 4. There was no difference on cancellous bone fractional volume, connectivity density, structural model index,

trabecular number, trabecular thickness, or trabecular separation between control and alcohol treatment groups.

Distal Femur

Cancellous Bone (μCT)

The effects of alcohol consumption on cancellous bone in the distal femur are shown in Table 5. There was no difference in bone fractional volume, connectivity density, structural model index, trabecular number, trabecular thickness, and trabecular separation in the epiphysis and metaphysis between the ethanol and control group.

Tibia

Total Bone (DXA)

The effects of chronic alcohol consumption on bone area, BMC and BMD in the tibiae/fibulae were analyzed using DXA. There were no significant differences seen in bone area, BMC and BMD in the tibiae/fibulae (Table 6).

Cortical Bone (μCT)

Significant difference between the control and alcohol group was not observed in the cortical architecture parameters of cross-sectional volume, cortical volume, marrow volume, cortical thickness, I_{max}, I_{min}, or I_{polar} (Table 7).

Histomorphometry

The number of incomplete osteons was lower in the alcohol group compared to the control group ($P < 0.05$) (Figure 6A). In addition, there was a trend ($P = 0.08$) for a lower number of labeled osteons in the alcohol group compared to the control group (Figure 6B).

Figure 3. Bone volumes quantified by µCT. (A) µCT cancellous volume of interest in the distal femur metaphysis and epiphysis. (B) µCT cancellous volume of interest of the 3rd lumbar vertebra. (C) µCT cortical volume of interest of the distal tibia diaphysis.

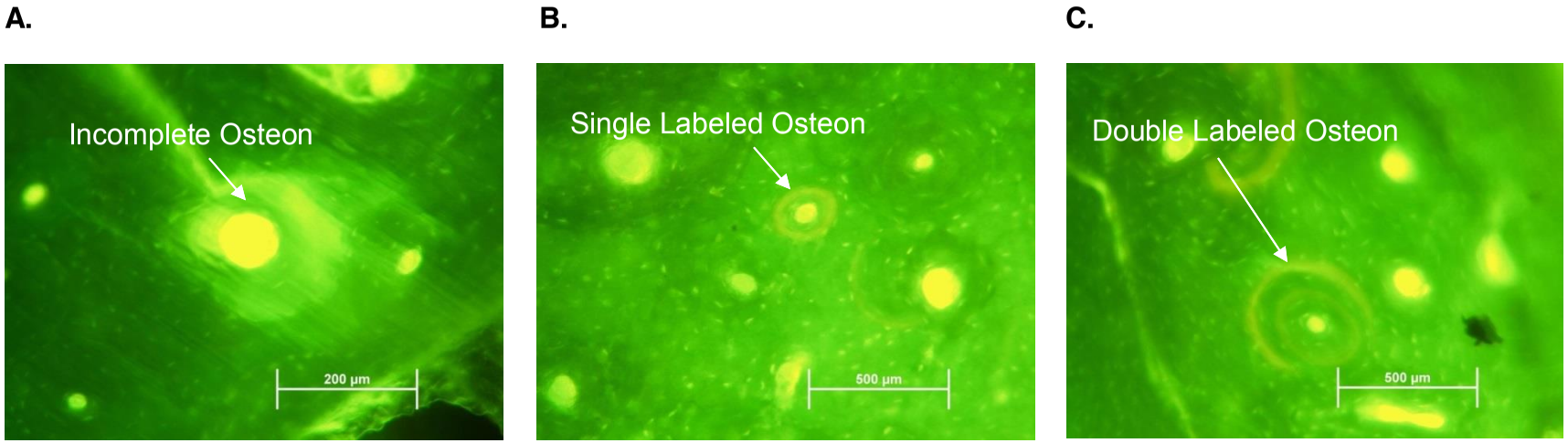

Figure 4. Photomicrograph of quantitative intracortical bone histomorphometry: (A) incomplete (open) osteon, (B) single labeled osteon, (C) double labeled osteon.

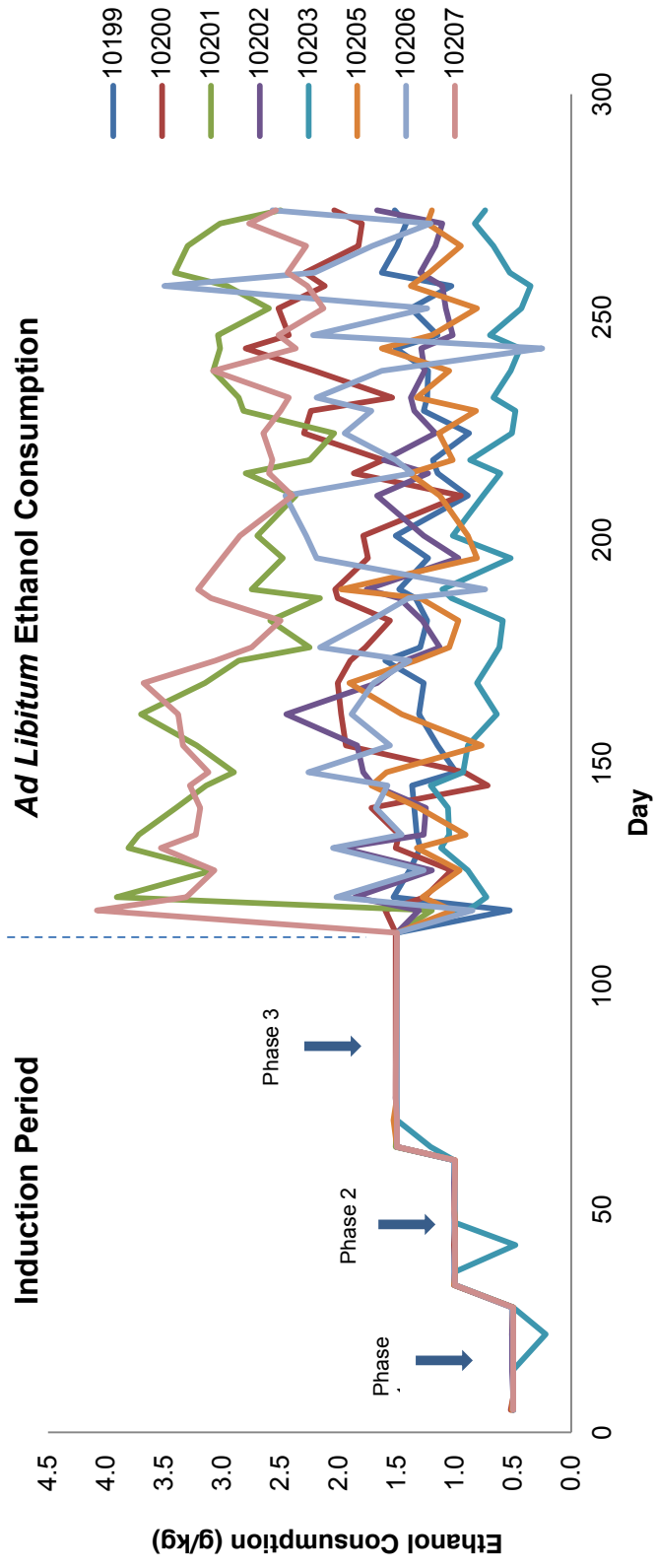

Figure 5. Daily ethanol consumption pattern in ethanol-treated monkeys.

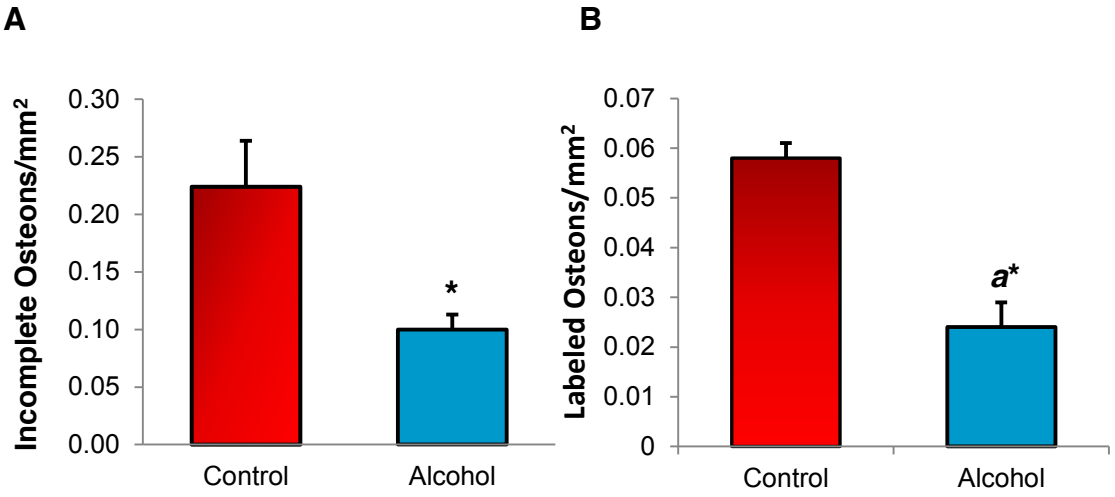

Figure 6. Number of complete and label osteons in control and alcohol treated monkeys. (A) Number of incomplete osteons per square millimeter in control and alcohol monkeys. (B) Number of labeled osteons per square millimeter in control and alcohol monkeys. Data are mean ± SE. * P<0.05 compared to control. *a** P< 0.1 compared to control.

Table 1. Study Population

	Control	Alcohol	t-test, p
Terminal Age (years)	7.5 ± 0.0	7.0 ± 0.1	0.05
Initial Weight	6.5 ± 0.8	6.5 ± 0.6	0.94
Terminal Weight	7.7 ± 0.5	7.9 ± 0.9	0.64
6 mo. avg. ethanol intake (g/kg/d)	NA	1.83 ± 0.3	
Blood Alcohol Concentration (mg/dL)	NA	50.8 ± 49.8	

Data are mean ± SE

Table 2. Total Ethanol Intake

Monkey #	Body Weight (kg)	Total EtOH Intake (mL)	Total EtOH Intake (g/kg)
10199	7.08	68355	386
10200	6.32	78444	496
10201	7.25	132909	733
10202	6.75	71602	424
10203	8.32	49727	239
10205	7.74	74127	383
10206	9.09	110313	485
10207	8.60	149667	696
Mean	7.65	91893	480

Table 3. Effects of Alcohol on Bone in Lumbar Vertebra 1-4

Endpoint	Control	Alcohol	t test, p
Lumbar Vertebrae (1-4)			
Bone Area (cm^2)	15.6 ± 0.1	16.1 ± 0.4	0.48
BMC (g)	9.4 ± 0.6	10.5 ± 0.4	0.15
BMD (g/cm^2)	0.61 ± 0.04	0.66 ± 0.02	0.24

Data are means ± SE. BMC=Bone Mineral Content. BMD=Bone Mineral Density

Table 4. Effects of Alcohol on Cancellous Bone in the 3rd Lumbar Vertebra

Endpoint	Control	Alcohol	t-test, p
3rd Lumbar Vertebra (Cancellous Bone)			
Bone Volume/Tissue Volume (%)	20.1 ± 2.4	21.9 ± 1.1	0.43
Connectivity-density (1/mm^2)	9.9 ± 1.0	8.7 ± 0.8	0.41
Structural Model Index	1.4 ± 0.3	1.0 ± 0.1	0.16
Trabecular Number (1/mm)	1.6 ± 0.0	1.6 ± 0.1	0.77
Trabecular Thickness (μm)	146 ± 13	155 ± 4	0.86
Trabecular Separation (μm)	598 ± 16	609 ± 25	0.81

Data are means ± SE

Table 5. Effects of Alcohol on Cancellous Bone in the Distal Femur Epiphysis and Metaphysis

Endpoint	Control	Alcohol	t test, p
Distal Femur Epiphysis (cancellous bone)			
Bone Volume/Tissue Volume (%)	30.6 ± 0.71	34.0 ± 2.8	0.50
Connectivity-density (1/mm2)	6.5 ± 2.0	4.4 ± 0.7	0.23
Structural Model Index	0.3 ± 0.2	-0.4 ± 0.1	0.15
Trabecular Number (1/mm)	1.6 ± 0.2	1.5 ± 0.1	0.49
Trabecular Thickness (μm)	212 ± 18	245 ± 13	0.19
Trabecular Separation (μm)	591 ± 58	655 ± 59	0.56
Distal Femur Metaphysis (cancellous bone)			
Bone Volume/Tissue Volume (%)	13.5 ± 2.7	20.6 ± 2.0	0.09
Connectivity-density (1/mm2)	2.9 ± 0.8	3.8 ± 0.5	0.40
Structural Model Index	1.7 ± 0.3	1.1 ± 0.2	0.08
Trabecular Number (1/mm)	1.2 ± 0.1	1.3 ± 0.1	0.53
Trabecular Thickness (μm)	179 ± 20	204 ± 9	0.21
Trabecular Separation (μm)	826 ± 64	772 ± 57	0.61

Data are means ± SE

Table 6. Effects of Alcohol on Tibia Bone Mineral Content and Bone Mineral Density

Tibia/fibula	Control	Alcohol	t-test, p
Bone Area (cm2)	20.6 ± 1.2	19.7 ± 1.2	0.69
BMC (g)	6.9 ± 0.4	6.9 ± 0.8	0.97
BMD (g/cm2)	0.34 ± 0.00	0.34 ± 0.02	0.88

Data are means ± SE. BMC=Bone Mineral Content. BMD=Bone Mineral Density

Table 7. Effects of Alcohol on Tibia Skeletal Architecture

Endpoint	Control	Alcohol	t test, p
Tibia Length (mm)	151 ± 4	147 ± 2	0.32
Tibia Diaphysis (cortical bone)			
Cross-Sectional Volume (mm^3)	57 ± 5.3	55.3 ± 2.2	0.73
Cortical Volume (mm^3)	40.3 ± 2.6	38.5 ± 1.6	0.57
Marrow Volume (mm^3)	16.7 ± 2.8	16.8 ± 1.9	0.97
Cortical Thickness (μm)	1919 ± 39	1831 ± 97	1
Imax (μm^4)	290 ± 45	258 ± 17	0.42
Imin (μm^4)	195 ± 38	188 ± 15	0.83
Ipolar (μm^4)	485 ± 81	446 ± 31	0.58

Data are means ± SE

DISCUSSION

This study is unique because of the study length, species of monkeys, age of the monkeys, and amount of alcohol consumed by the monkeys. This study was conducted to determine the effects of 6 months of chronic alcohol consumption on bone mass, microarchitecture, and turnover in male cynomolgus monkeys. On average, the animals in the alcohol treatment group consumed 1.83g of ethanol per kg of body weight per day. That is approximately equivalent to 10 standard drinks a day for a 75kg person.

Six months of heavy alcohol consumption had no effect on body weight, bone mass, or most indices of bone microarchitecture. However, monkeys in the alcohol treatment group exhibited lower labeled osteon density and fewer incomplete osteons than non-drinking monkeys. These findings provide evidence that 6 months of heavy alcohol consumption lowers intracortical bone remodeling.

Many studies have been conducted to determine how alcohol affects skeletal health. General findings from experimental research have shown that large amounts of alcohol consumption are correlated with impairment in bone health. In a 12-month study, male rhesus macaques underwent voluntary self-administration of ethanol. Results from this study showed that there were no differences between the alcohol and control group in any indices of bone mass or bone microarchitecture. The alcohol consuming monkeys did show significantly lower intracortical bone formation and bone resorption (32). The data from this 6 month study reflected the data collected from the 12 month study. This is significant because it provides evidence that alcohol influences bone metabolism

in a time period as short as 6 months. In addition, it shows that the impact of heavy alcohol consumption is seen across different primate species.

Other studies have also shown a correlation between heavy alcohol consumption and negative impact on bone. When 36% of daily caloric intake is comprised of alcohol in male rats a decrease in bone strength, bone density, and cancellous bone volume is seen (33). In a 10-month alcohol consumption study performed in rats, alcohol resulted in reduced cortical bone accrual and osteopenia due to decreased bone formation (34). Another study was conducted to determine how bone health was impacted with alcohol abstinence post heavy chronic consumption. Results show insufficient catch-up repair growth to restore bone back to original mass (36).

The finding that heavy alcohol consumption is a risk factor for osteoporosis has been consistently reinforced by past studies. This 6 month study reflects this pattern. In human studies, the effects of alcohol on bone have been evaluated during much longer periods. This study demonstrates that the effects of heavy alcohol consumption on intracortical bone remodeling in non-human primates are detectable in the bone in a time period as little as 6 months.

Most studies in animal models have been conducted in young growing rodents and demonstrated that bone growth is inhibited by heavy alcohol consumption. However, in humans, late adolescent males are the most likely age for chronic heavy alcohol consumption (1). The monkeys used in this study reflect this specific age group and gender.

Limitations of this study include small group size and only having late adolescent young adult male samples. The small sample size in this study potentially limited the statistical power to detect differences in the architectural parameters. Nevertheless, this study detected a significant decrease in intracortical bone turnover.

CONCLUSION

This study demonstrates that 6 months of chronic heavy alcohol consumption suppressed intracortical bone remodeling in monkeys. These findings suggest that chronic high levels of alcohol consumption act to lower bone quality in humans by reducing normal bone turnover. These findings provide a potential explanation to why in humans, alcohol abuse is associated with increased fracture risk even when BMD is normal.

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