# Voluntary Alcohol Consumption in Male Rhesus Macaques Suppresses Cancellous Bone Remodeling

by
Arianna Kahler-Quesada

A THESIS

submitted to

Oregon State University

Honors College

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the requirements for the
degree of

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

Presented June 2, 2017
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AN ABSTRACT OF THE THESIS OF

Arianna Kahler-Quesada for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics presented on June 2, 2017. Title: Voluntary Alcohol Consumption in Male Rhesus Macaques Suppresses Cancellous Bone Remodeling.

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**Abstract**

**Background:** Chronic heavy alcohol consumption is a risk factor for bone fracture. Using a non-human primate model of voluntary alcohol self-administration, we have recently shown that alcohol consumption suppresses intracortical bone remodeling. The current study extends this investigation to asses the effect of alcohol consumption on cancellous bone mass, architecture, and formation in lumbar vertebrae.

**Methods:** Following a 4-month induction period, male rhesus macaques (n=5) voluntarily self-administered water or alcohol (4% w/v) for 22h/d, 7d/wk for a total of 12 months. Control animals (n=4) consumed an isocaloric maltose-dextrin solution. Tetracycline hydrochloride was administered orally 17 and 3 days prior to sacrifice to label mineralizing bone. The response to alcohol in lumbar vertebrae was evaluated using densitometry, microcomputed tomography, and histomorphometry.

**Results:** Monkeys in the alcohol group consumed an average of 2.0 ± 0.2 (mean ± SE) g/kg/d of ethanol, resulting in an average blood ethanol concentration of 67± 9 mg/dl. However, average consumption and blood ethanol concentration varied widely from day to day, as well as from subject to subject. Significant differences in lumbar

vertebrae 1-4 bone area, bone mineral content, and bone mineral density were not detected with treatment. Significant differences in cancellous bone architecture (bone volume/tissue volume, trabecular thickness, trabecular number, and trabecular spacing) were likewise not detected with treatment. However, bone formation rate/bone perimeter and bone formation rate/tissue area were lower in the alcohol-consuming monkeys compared to controls (46.5 ± 9.5 versus 23.0 ± 5.9 %/y and 20.6 ± 3.5 versus 9.6 ± 2.3 %/y, respectively).

**Conclusions:** These results suggest that chronic alcohol consumption reduces cancellous bone formation in lumbar vertebrae.

264/300

Key Words: Computed tomography, bone histomorphometry, non-human primate, DXA

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

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**Methods:** Following a 4-month induction period, male rhesus macaques (n=5) voluntarily self-administered water or alcohol (4% w/v) for 22h/d, 7d/wk for a total of 12 months. Control animals (n=4) consumed an isocaloric maltose-dextrin solution. Tetracycline hydrochloride was administered orally 17 and 3 days prior to sacrifice to label mineralizing bone. The response to alcohol in lumbar vertebrae was evaluated using densitometry, microcomputed tomography, and histomorphometry.

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## Introduction

It is estimated that just over half of all US adults consume alcohol, with 48% of the drinking population classified as binge drinkers or heavy drinkers (Center for behavioral Health Statistics and Quality, 2016). While studies have shown that moderate alcohol consumption may slow age related bone loss by slowing bone turnover (Sripanyakorn et al., 2009; Felson et al., 1995; Ganry et al., 2000). Chronic alcohol abuse has been linked to a lower bone mineral density (BMD) (Chappard et al., 1991; Gonzalez-Calvin et al., 1993; Malik et al., 2009) and increased risk of fracture (Berg et al. 2008; Spencer et al. 1986).

Many studies suggest that chronic alcohol abuse lowers the rate of bone formation (Gonzalez-Reimers et al., 2013; Maurel et al. 2012; Gonzalez-Calvin, 1993) with variable effects on bone resorption (Cheung et al. 1995). Rapid changes observed in bone turnover markers suggest that alcohol can alter the activity and/or number of both osteoclasts and osteoblasts. However, widely varying impacts of alcohol based on gender, age, and pattern of consumption have been reported (Gaddini et al., 2016).

Variable study outcomes and the presence of co-morbidities (Kanis et al., 1999) associated with chronic alcohol abuse have made the specific effect of alcohol on the human skeletal system difficult to define. Due to the difficulty in conducting alcohol studies in humans, and measuring the direct impact of alcohol on bone structure and turnover, animal models have been widely used. Studies in rats have demonstrated detrimental effects of high levels of alcohol consumption on bone accrual during growth (Gaddini et al., 2016; Court-Brown, 2012). However, few animal studies have been performed following skeletal maturity.

Bone loss in adults can be due to a number of factors 1) increased bone remodeling where resorption exceeds formation 2) reduced remodeling where resorption exceeds formation or 3) uncoupled remodeling where resorption increases and formation decreases. While loss of bone and BMD is detrimental to bone strength, the quality of existing bone also impacts the risk of fracture. When the trabecular struts in cancellous bone are compromised, bone strength can decrease significantly independent of BMD. Cancellous bone, the spongy bone present in the vertebrae and end of long bones, remodels in order to cope with mechanical strain and to heal micro-damage. Cancellous bone remodeling occurs on the surface of trabeculae, and works through the basic cellular unit (BMU), which is initiated by osteoclast resorption (Eriksen, 2010). In normal bone, the resorption trench is completely filled with osteoid by osteoblasts, which then mineralizes to form bone. Chronic alcohol abuse may interfere with this process, leading to weakened bone.

We have recently shown that chronic heavy alcohol consumption suppresses intracortical bone remodeling in a non-human primate model of voluntary alcohol self-administration (Gaddini et al., 2015). The present study aims to expand on that investigation by evaluating the effect of chronic alcohol consumption on cancellous bone mass, architecture, and turnover in the lumbar vertebrae of monkeys.

## Materials and Methods

### Animals

The study population was comprised of a total of 9 (n = 4 control, 5 alcohol) young adult (7.3 ± 0.1 years old at initiation of alcohol protocol) male rhesus macaques

(*Macaca mulatta*). This age corresponds to early skeletal maturity, as the epiphyseal growth plate closes at 6.5 years in male rhesus macaques (Cheverud, 1981). Animals were born and reared in captivity at the Oregon National Primate Research Center at Oregon Health and Sciences University. All animals were subjected to the same experimental design (below). Monkeys were individually housed at a constant humidity (65%), temperature (20-22 ºC), and an 11-hour light cycle (light 0700-1800 hrs) in a room that allowed visual, auditory, and olfactory contact with other monkeys. Alcohol intake was recorded, blood ethanol concentration was measured every 5 days, and body weights recorded weekly (Helms et al., 2014; Kroenke et al., 2014).

**Experimental Design**

The experimental design is described in detail elsewhere (Grant et al., 2008). In brief, monkeys were trained to self-administer food and drink (using a touch-screen panel on the side of their cage) and to present their leg for blood sampling.

**Induction phase:** To induce drinking, a 1 g flavored food pellet was delivered every 5 min and water was the only fluid available. After water consumption became associated with the delivery of food, monkeys in the treatment group were induced to drink increasing volumes of an ethanol solution (4% w/v ethanol mixed in deionized water) in a step-wise fashion over 4 consecutive 30-day periods for a total of 120 days. First, the monkeys underwent a 30-day session where water was the only drinking fluid provided. During the second 30-day interval, animals drank a predetermined volume of ethanol solution corresponding to 0.5 g/kg/d ethanol, followed by volumes of alcohol corresponding to 1.0 and 1.5 g/kg/d during the third and fourth 30-day intervals, respectively. Monkeys were allowed to drink 4% w/v ethanol until the required dose of

ethanol was reached (e.g., 0.5 g/kg/d), at which point only water was provided. This step-wise increase in ethanol consumption was done to allow subjects to associate ethanol with its intoxicating effects and prevent taste aversion.

**Voluntary drinking phase:** Following the 120-day induction, monkeys in the alcohol group (n = 5) were given free access to water and/or ethanol for 22 h/d (1100-0900 hrs each day), 7 d/wk for 12 months. Control animals (n = 4) were allowed to self-administer a volume of maltose-dextrin solution isocaloric to the average volume of alcohol consumed by the alcohol group. Consumption was recorded daily by recording the change in mass of the containers used to dispense the solutions (Ohaus Corp., Parsippay, NJ).

Every 5 days, blood samples were collected from the saphenous vein of monkeys in the alcohol group 7 hours into the 22h sessions, just before the lights were turned off (between 1800 and 1900 hrs). Samples were sealed in airtight vials in 0.5 ml of distilled water and 0.02 ml of 10% isopropanol (internal standard), and stored at 4ºC until analysis.

Food consisted of 1g banana-flavored pellets (carbohydrate, 63%; fat, 4%; protein, 22%; PJ Noyes, Lancaster, NH). Over the 12-month duration of the experiment, the monkeys were required to eat at least three meals per day, with at least 2 hours between each meal. The meal ended if one-third of the daily food allotment was consumed, or if the monkey took longer than 2 minutes to obtain a pellet.

Fluorochrome tetracycline hydrochloride (20 mg/kg) was administered orally (17 and 3 days) prior to sacrifice for determination of active mineralization sites and rates of bone formation. At necropsy, lumbar vertebrae 1-4 (LV 1-4) were harvested from each animal, placed into 70% alcohol, and stored at 4ºC until analysis.

**Blood Ethanol Concentrations**

Blood ethanol concentrations were assayed in plasma using gas chromatography (Gaddini et al., 2015). In the alcohol group, subjects were divided between classifications of heavy drinking (HD, n=1), binge drinking (BD, n=1), and low drinking (LD, n=3). The development of these classifications are described in detail elsewhere (Baker et al, 2014; Baker et al., 2017). In brief, heavy drinking animals consume more than 3g/kg of alcohol for more than 20% of access days, binge drinkers consume greater than 2g/kg for more than 55% of access days (with at least one occasion of BEC higher than 80mg/dl), and low drinkers generally consume less than 2g/kg/d.

**Dual-Energy X-Ray Absorptiometry**

Bone area ($cm^2$), bone mineral content (BMC, g), and areal BMD ($g/cm^2$) in LV 1-4 were determined *post mortem* using a dual-energy X-ray absorptiometry (DXA) scanner (Hologic Discovery A, Waltham, MA) and Hologic APEX System Software, Version 3.1.1. Quality control check was performed against the Anthropomorphic Spine Phantom and Small Animal Step Phantom provided by the manufacturer. The coefficient of variation evaluating test-retest reliability for BMC, area and BMD for DXA scans in our laboratory is 1.0%. The least significant difference is 0.003-0.006 $g/cm^2$, depending on skeletal site, at the 95% confidence level.

**Microcomputed Tomography**

Microcomputed tomography (μCT) was used to perform a nondestructive 3-dimensional evaluation of cancellous bone. To evaluate cancellous bone architecture, the 3$^{rd}$ lumbar vertebra was scanned in 70% ethanol at a voxel size of 36 x 36 x 36 μm (55 $kV_p$, 145 μA, and 200 ms integration time, 500 projections/rotation) on a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland). For evaluation, filtering

parameters sigma and support were set to 0.8 and 1, respectively. Cancellous bone within the vertebral body (409 ± 5 slices) was analyzed at a threshold of 175 (gray scale of 0-1000) determined empirically. Cancellous bone measurements included (1) cancellous bone volume fraction (bone volume/tissue volume %) (2) connectivity density ($1/mm^3$) (3) trabecular thickness (μm) (4) trabecular spacing (μm) and (5) trabecular number (1/mm). The region of interest is shown in Figure 1A.

**Quantitative Bone Histomorphometry**

Methods for measuring dynamic bone histomorphometry have been described previously (Iwaniec et al, 2008). In brief, cores (diameter of 7.1 mm) of the 3$^{rd}$ lumbar vertebrae were removed from the center of the vertebral body using a Ryobi DP102 drill (Figure 1B). The cores were dehydrated in a graded series of ethanol and xylene, and then embedded in modified methyl methacrylate. 4μm thick sections were then cut using a vertical bed microtome (Leica 2065) and affixed to gel slides (pre-coated with 1% gelatin solution). One section/animal was mounted unstained for measurement of flurochrome labels.

Fluorochrome-based measurements of cancellous bone remodeling included (1) mineral apposition rate (the distance between double fluorochrome labels, divided by the 14 day period between label administration, μm/day) (2) mineralizing perimeter (mineralizing perimeter/bone perimeter: perimeter covered with double label plus half single label, normalized to bone perimeter, %) (3) bone formation rate (the mineralizing perimeter multiplied by mineral apposition rate and expressed per bone perimeter ($\mu m^2/\mu m/y$), bone area (%/y), or tissue area (%/y)). Trabecular thickness, trabecular spacing and trabecular number were also determined.

All histomorphometric data were collected using an Olympus BH2 Microscope (Olympus, Shinjuku, Tokyo, Japan) equipped with an Olympus DP71 microscope digital

camera (Olympus, Shinjuku, Tokyo, Japan) and attached to a computer system with OsteoMeasure software (OsteoMetrics, Atlanta, GA).

### Statistical Analysis

A two-tailed *t*-test was used to evaluate the effects of alcohol on bone mass, cancellous architecture, and parameters of cancellous bone remodeling. Data analysis was conducted using SPSS. A *p*-value of ≤ 0.05 was considered significant. All data are expressed as mean ± SE.

## Results

Characteristics (age, body weight, ethanol consumption and blood ethanol concentrations) are presented in Table 1. Age and body weight did not differ between control and ethanol consuming monkeys. Monkeys in the ethanol group consumed, on average, 2.0 g ethanol/kg/d resulting in an average blood ethanol concentration of 67 (mg/dl). Three of the animals were classified as low drinkers (consumed <2g/kg/d), one as a heavy drinker (consumed >3g/kg of alcohol for more than 20% of access days), and one as a binge drinker (consumed >2g/kg for more than 55% of access days) (Figure 2). The development of these classifications are described in detail elsewhere (Baker et al., 2014).

The effect of ethanol on bone in lumbar vertebrae 1-4 was by evaluated by DXA (figure 3). Significant differences in vertebral bone area (Figure 3 A), BMC (Figure 3B), or BMD (Figure 3C) were not detected with treatment.

The effect of ethanol on cancellous bone volume and architecture in the third lumbar vertebra (LV3) was evaluated by microCT (Figure 4). Significant differences in bone volume/tissue volume (Figure 4A), connectivity density (Figure 4B), trabecular number (Figure 4C), trabecular thickness (Figure 4D) or trabecular spacing (Figure 4E) were not detected with treatment.

The effect of ethanol on cancellous bone and dynamic indices of bone formation was subsequently evaluated by histomorphometry in a subsample (core biopsy) of the third lumbar vertebra (Figure 1A). Consistent with microCT evaluation of the entire cancellous envelope, significant differences in bone area/tissue area (Figure 5A), trabecular thickness (Figure 5B), trabecular number (Figure 5C), and trabecular spacing (Figure 5D) were not detected with treatment in the vertebral biopsy. In addition, significant treatment differences were not detected for mineralizing perimeter/bone perimeter (Figure 5E) or mineral apposition rate (Figure 5F). However, bone formation/bone perimeter and (Figure 5G) and bone formation/tissue area (Figure 5H) were lower in alcohol-consuming monkeys compared to controls. Significant differences in bone formation rate/bone area (Figure 5I) were not detected with treatment. An example of a histological image containing a double fluorochrome label is shown in Figure 1C.

## Discussion

In this study, we investigated the effects of 12 months of voluntary alcohol consumption on vertebral bone density, cancellous bone architecture, and cancellous bone formation in young adult male rhesus macaques. Average consumption of 2.0 g

ethanol/kg/d (average blood ethanol content of 66.9 mg/dl) resulted in no significant changes in BMD, cancellous bone mass or bone micro-architecture in lumbar vertebrae. However, cancellous bone formation (bone formation rate/bone perimeter and bone formation rate/tissue area) was suppressed in alcohol consuming monkeys compared to controls.

Macaques share many physiological similarities with humans, including bone physiology, and alcohol absorption and metabolism. In addition, macaques display voluntary alcohol consumption patterns remarkably similar to humans (Vivian et al., 2001; Mello et al., 1971). This includes marked variability among subjects in amount and pattern of alcohol consumption, reflected by a distribution of drinking patterns which includes heavy, binge, and low drinkers. Due to their high homology to humans, rhesus macaques are likely to prove an excellent model to evaluate the effects of alcohol on the skeleton.

In a previous study, 12 months of voluntary heavy alcohol consumption in male rhesus macaques resulted in suppressed intracortical bone remodeling in tibia that led to decreased cortical bone porosity (Gaddini et al., 2015). These findings suggest that alcohol has a negative effect on bone not detected by measurement of BMD. However, this study did not investigate the effect of alcohol on cancellous bone, which has important implications in bone health and is the main subject of investigation in the current study. Specifically, the majority of fractures in adults occur at skeletal sites rich in cancellous bone.

Cancellous bone is found mainly in the ends of long bones, the vertebrae, ribs, and iliac crest. In comparison to the cortical (compact) bone found in the shaft, cancellous bone derives its strength from highly interconnected plates and rods of bone.

Therefore, bone strength is a function of trabecular number, trabecular thickness, and connectivity of rods and plates. Because of its location at the ends of bone, cancellous bone functions mainly to direct forces to the cortical bone (Burr et al. 2014). Modeling and remodeling of cancelllous bone occurs on the surface of trabeculae, and functions to mediate adaptation of bone to prevailing mechanical strain and to heal micro-damage. In normal bone, bone resorption by osteoclasts is matched by osteoblast-mediated bone formation, but disease states, age, and nutritional deficiencies can interfere with this process.

Histological evaluation of bone biopsies – though invasive – is the only direct measure of cancellous remodeling in humans. Previous studies have reported decreased indices of bone formation associated with chronic heavy alcohol consumption. Histomorphometric results are supported by decreased blood levels of biochemical markers of bone formation. The effects of alcohol on biochemical markers of bone resorption are more varied, though the pattern of gradual bone loss generally supports the conclusion that resorption is decreased in alcoholics, leading to an overall reduction in bone remodeling (Diez et al., 1994; Laitinen et al., 1992; Gonzalez-Reimers et al., 2013; Maurel et al., 2012; Gonzalez-Calvin et al., 1993). However, studies often include a range of subjects that present comorbidities and disease states associated with heavy alcohol consumption (cirrhosis, diabetes, pancreatitis, etc) that may influence bone metabolism and confound the interpretation of results.

Thus far, rodents have been instrumental in modeling the effects of alcohol consumption on bone. While most studies have been performed on growing rodents, the few studies that have been performed in skeletally mature rodents demonstrate a remodeling imbalance that leads to progressive bone loss. Some studies have shown that long exposure to alcohol may lead to trabecular thinning, reduced mechanical

strength (Hogan et al., 2001) and reduced indices of bone formation and resorption (Turner et al., 2001a).

One year of voluntary alcohol consumption had no effect on cancellous bone mass or architecture. However, bone formation was reduced. Gaddini et al (2015) reported similar findings for cortical bone. Additionally, Gaddini et al reported a reduction in serum CTx, a biochemical marker of bone resorption. Taken together, these findings suggest that chronic alcohol consumption reduces cancellous as well as cortical turnover.

While the specific effect of alcohol on the bone is still debated, it has an effect likely mediated by both direct and direct mechanisms. In culture, alcohol increases indices of bone resorption by osteoclasts and decreases markers of bone formation by osteoblasts (Cheung et al., 1995; Chavvassieux et al., 1993; Turner, 2000). In addition, alcohol has been associated with changes in various hormones that impact bone metabolism. These include calcium-regulating hormones that mediate mineral homeostasis (eg. Vitamin D and PTH), growth (eg growth hormone), and reproductive hormones (Turner et al, 2012; Turner, 2000). In addition, malnutrition is often associated with chronic alcohol abuse, and may lead to further mineral and hormone imbalances (Gonzales-Reimers et al., 2011). Overall, the indirect effect of alcohol on bone is multifaceted and difficult to assess. Further study is needed to determine the specific effects of alcohol on cellular processes and hormone production in the non-human primate model of voluntary ethanol consumption.

In summary, in this study we investigated the effects of voluntary alcohol consumption in male rhesus macaques over the course of 12 months. Our findings

suggest that heavy alcohol consumption suppresses cancellous bone formation, which long term may have a significant negative impact on bone strength and risk of fracture.

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**Table 1. Study population.**

| | Control (n=4) | Alcohol (n=5) |
|---|---|---|
| Age (years) | 7.3 ± 0.1 | 7.2 ± 0.1 |
| Body weight (kg) | 10.3 ± 0.8 | 8.6 ± 0.7 |
| Ethanol intake (g/kg/d) | - | 2.0 ± 0.2 |
| Blood ethanol concentration (mg/dl) | - | 66.9 ± 9.01 |

Data are mean ± SE

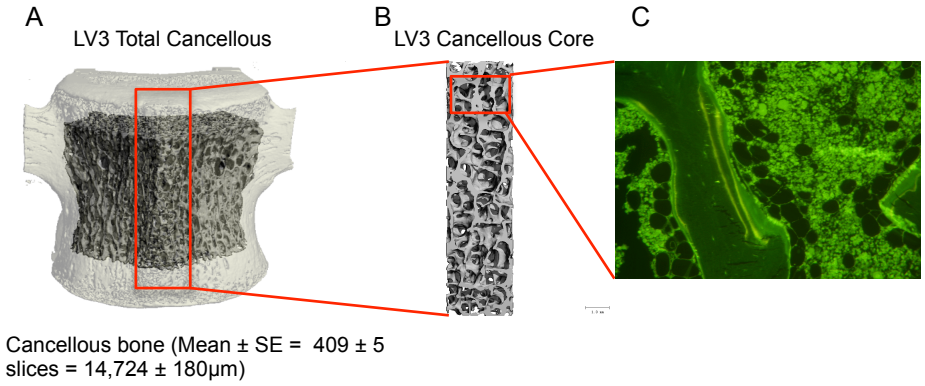


**Figure 1:** Lumbar vertebrae locations at which data was collected using µCT (A) and histology. Cancellous bone was analyzed within the vertebral body (A), and then a core was removed from the center of the vertebral body (B) and prepared for measurement of flurochrome labels (C).

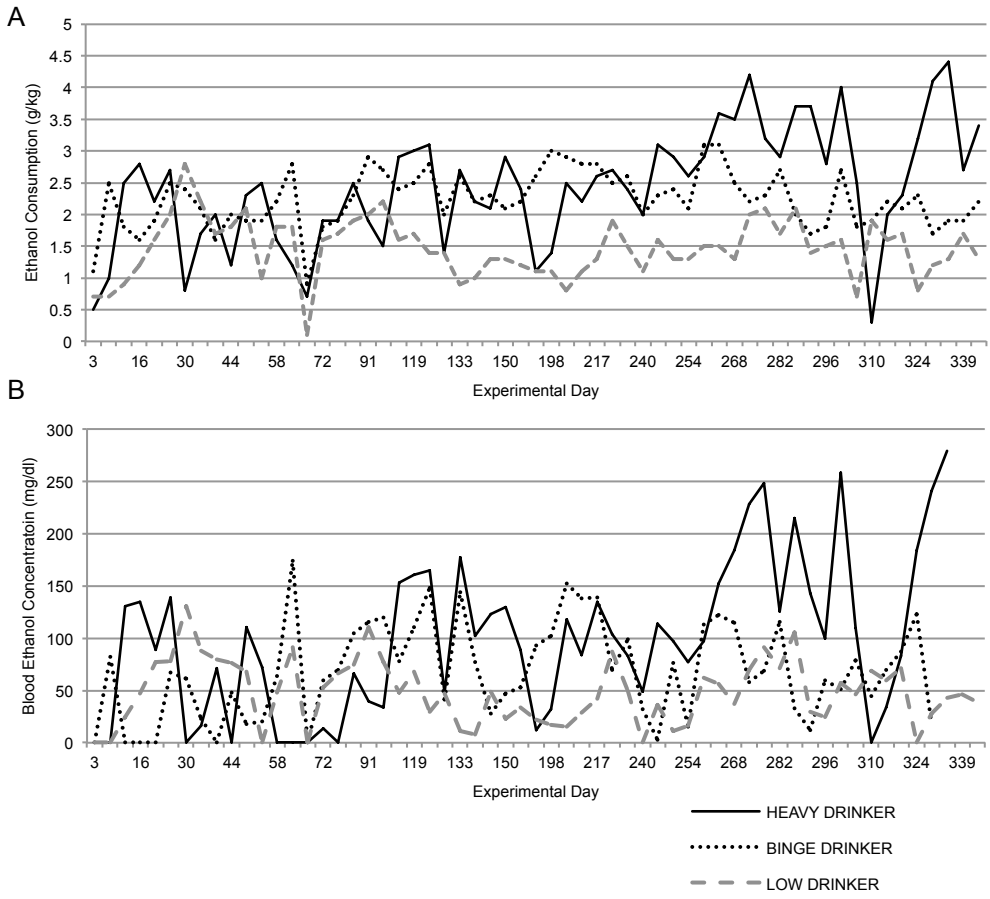


**Figure 2:** Ethanol consumption (A) and blood ethanol concentration (B) of a heavy drinker (solid), binge drinker (dots), and low drinker (dashes). The alcohol group consisted of one heavy drinker, one binge drinker, and three low drinkers.

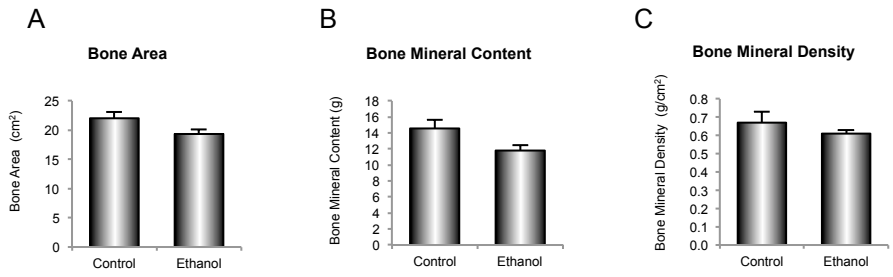


**Figure 3:** Effect of ethanol consumption on total lumbar vertebrae 1-4 bone area (A), bone mineral content (B) and bone mineral density (C) in rhesus macaques as measured by densitometry. Significant differences were not detected. Data are mean ± SE.

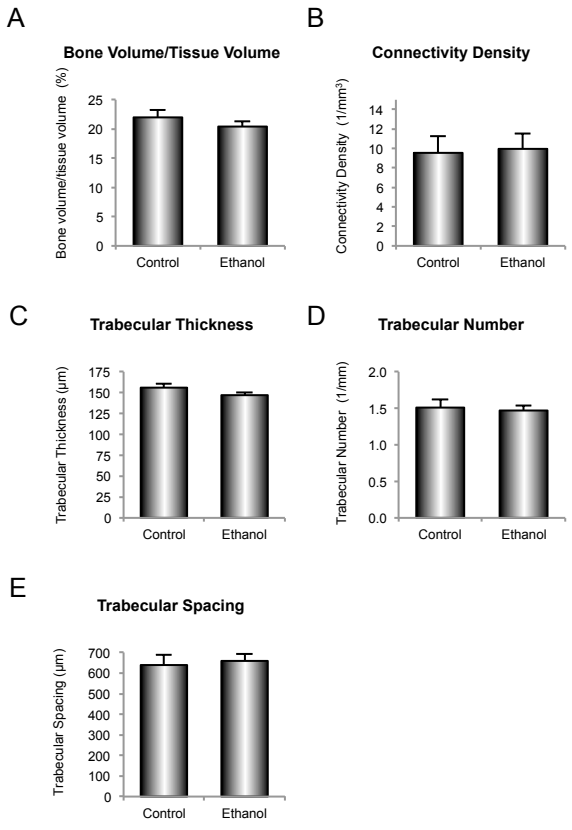


**Figure 4:** Effect of ethanol consumption on cancellous bone in lumbar vertebrae 3 as evaluated by µCT. Significant differences in bone volume/tissue volume (A), connectivity density (B), trabecular thickness (C), trabecular number (D), trabecular spacing (E) were not detected in µCT. Data are mean ± SE.

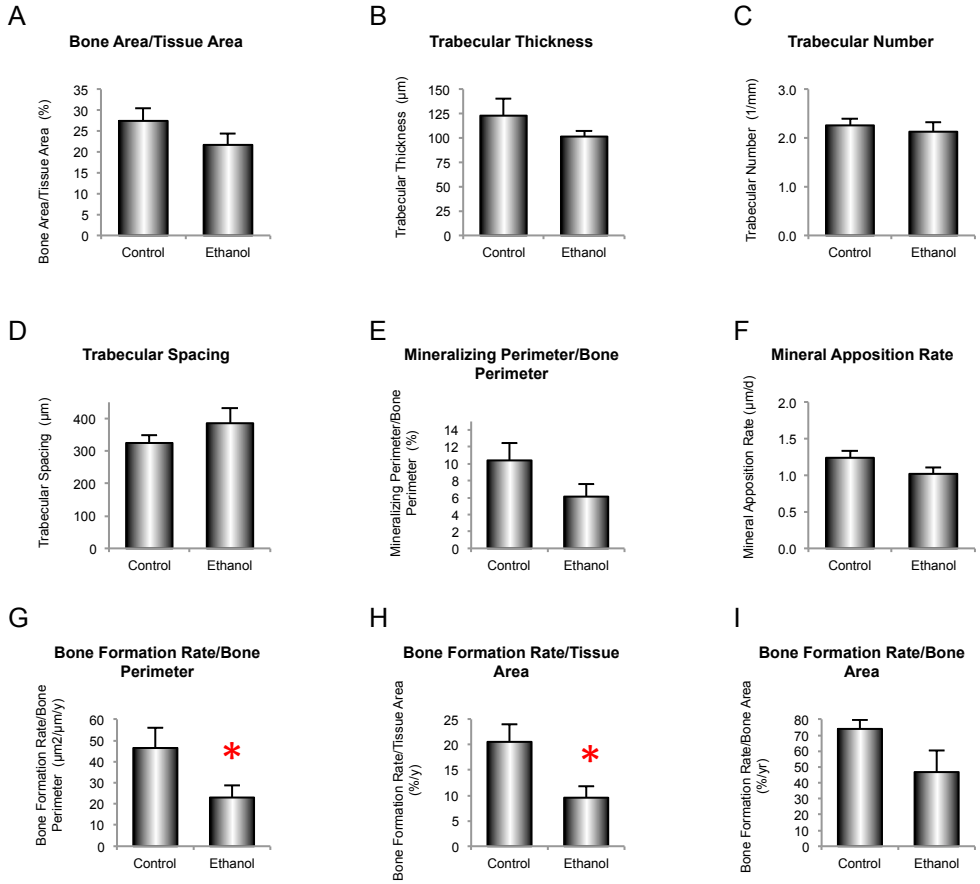


**Figure 5:** Effect of ethanol consumption on cancellous bone in lumbar vertebrae 3 as evaluated by histomorphometry. Significant differences in bone area/tissue area (A), trabecular thickness (B), trabecular number (C), trabecular spacing (D), mineralizing perimeter/bone perimeter (E), mineral apposition rate (F), and bone formation rate/bone area (I) were not detected by histomorphometry. However, bone formation rate/bone perimeter (G) and bone formation rate/tissue area (H) were lower in ethanol consuming monkeys compared to controls. Data are mean ± SE. *Different from Control, $p<0.05$