AN ABSTRACT OF THE THESIS OF

Gino W. Gaddini for the degree of Master of Science in Nutrition presented on August 1, 2013.

Title: Effects of 12 Months of Voluntary Alcohol Self-Administration on Intracortical Bone Remodeling in a Rhesus Macaque Model

Abstract approved:

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Background: Alcohol is a nonessential nutrient widely consumed throughout the world. Chronic alcohol abuse is associated with numerous adverse skeletal effects. However, prior to this study, the effects of alcohol abuse on intracortical bone remodeling in an animal model had not been investigated. Reduced intracortical bone remodeling could impair repair of microdamage accrued during activities of daily living and lead to increased risk of bone fractures.

Methods: To fill this gap in knowledge, we investigated the effects of 12 months of voluntary self-administration of ethanol (4% v/v) on bone mass, bone microarchitecture, and intracortical bone remodeling in male rhesus monkeys approaching skeletal maturity (mean age 6.7 years).

Results: Alcohol-fed monkeys consumed a mean of 2.8 g/kg ethanol per day. No significant difference was found between alcohol and control monkeys for body weight, age, or any indices of bone mass or bone microarchitecture. However, monkeys in the alcohol group had significantly lower intracortical bone formation and bone resorption compared to the control group.

Conclusions: These results suggest that 12-months of self-administration of alcohol results in significantly reduced intracortical bone remodeling without affecting bone mass or bone microarchitecture; thus, evidence from this study indicates that alcohol abuse can reduce bone quality without affecting bone quantity.

Key Words: Alcohol abuse, rhesus macaque model, histomorphometry, cortical bone remodeling.

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Effects of 12 Months of Voluntary Alcohol Self-Administration on Intracortical Bone Remodeling in a Rhesus Macaque Model

by
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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Gino W. Gaddini, Author

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1- Introduction

Osteoporosis (from Greek, "porous bone") is a disease characterized by reduced bone mass and altered microarchitecture, which results in diminished bone strength and increased fracture risk. Osteoporosis is highly prevalent, afflicting approximately 1 in 6 Americans (44 million people) [1, 2]. In the year 2000, over 9 million osteoporotic fractures were reported worldwide [3]; moreover, 54% of women in the U.S. over 50 years old will suffer an osteoporotic bone fracture during their lifetime [4]. Osteoporotic bone fracture can result in significant reductions in quality of life (debilitating pain, functional impairment, depression, and disability), as well as significant mortality [3, 5]. Roughly 24% of individuals over 50 who suffer a hip fracture will die within one year of the fracture [4]. Osteoporosis places a significant burden on society, with estimated direct medical costs of $20 billion in 2005, which are expected to rise to $25 billion by 2025 [5], as well as significant additional indirect costs related to poor health and need for social support. With an aging population, these numbers are expected to grow.

Throughout life, bone undergoes "ceaseless activity" [6] and is constantly being broken down and reformed in a coupled process known as bone remodeling (reviewed in Robling et al (2006) [7]). Imbalance between the relative and absolute rates of bone formation and bone resorption can affect bone quantity and bone quality, both of which are important for the structural integrity of bone. There are many factors that collectively contribute to modulation of bone remodeling and thus the risk of developing osteoporosis and risk of bone fracture, including genetics [8, 9], autoimmune disorders [10-13], endocrine disorders [14], chronic diseases (such as hepatic and renal disease [15-17]), and gastrointestinal disorders [18], as well as modifiable lifestyle factors such as diet [19], sunlight exposure [20], corticosteroid medication use [21], physical activity [22-24] and tobacco smoking [25-27]. Alcohol consumption is another important modifiable lifestyle factor that can influence skeletal health.

Alcohol consumption is highly prevalent throughout the world. In the U.S., over half of adults (roughly 131 million people) drink alcoholic beverages on one or more occasions per month [28], and around 3.8% of Americans are classified as alcohol-dependent [29]. Alcohol consumption and general health can be described as exhibiting a J-shaped curve; that is, compared to abstinence, moderate alcohol

consumption is associated with various health benefits, including improved blood lipid profiles [30-33] and improved insulin sensitivity [34-36], as well as reduced risk of cardiovascular disease [37-40], type II diabetes mellitus [41-43], Alzheimer's disease and other forms of cognitive decline [44-46], and reduced all-cause mortality [47, 48]. Conversely, chronic, heavy alcohol consumption is related to numerous negative health consequences, including deleterious effects on the cardiovascular [49], gastrointestinal [50], immune [51], nervous [52], and reproductive systems [53], as well as increased risk of certain cancers [54, 55] and a wide array of psychiatric disorders [56].

Alcohol consumption and human skeletal health also exhibit a J-shaped curve. Compared to alcohol abstention, moderate alcohol consumption is reported to result in higher BMD [60-70] and lower fracture risk in certain populations (especially elderly men and postmenopausal women). However, other studies report no beneficial or detrimental effects of moderate alcohol on bone mineral density (BMD) or fracture risk [57-59]. In contrast, chronic heavy alcohol consumption is associated with lower BMD [57, 71-78] and increased fracture risk in both men and women [73, 78-80].

While BMD is a predictor of bone fracture risk in the general population [81-84], the increased fracture risk associated with alcohol abuse in humans is greater than what is explained by decreased BMD alone [85], indicating that alcohol abuse negatively affects bone quality. Histological analyses of cancellous bone (spongy bone) in the iliac crest of human alcoholics have demonstrated that the percentage of cancellous bone covered by osteoblasts (bone forming cells) or incorporating tetracycline label (reflecting bone mineralization) are significantly decreased in alcoholics [76, 86-89], indicating that heavy alcohol consumption reduces cancellous bone formation. These findings are corroborated by reports of reduced serum biomarkers of bone formation in alcoholics compared to controls [58, 71-74, 76, 90-93]. The effects of heavy alcohol consumption on histological and biochemical markers of bone resorption in humans are less clear however, with some groups reporting lower resorption [86], others reporting higher resorption [87, 90, 92, 94], and some reporting no effect [76]. Findings in humans are replicated in rat models for alcohol abuse, which have shown that chronic, heavy alcohol consumption is associated with lower BMD

[95-98], as well as lower histological indices of cancellous bone remodeling at multiple skeletal sites [95, 96, 99]. Additionally, findings in animals have suggested that alcohol abuse is associated with altered microarchitecture in cancellous and cortical bone [95-97, 101] and lower compressive and tensile breaking strength [99, 101-104]. Thus, there is good evidence substantiating that alcohol does indeed disrupt global bone remodeling, and there is strong evidence that alcohol reduces cancellous bone remodeling; however, the effects of alcohol abuse on intracortical bone remodeling are not known. Since cortical bone comprises nearly 80% of skeletal mass [105] and is crucial for structural support, alcohol-induced alterations in intracortical bone remodeling may play a critical role in fracture risk.

Studies in humans are complicated by numerous factors, including alcoholism-associated comorbidities with alcohol-independent skeletal effects (such as malnutrition, tobacco smoking, illicit drug use, and chronic disease), the validity and reliability of alcohol consumption assessment, and the invasiveness of analysis of intracortical bone remodeling. Additionally, prospective alcohol intervention studies are difficult to conduct in humans for ethical reasons. Thus, it is challenging to accurately assess the effects of alcohol, especially alcohol abuse, on bone in humans. Rodent models allow circumvention of many of these limitations; however, rodents do not exhibit intracortical (Haversion or osteonal) bone remodeling (the primary process through which cortical bone is remodeled in humans); thus, the effects of alcohol on intracortical bone remodeling cannot be examined in rodents.

To understand the effect of alcohol on intracortical bone remodeling, and how this may relate to fracture risk in alcohol consumers, we used a rhesus macaques (*Macaca mulatta*), non-human primate model for chronic alcohol abuse. Unlike rodents, rhesus monkeys exhibit intracortical remodeling that closely resembles bone remodeling in humans [106]. Thus, this is a very relevant model for the skeletal effects of alcohol abuse. The primary purpose of this investigation was to evaluate the effects of alcohol abuse on cortical bone mass, density, and intracortical remodeling in young adult rhesus monkeys.

2- Literature Review

2.1 Osteoporosis

Osteoporosis is a major public health concern, which can lead to compromised bone strength and increased fracture risk. Nearly 1 in 6 Americans (44 million people) are affected by osteoporosis [1, 2], and with an aging population this number will only increase. Osteoporotic bone fracture can lead to significant reductions in quality of life (such as debilitating pain, functional impairment, depression, and disability [5]), as well as significantly increased risk of mortality [3]. In 2000, it was estimated that there were 9 million osteoporotic fractures worldwide [3]. In the U.S., about 54% of women over 50 will have an osteoporotic bone fracture; further, 24% of individuals over 50 who suffer a hip fracture will die within one year of the fracture [4]. Osteoporosis places a significant financial burden on society, with estimated direct medical costs of $20 billion in 2005, which are expected to rise to $25 billion by 2025 [5].

2.2 Bone Remodeling

Throughout life, bone undergoes “ceaseless activity” [6] and is constantly being broken down and reformed in a coupled process known as bone remodeling. A comprehensive review of bone remodeling can be found in Robling et al (2006) [7]. Briefly, at the initiation of bone remodeling, bone-resorbing cells (osteoclasts) are recruited to previously quiescent bone surfaces, where they degrade bone via secretion of protons and pH-sensitive enzymes. Following resorption, growth factors released from degraded bone facilitate the recruitment and activation of bone forming cells (osteoblasts) to the resorption site. Osteoblasts secrete a collagenous matrix and then facilitate the mineralization of this matrix to form bone. The end result of each remodeling cycle is the production of a new Basic Multicellular Unit (BMU), which is the functional unit of bone remodeling.

Proper regulation of bone remodeling is integral for maintaining the structural integrity of bone, as well as maintaining mineral homeostasis. An increased rate of bone resorption relative to formation will eventually result in a loss of bone mass, which is associated with increased risk of traumatic and non-traumatic bone fractures [81-84]. Moreover, bone quality can be negatively impacted when bone remodeling becomes abnormally low or high. Continuous bone remodeling is necessary for repair of

microfractures generated by activities of daily living. Impaired bone remodeling can lead to an accumulation of microfractures. An accumulation of microfractures reduces mechanical strength and eventually increases the probability of suffering a more severe bone fracture [107].

There are two types of bone, (1) cortical (compact) bone and (2) cancellous (trabecular or spongy) bone. Cortical bone turns over at a rate of 2-3% per year, while cancellous bone turns over slightly faster [7]. Due to the slow rate of turnover in bone tissue (relative to other tissues such as dermal and intestinal tissue), pathological (or beneficial) alterations in the bone remodeling process may take months to years to manifest changes in mass, density or architecture.

There are many factors that collectively contribute to skeletal health, including genetics [8, 9], autoimmune disorders [10-13], endocrine disorders [14], chronic diseases (such as hepatic and renal disease [15, 16]), and gastrointestinal disorders [18], as well as modifiable lifestyle factors such as diet [19], sunlight exposure [20], corticosteroid medication use [21], physical activity [22-24], tobacco smoking [25-27], and alcohol consumption.

2.3 Background on Alcohol Consumption in Humans

Humans have been consuming alcohol for a long time. Evidence of intentionally fermented alcoholic beverages dates back to the Neolithic period (circa 10,000 B.C.) [108]. Historically, alcoholic beverages have been consumed for numerous reasons. Alcoholic beverages have served as a source of nutrients and as a safe thirst quencher, and have been used for their antiseptic, analgesic, and mood altering properties [108]. Today alcohol is consumed throughout the world regularly.

In the U.S., a standard alcoholic drink is defined as 14 g (or 17.7 mL) of pure ethanol. This is roughly equivalent to 12 fluid ounces of beer, 8 fluid ounces of malt liquor, 5 fluid ounces of wine, or 1.5 fluid ounces (a “shot”) of 80-proof distilled spirits (e.g. vodka, whiskey, rum, or gin) [109]. According to the U.S. *Dietary Guidelines for Americans*, it is recommended that otherwise healthy adults limit alcohol consumption to moderate levels, which are defined as 1 standard drink (14 g) per day for women and 2 standard drinks (28 g) per day for men [110]. In the U.S, purchasing alcohol is

illegal for individuals under 21 years of age, and alcohol abstinence is recommended for pregnant and breastfeeding women [110]. The definition of a standard drink and alcohol consumption recommendations vary among nations; for example, in the United Kingdom, Australia, Finland, and Japan a standard drink is defined as 8, 10, 12, and 19.75 g of pure ethanol respectively [111]. Furthermore, in the United Kingdom it is recommended that women limit alcohol consumption to 16-24 g/d and men limit alcohol consumption to 24-32 g/d. Additionally, alcohol recommendations in the United Kingdom allow for some consumption of alcohol by minors (≤1 drink/wk for 15-17 year olds while under adult supervision) and pregnant women (≤2 units on ≤2 d/wk after the first 3 months of pregnancy).

Alcohol consumption is highly prevalent and bridges social, economic, racial, and educational gaps. According to data from several recent national surveys, approximately two thirds of U.S. adults have consumed alcohol during the past year, and over half of adults (or around 131 million people) have reported drinking during the month prior to being surveyed [28]. Approximately 1 in 4 adult Americans reported engaging in at least one binge-drinking episode in the past month (consuming ≥5 drinks within a period of several hours). Approximately 1 in 6 adults in the U.S. reported having 4 binge-drinking episodes per month, consuming an average of 8 drinks per binge [112]. Roughly 16.9 million Americans (6.7% of the population) have engaged in heavy drinking in the past 30 days (consuming ≥5 drinks on the same occasion on ≥5 days out of the past 30 days) [112], and around 3.8% of Americans meet the criteria of alcohol dependence [29].

Demographically, in the U.S., men are more likely to drink than women (~60% of men versus ~44% of women consumed alcohol in the past month) [28, 112]. Drinking prevalence is greater with more education (36.8% of adults with less than a high school education versus 69.1% of college graduates drink). Individuals in the 21-25 year old age group were the most likely to consume alcohol (70% of people in this group), and the prevalence of alcohol use decreases with age from 65.3% in 26-29 year olds to 51.6% among 60-64 year olds and 38.2% in individuals older than 65 [112]. Several 2011 national surveys found that 25-40% of youth (aged 12-17 years old) have consumed alcohol. Although the rate of alcohol use in youths has decreased

in the past several years, the 2010 National Survey on Drug Use and Health reported that 13.6% of youths consumed alcohol in the past 30 days, 7.8% engaged in binge drinking, and 1.7% were classified as heavy drinkers [112, 113]. These surveys support the conclusion that alcohol consumption is pervasive in American society, and that alcohol abuse is a major public health concern in the United States.

Detrimental health effects of alcohol abuse have been known for a long time; documented negative physiological consequences of alcohol abuse date back to the 11th century A.D. [108]. Research has demonstrated that excessive alcohol consumption is associated with deleterious effects on the cardiovascular [49], gastrointestinal [50], immune [51], nervous [52], and reproductive systems [53], as well as increased risk of certain cancers [54, 55] and a wide array of psychiatric disorders [56]. On the other hand, compared to alcohol abstinence, moderate drinking is associated with numerous health benefits. Moderate alcohol consumption has been shown to decrease serum LDL and triglyceride concentrations, lower blood pressure, decrease collagen-induced platelet aggregation, and increase plasma HDL levels in both men and women [30-33]. Numerous reviews and meta-analyses have concluded that moderate alcohol intake is associated with significantly decreased risk of heart disease [37, 38] and stroke [39, 40]. Moderate alcohol consumption may even negate the negative effects of high saturated fat diets on cardiovascular disease risk [114]. Moderate alcohol consumption is also associated with enhanced insulin sensitivity [34-36] and reduced risk of type II diabetes [41-43]. Additionally, light-to-moderate drinking is linked to a reduced risk of Alzheimer's disease and other forms of cognitive decline [44-46], as well as reduced risk of all-cause mortality [47, 48]. However, these protective effects of moderate alcohol may not extend to all populations, such as people with liver disease, recovering alcoholics or drug users, or people with mental disorders [115]. Additionally, while there is considerable evidence for the beneficial effects of alcohol on general health in humans, this conclusion is not unanimous in the literature. Shaper et al (1988) proposed the "sick quitter" hypothesis, in which formerly moderate drinkers cease drinking alcohol due to health issues or potential prescription drug interactions, thus inflating the number of "sick" abstainers and skewing the results of epidemiological studies [116]. Overall, the relationship between alcohol use and overall human health can be described as having a U- or J-shaped curve; however,

there is insufficient evidence to make a clear public health recommendation regarding the optimal amount of alcohol consumption, and this optimal level likely among between individuals.

2.4 The Effects of Alcohol on Skeletal Health in Humans

Similar to the relationship between drinking and general health, evidence in the literature suggests that chronic, heavy alcohol consumption in humans has serious negative skeletal consequences, while low-to-moderate drinking has no detrimental skeletal effects, and may even be beneficial in certain populations.

2.4.1 Chronic Heavy Alcohol Consumption

The general consensus in the scientific literature is that chronic, excessive alcohol consumption is associated with poor skeletal health. BMD is commonly assessed in clinical settings using dual X-ray absorptiometry (DXA) and is often used to predict fracture risk. Epidemiological and case-control studies using DXA to assess bone in alcoholic patients have associated chronic, heavy alcohol consumption with reduced BMD at multiple weight bearing and non-weight bearing skeletal sites [57, 71-78], as well as increased risk of bone fracture in both men and women [73, 78-80].

Histomorphometric analyses of transiliac biopsies from alcoholic hospital patients have shown that chronic heavy alcohol consumption is negatively associated with histological indices of bone formation [76, 86-89]. A case-control study by Crilly et al (1988) compared transiliac biopsies from male alcoholic patients admitted to a Veterans' Administration hospital (n=16, age = mean±SD = 65.3±7.3) to transiliac biopsies from age-matched volunteers who abstained from drinking (n=9, age = 63.3±6.0). They found that the percentage of bone covered in osteoblasts or incorporating tetracycline label (signifying bone formation) was significantly decreased in alcoholics, suggesting reduced bone formation [88]. Another case-control study by Schnitzler et al (2010) compared 16 male chronic alcoholic patients with pancreatitis (age = 41±9 years, duration of alcohol abuse=7-26 years, mean alcohol consumption=170 g/day) to 37 healthy, age-matched male volunteers. Analysis of transiliac biopsies showed that endocortical osteoid surface, mineral apposition rate, and bone formation rate were decreased, and endocortical eroded surface was

increased in the cortical bone of alcoholics compared to controls; however, no statistical differences were found in intracortical osteoid thickness or surface, eroded surface, mineral apposition rate, or bone formation rate, suggesting that in cortical bone alcohol may reduce formation and increase resorption on endocortical surfaces but have no effect on intracortical remodeling. Additionally, this group also showed that trabecular bone volume and trabecular thickness were significantly decreased in alcoholics compared to controls [89]. However, it should be noted that the alcoholic patients in this study had multiple comorbidities that could independently affect bone metabolism, including pancreatitis and type II diabetes.

In general, histological findings of reduced bone formation are corroborated by reports of reduced serum and urinary bone formation biomarkers (e.g. osteocalcin) in alcoholic patients compared to controls [58, 71-74, 76, 90-93]. However, the effects of heavy alcohol consumption on histological and biochemical markers of bone resorption in humans are less consistent, with some groups reporting lower resorption [86], others reporting higher resorption [87, 90, 92, 94], and some reporting no effect [76]. Furthermore, in a number of studies of former heavy alcoholics (>84 g/d for >5 years), BMD and biomarkers of bone formation were compared prior to and after periods of abstention (thus, subjects served as their own controls). Results showed that BMD was higher after abstention for 6 months [71] to 2 years [117], and that abstaining for periods of 7 days to 2 years resulted in significant increases in serum bone formation markers [58, 92, 94].

2.4.2 Moderate Alcohol Consumption

In contrast to alcohol abuse, numerous studies have associated light-to-moderate drinking with beneficial skeletal effects compared to abstention. However, this association may not be true for all populations. Epidemiological studies have shown that in post-menopausal women and elderly men, moderate alcohol consumption is associated higher bone mass and with smaller decreases in BMD and BMC at multiple weight-bearing and non-weight-bearing skeletal sites compared to non-drinkers [60-69]. In younger and middle-aged men, the effects of moderate alcohol consumption on BMD are less clear, with some studies showing a positive relationship [59, 62, 69, 70] but others showing no effect [57]. A lack of a relationship between

moderate alcohol intake and bone mass has also been reported in premenopausal women [58, 59]. This discrepancy could potentially be due to an insufficient duration of alcohol consumption in younger individuals to manifest alterations in BMD. Or, alcohol may be counteracting the skeletal effects of disrupted endocrine function resulting from advancing age. For instance, estrogen deficiency in postmenopausal women results in increased bone remodeling such that resorption outpaces formation [118]. Moderate alcohol consumption is associated with decreased serum and urinary markers of bone resorption and formation in post-menopausal women [67]. A prospective study by Marrone et al (2012) found that in moderate drinking (19±1 g/d) postmenopausal women (age 56.3±0.5 years), serum osteocalcin and carboxy-terminal collagen crosslinks (CTX) levels were increased after two weeks of alcohol abstention, suggesting that moderate alcohol consumption can decrease the rate of bone turnover in postmenopausal women [65]. Thus, moderate alcohol intake may be beneficial in postmenopausal women, in part, by counteracting the effects of estrogen deficiency on bone turnover rate.

2.4.3 Limitations of Human Studies

Although results of studies in humans are very valuable, there are numerous issues that affect interpretation of results. For example, many of the studies are retrospective. The validity and reliability of assessing alcohol consumption retrospectively is questionable, especially in alcoholics, who may not be able to accurately recall the quantity of alcohol they regularly consume. Additionally, alcoholism is associated with numerous factors known to have alcohol-independent skeletal effects: irregular feeding behavior and malnourishment are common in alcoholics [74, 119], many suffer from chronic diseases (e.g. liver, kidney, and pancreatic disease), and many engage in lifestyle factors such as cigarette smoking, illegal drug use, and/or physical inactivity. Furthermore, studies in humans may not be generalizable. Most studies have been done in middle aged and elderly white men and women, and skeletally immature individuals have been under-investigated. Also, there is a lack of consensus on the exact definition of light, moderate, and heavy alcohol consumption among studies. There is a large discrepancy in the amount of alcohol consumption defined as moderate. Additionally, there is evidence that the type of alcoholic beverage (e.g. beer, wine, or spirits) may potentially differentially affect the

skeleton [61, 66, 69, 120]. Furthermore, most studies in humans have reported alcohol consumption as a volume (or mass) of alcohol consumed per day. However, factors such as the rate and concentration of alcohol consumed, the amount and composition of foods present in the gastrointestinal tract with imbibed alcohol can affect the ethanol concentrations experienced at the cellular level independent of the volume of alcohol consumed. Blood alcohol concentrations would likely be a better measure to assess the skeletal effect of alcohol compared to the volume or duration of alcohol consumption; however, there is presently limited data on blood alcohol concentrations from studies designed to evaluate skeletal endpoints. Also, prospective studies investigating the effects of chronic, heavy alcohol consumption are difficult due to the long duration needed to see significant alterations in bone microarchitecture and bone mass, as well as ethical reasons (due to the documented negative health effects of alcohol abuse). Fortunately, several animal models for the effects of alcohol on bone have been effectively used to circumvent many of the limitations of human studies [121].

2.5 Effects of Alcohol on Skeletal Health in Rodents

A variety of animal models, including rodents, rabbits, dogs, and primates, have been used to model the skeletal effects of alcohol (the strengths and weaknesses of animal models for assessing skeletal health are reviewed by Iwaniec et al (2008) [121]). In the past decade, the laboratory rat has been the preferred animal for the majority of researchers. The benefits of rat models are that they have a relatively short lifespan, grow rapidly, are inexpensive, allow for strict control of diet and other variables, and have well characterized skeletons that serve as good models for a number of skeletal conditions [122]. Additionally, established protocols exist for hormonal interventions, immobilization, fracture healing, dietary manipulations, and numerous well characterized techniques are available to assess the effects of alcohol on bone, including DXA [123], peripheral quantitative computed tomography (pQCT) [124], micro computed tomography (μCT) [125], histomorphometry [121], and mechanical load tests.

2.5.1 Comparison of Bone Physiology Between Rodents and Humans

Bone growth and remodeling between humans and rodents (especially rats) share many similarities, which allow these animals to be good models for various aspects of the effects of alcohol on skeletal health. Similar to humans, rats undergo BMU-based endocortical and cancellous bone remodeling [126, 127]. Both rats and humans exhibit slow increases in cross-sectional bone area throughout the lifetime, resulting in life-long periosteal expansion [128, 129]. Additionally, both humans and rats exhibit endochondral ossification that slows in magnitude and ultimately ceases [130]. Like in humans, bone remodeling rate in rodents is not constant throughout life; thus, the age of the animals used is important for assessing the effects of alcohol on bone turnover. Rats undergo a rapid rate of bone growth during the first 3 months of life, which peaks around 6 weeks of age and then rapidly declines with increasing age [122]. Following this rapid growth phase, endochondral ossification slows and eventually ceases [130] while periosteal bone formation continues slowly throughout life. By 7.4 months of age in males and 6.5 months in females, longitudinal bone growth ceases through epiphyseal closure mediated by bone bridging [130], and there is a marked decrease in bone turnover rate during skeletal maturation in rats [126]. A study by Baron et al (1984) showed that, despite having similar trabecular bone volume, 8-week old rats had 5 times higher bone formation rate and 5 times more osteoclasts than 12-week old rats [126]. Most studies investigating the effects of ethanol consumption in rats during the past decade have been done in rapidly growing animals (See Table 2.1).

In contrast to humans, intracortical remodeling (the process through which cortical bone is remodeled in humans) is largely absent in rodents [131, 132]. Instead, bone remodeling in rodents is limited to endocortical and cancellous bone surfaces. Thus, rodents are poor models for investigating the effects of alcohol on intracortical bone remodeling [122]. Cortical bone is critically important in the skeletal system, providing much of the mechanical strength of bone. To date, due to lack of appropriate animal models, there has been no investigation into the effects of alcohol on intracortical bone remodeling. Primates, on the other hand, undergo intracortical remodeling similar to that in humans making them a good model for studying

intracortical bone remodeling; however, due to limitations (such as financial cost) relatively few alcohol studies have been done in non-human primates.

2.5.2 Methods for Delivery of Alcohol to Model Chronic Alcohol Abuse

To model chronic alcohol abuse, ethanol has been administered through several different methods, including mixing ethanol in drinking water, alcohol-containing liquid diets, and total enteric nutrition. The simplest method of alcohol delivery is through mixing ethanol in water (usually 4-5% v/v); however, animals show aversion to consuming higher concentrations of ethanol in water, which can cause decreased fluid intake and dehydration [133]. Additionally, macronutrient and micronutrient imbalance between treatment and control groups may become an issue as treatment animals receive nutrition from both chow and alcohol while control animals only receive nutrition through chow [133]. Thus, changes observed in bone may be the result of micronutrient or caloric differences and not from ethanol. To circumvent some of the limitations of administering ethanol through drinking water, the majority of animal studies in the last decade have administered alcohol through standardized liquid diets (see Table 2.1), such as the Lieber-DeCarli diet [133], which contain either ethanol or an isocaloric amount of simple carbohydrate. However, a study by Maddalozzo et al (2009) showed that when given *ad libitum* access to liquid diets containing either 35% of kcals as (1) ethanol or (2) maltose-dextrin (control), ethanol-fed animals gained significantly less weight than control animals [96]. When control animals were pair-fed to alcohol animals, body weight differences were resolved; however, pair-fed controls had significantly reduced bone volume and bone formation compared to *ad libitum* controls, suggesting that caloric restriction in rats fed ethanol-containing liquid diets may have skeletal effects independent of alcohol. In many studies using liquid diets, animals were not pair fed and the body weights of ethanol-fed animals were either not reported or were significantly lower than controls [103, 104, 134, 135]; thus, in these studies it is difficult to separate the effects of alcohol from the potential effects of malnourishment on skeletal phenotypes.

Total enteric nutrition (TEN) has also been used to administer ethanol in rats [136-138]. TEN involves implantation of a tube into the wall of the stomach; this tube is tunneled subcutaneously to the head and attached to a headpiece through which the

animal receives infusion of a liquid diet. Compared to ethanol mixed in drinking water or liquid diets, TEN allows for exquisite control of (1) caloric intake and dietary composition, (2) the volume, duration, and rate of alcohol ingestion, and (3) blood alcohol concentrations; additionally, TEN is an effective method to administer very high concentrations of ethanol. However, TEN is invasive, and it is conceivable that stress on an animal due to placement of the feeding tube may affect bone independent of alcohol, although this has not yet been thoroughly examined.

2.5.3 Effects of Alcohol on Bone in Rodents

Compared to controls, in skeletally immature (6- to 12-week old) rats administered 26-35% of kcals as ethanol for 6 to 16 weeks, (1) gains in BMD are reduced in the femur, tibia, lumbar vertebra, and in the whole body [96-98, 101, 103, 104, 139], (2) cancellous bone volume/tissue volume ratio and trabecular thickness are lower, and trabecular separation is generally higher [96, 97, 103, 140] (although some studies show no significant change in trabecular separation [141]), (3) cortical medullary area, cortical porosity, and pore number are higher and cortical thickness is lower in some but not all studies [96, 97, 142, 143], and (4) compressive and tensile breaking strength is lower [99, 101-104]. In skeletally mature rats (>8 month old), studies modeling alcohol abuse have reported bone loss. Administration of 35% of kcals as ethanol for 12-16 weeks decreases the ratio of bone volume to tissue volume [100, 144], causes thinning of the trabeculae [100, 135], thinning of cortical bone [135], and compromises mechanical strength [144].

Furthermore, studies have shown that alcohol administration in rats reduces histomorphometric indices of bone formation (i.e. osteoblast perimeter and mineralizing perimeter/bone perimeter ratio) in both skeletally immature [95, 96, 99] and skeletally mature rats [100]. Turner et al (2001) showed that bone formation in skeletally mature 8-month old rats can be reduced dose-dependently by administration of as little as 3% of kcal as ethanol [100]. Furthermore, administration of alcohol (1) impairs fracture healing in rats [136, 137, 145, 146] with as little as 7.2% of kcal as ethanol [147], (2) impairs osteoinduction in demineralized allogeneic bone matrix (DABM) implants [142, 148], and (3) reduces the mechanical strength during fracture repair [149-151].

2.5.4 Methods for Delivery of Alcohol to Model Binge Drinking

Rats have also been used to model the skeletal effects of binge drinking. Many studies have attempted to model binge drinking via daily intraperitoneal (IP) injections of large doses of ethanol. Studies have shown that once daily injections of 3 g ethanol/kg body weight on 3 consecutive days per week for 4 weeks in skeletally immature (7-16 week old) rats results in significantly reduced whole body BMD and cancellous BMD in the tibia and lumbar vertebra (assed via peripheral quantitative computed tomography) [152-155], but no change in cortical BMD in the tibia or lumbar vertebra compared to controls [154, 156]. The same alcohol administration regimen is also associated with altered bone microarchitecture [152], lower compressive strength in the lumbar vertebra [153-156], reduced serum osteocalcin (a biomarker of bone formation) [154], and suppressed expression of numerous genes involved in bone formation [153, 156, 157]. However, IP injection may have alcohol-independent effects that could preclude accurate extrapolation of results. Once daily IP injection of 1.2 g ethanol/kg body weight for 7 days in 4-month old female rats causes reduced body weight, suppressed periosteal and cancellous bone formation, and decreased expression of bone matrix proteins compared to oral gavage of the same dose of ethanol [158].

2.5.5 Limitations of Rodent Models

Modeling human drinking behavior in animals can be challenging, as many human drinkers have irregular patterns of alcohol consumption that can range from occasional, to regular, to binge or combinations of regular and binge drinking. While administration of alcohol via a liquid diet, mixed in drinking water, or IP injection allows control of pattern of alcohol administration, it does not necessarily model the voluntary patterns of human drinking. Additionally, most studies done in rodents have been done in skeletally immature, male rats given very high doses of ethanol. See Turner et al (2001) for review of studies prior to the year 2000 [122] and Table 2.1 for review of studies post 2000. These studies in rodents essentially model excessive, continuous alcohol consumption in young, growing individuals. As a result, it is unclear whether involuntary consumption of alcohol or involuntary caloric restriction may impact the skeleton. However, reduced caloric intake, which is common with alcohol abuse, has negative effects on bone [159, 160]. Furthermore, rodents are not humans. As

previously mentioned, rodents do no not exhibit intracortical remodeling, which is the process through which humans remodel cortical bone; thus, rodents are poor models for human cortical remodeling. Cortical bone comprises nearly 80% of bone mass and provides much of the structural support. The effects of alcohol on cortical bone remodeling are poorly understood, yet they are of significant clinical relevance. Due to the prevalence and severity of osteoporosis and osteoporotic fractures there is a critical need to understand the effects of alcohol on cortical remodeling.

To circumvent limitations of rodent models we have used a primate model of voluntary self-administration of alcohol. By undergoing intracortical remodeling similar to humans, primates are a physiologically relevant model. Importantly, this model allows investigation of intracortical bone remodeling in response to alcohol consumption patterns similar to those of human alcoholics [161].

2.6 Possible Mechanisms for the Skeletal Effects of Alcohol

The cellular and molecular mechanisms underlying the effects of alcohol on bone are not fully understood. It is likely that the actions of alcohol on bone are mediated through a combination of several mechanisms that act on numerous physiological systems. Cell culture studies have shown that high concentrations of ethanol can (1) act directly on osteoblasts to decrease their activity [162, 163], inhibit differentiation from progenitor cells [162, 164-168], induce senescence pathways [169], and stimulate expression of receptor activator of nuclear factor kappa-B ligand (RANKL, an osteoclastogenic factor) [170], (2) act directly on osteoclasts to increase their activity and differentiation [162, 171], and (3) directly induce apoptosis of osteocytes [97, 172, 173]. Several *in vitro* studies have shown that alcohol directs differentiation of mesenchymal stromal cells (which are progenitors for both the adipocyte and osteoblast lineages [174]) into adipocytes rather than osteoblasts [163, 173, 175-177], which could explain the increased marrow adiposity and decreased bone mass observed after chronic heavy alcohol consumption in rat models [96, 139]. Additionally, acetaldehyde, the main metabolite of ethanol has also been shown to decrease osteoblast activity and differentiation in culture [178, 179]. There is also *in vitro* evidence that chronic high levels of ethanol exposure may act directly on bone cells to suppress the Wnt/B-catenin signaling pathway [153, 156, 180], which plays an

important role in the regulation of genes involved in the function, apoptosis, proliferation, and differentiation of bone cells [181]. However, most of these aforementioned *in vitro* studies have exposed bone cells to ethanol concentrations that are unlikely to be achieved in living organisms; thus, drawing the conclusion that alcohol might directly affect bone cells requires additional *in vivo* support.

In addition to possible direct effects of alcohol on bone cells, there is strong evidence that alcohol may act indirectly on the skeleton through combinatorial effects on numerous physiological systems. Alcohol may affect the intake, absorption, metabolism, and excretion of micronutrients that are critical to bone health (such as calcium, magnesium, and vitamin D). Human alcoholics often have poor diet and voluntarily replace nutrient dense foods with alcohol [182]; thus, many alcoholics may suffer from deficient intakes of nutrients critical to bone health.

Chronic alcoholism is also associated with kidney and liver disease in humans [15-17] and cynomolgus monkeys [183]. Kidney and liver disease can result in deregulated absorption, metabolism, and excretion of nutrients involved in bone health. There is also evidence that alcohol disrupts the concentrations and activity of calcium regulating hormones, including vitamin D, parathyroid hormone (PTH), and calcitonin. Alcoholism in humans has been associated with decreased serum 1,25 dihydroxyvitamin D [71, 184] and serum 25-hydroxy-vitamin D [57, 89], as well as decreased serum PTH [184]. Decreases in vitamin D and PTH can indirectly lead to decreased bone mass and bone formation rate through impaired osteoblast function and differentiation, as well as impaired intestinal calcium absorption [185]. However, not all studies have reported alterations in serum vitamin D or PTH due to alcoholism [57, 71, 86, 89]. Studies in rats have shown that although abnormal vitamin D metabolism is observed in alcohol-fed rats, changes in vitamin D levels are preceded by changes in bone metabolism; thus, it is uncertain whether abnormalities in vitamin D levels are a cause or consequence (or both) of alcohol abuse.

Alcohol abuse is often associated with irregular feeding behaviors and reductions in fat mass and lean tissue mass in humans and rats [73, 96]. Fat mass and lean mass are both positively correlated with BMD [186, 187]. Reduced lean tissue

mass may decrease the mechanical stimulus in exercised-induced bone gain through reduced muscle force production and theoretically contribute to lower BMD [188]. Reduced fat mass may alter signaling of bone active hormones secreted from adipocytes; in particular leptin, an adipocyte secreted cytokine involved in food intake and body weight regulation. Leptin may act both centrally and peripherally to regulate bone mass [189]. Leptin stimulates the differentiation of human marrow stromal cells into osteoblasts and inhibits differentiation into adipocytes [190]. Alcohol consumption has been shown to decrease leptin concentrations in rats [96, 97] and humans [191].

Alcohol may also indirectly affect bone by inducing skeletal resistance to growth hormone [192]. Growth hormone stimulates differentiation of stromal cells into osteoblasts and suppresses adipocyte differentiation [193]. Both alcohol and growth hormone deficiency result in similar skeletal responses: reduced bone growth and turnover and increased marrow adiposity [193, 194]. In a study investigating hypophysectomized rats simultaneously given alcohol and growth hormone, alcohol consumption was shown to impair the effects of growth hormone on both tibial growth and cancellous bone formation [192]. Many of the actions of growth hormone on cells are mediated through insulin like growth factor-I. Heavy chronic alcohol consumption is associated with decreased insulin like growth factor-I expression in bone [195], decreased hepatic synthesis of insulin like growth factor-I binding proteins [195, 196], and decreased serum insulin like growth factor-I in both rats [96, 142, 196, 197] and humans [198]. The disruption of growth hormone/insulin like growth factor-I signaling by chronic alcohol abuse could be achieved through either (1) decreased hormone bioavailability and/or (2) end organ resistance [192], and disruption of this signaling could lead to bone loss.

Alcohol-induced effects on sex hormones have also been found to influence bone metabolism. Numerous studies have associated testosterone deficiency with low BMD in male and female humans [199-209], and studies in orchidectomized male rats have shown that androgen deficiency results in altered bone metabolism [210], reduced periosteal bone formation [211], cancellous osteopenia [212], impaired radial and longitudinal growth in long bones [211, 213], and decreased BMC/BMD with aging [214]. There are conflicting results on the effects of chronic heavy alcohol consumption

on serum testosterone levels, with some studies reporting decreases in both humans [70, 73] and rats [103] and others finding no effect of chronic heavy alcohol consumption in either humans [86, 88, 90, 215] or rodents [95, 104]. However, there is evidence that serum testosterone levels are acutely diminished following bouts of alcohol consumption in humans [216-219], macaque monkeys [220], and rats [221-223]. Rodent work shows that acute alcohol administration results in drastic reductions in serum testosterone levels, which return to normal levels after 2 hours [222]. These reductions may be dose-dependent [220, 221]. Interestingly, moderate alcohol is associated with increased plasma total and free testosterone levels [224].

Estrogens influence bone physiology throughout life [225] and play a role in limiting the activation of new bone remodeling units, as well as the maintenance of proper balance between the activity of osteoblasts and osteoclasts. Estrogen deficiency results in increased amounts of bone actively undergoing remodeling. Since actively remodeling bone has reduced mechanical strength, estrogen-deficiency-induced reduction in bone quality can increase fracture risk [118]. Estrogen deficiency results in a remodeling imbalance in which resorption outpaces formation, which can cause reduced bone mass over time [118]. Lower serum estrogen is associated with lower BMD at multiple skeletal sites in elderly men and women [59, 207], and estrogen replacement therapy delays decreases in bone mass [226], and decreases fracture risk [227].There is evidence that moderate alcohol may modulate the effects of estrogen on bone through increased estrogen receptor alpha expression [228, 229], increased aromatization of testosterone to estrogens [230, 231], decreased clearance of estrogen [232] (via direct inhibition of estradiol catabolism by ethanol [233]), or a combination of these effects. In humans, moderate alcohol intake is associated with increased serum estrogen in post-menopausal women [215, 234-237]. Some studies show that moderate alcohol intake is linked to increased serum estrogens in men and pre-menopausal women [70, 238]; whereas, other studies have reported no differences in serum estrogen levels in pre-menopausal women [239] or middle-aged men or postmenopausal women [219]. Acute alcohol administration results in increased plasma estradiol within 25 minutes of administration [240, 241], suggesting that alcohol might acutely induce changes in hormone levels that affect bone. Thus, alcohol-

induced increase in serum estrogen may in part explain the beneficial skeletal effects of moderate alcohol consumption.

Voluntary alcohol self-administration in cynomolgus monkeys has been demonstrated to alter the expression and activity of several neurological factors including gamma-aminobutyric acid receptors [242-245], ionotropic glutamate receptor complex subunits (e.g. *N*-methyl-*D*-aspertate receptors) [246, 247], and serotonin receptors [248], all of which have been shown to influence bone remodeling [249]. Furthermore, alcohol abuse in cynomolgus monkeys has been associated with altered activity and levels of stress-related proteins, including adrenocorticotropic hormone, cortisol, nuclear factor κ-light-chain-enhancer of activated B cells, and Janus kinase/signal transducer and activator of transcription [250, 251], all of which also are involved in regulation of bone remodeling [252-254].

Overall, it is likely that alcohol does not affect bone through any one mechanism, but rather through multiple mechanisms simultaneously. The effects of alcohol on bone are likely due to the concentration, duration, and rate of ethanol consumption. Factors such as the amount and composition of foods present in the gastrointestinal tract with imbibed alcohol can affect cellular exposure to alcohol independent of the volume of alcohol consumed.

2.7 Purpose and Hypothesis

The purpose of the present study was to investigate the effects of alcohol abuse on bone mass, bone microarchitecture, and intracortical bone remodeling. Our hypothesis was that one year of voluntary alcohol consumption would be associated lower bone mass, altered bone microarchitecture, and reduced intracortical bone remodeling in a non-human primate model for alcohol abuse.

Table 2.1 Summary of Animal Studies Investigating the Skeletal Effects of Alcohol

Paper	Model	Sex	Quantity of Ethanol	Administration Method	Duration	Start Age
Broulik *et al*, 2009	Rat (Valaz Prague)	M	7.6 g/kg/day	Drinking water	3 mo	2 mo
Callaci *et al*, 2006	Rat (Sprague-Dawley)	F	3 g/kg/d for 3 consec d/wk	IP injection	2 or 4 wk	Unspecified
Callaci *et al*, 2008	Rat (Sprague-Dawley)	M	3 g/kg/d for 3 consec d/wk	IP injection	1 or 4 wk	16 wk
Callaci *et al*, 2009	Rat (Sprague-Dawley)	M	3 g/kg/d for 3 consec d/wk	IP injection	1 or 4 wk	7 wk
Chakkalakal *et al*, 2002	Rat (Sprague-Dawley)	M	36% of calories	Liquid diet	13 wk	8-10 wk
Chakkalakal *et al*, 2005	Rat (Wistar)	M	26 or 36% of calories	Liquid diet	13 wk	11-12 wk
Dai *et al*, 2000	Mouse (C57BL/6)	M	26% or calories	Liquid diet	4 mo	8 wk
Elmali *et al*, 2002	Rat (Wistar)	M	17 g/kg/day	Liquid diet	8 wk	Unspecified
Himes *et al*, 2008	Rat (Sprague-Dawley)	M	3 g/kg/d for 3 consec d/wk	IP injection	4 wk	16 wk
Hogan *et al*, 2001	Rat (Sprague-Dawley)	F	35% of calories	Liquid diet	8 or 14 wk	9 mo
Howe *et al*, 2010	Rat (Sprague-Dawley)	M	35% of calories	Liquid diet	6 wk	3 mo
Irie *et al*, 2008	Rat (Wistar)	M	36% of calories	Liquid diet	4 wk	10 wk
Iwaniec *et al*, 2008	Rat (Sprague-Dawley)	M	35% of calories	Liquid diet	6 wk	3 mo
Lauing *et al*, 2008	Rat (Sprague-Dawley)	M	3 g/kg/d for 3 consec d/wk	IP injection	1 or 4 wk	7 wks
Lima *et al*, 2010	Rat (Wistar)	M	30.3±1.5 mL/d of 15% (v/v)	Drinking water	90 d	45 d
Maddalozzo *et al*, 2008	Rat (Sprague-Dawley)	M	35% of calories	Liquid diet	6 wk	10 wk
Maurel *et al*, 2011	Rat (Wistar)	M	18±3 mL/d of 35% (v/v)	Drinking water	19 wk	8 wk
Nyquist *et al*, 2002	Rat (Sprague-Dawley)	M	3.4±0.2 g/d of 15% (v/v)	Drinking water	6 wk	70 d
Perrien *et al*, 2004	Rat (Sprague-Dawley)	M	12 g/kg/d	Total enteric nurition	6 wk	3 mo
Perrien *et al*, 2002	Rat (Sprague-Dawley)	F	12 g/kg/d	Total enteric nurition	6 wk	3 mo
Rai *et al*, 2008	Rat (Sprague-Dawley)	M	Unspecified vol. of 20 or 30% (v/v)	IP injection	5 d or 6 wk	Unspecified
Reed *et al*, 2002	Rat (Sprague-Dawley)	M	35% of calories	Liquid diet	16 wk	6 mo
Sibonga *et al*, 2007	Rat (Sprague-Dawley)	M	35% of calories	Liquid diet	16 wk	8 mo
Trevisol *et al*, 2007	Rat (Sprague-Dawley)	M	35% of calories	Liquid diet	6 wk	3 mo
Turner *et al*, 2001	Rat (Sprague-Dawley)	F	3,6,13, or 35% of calories	Liquid diet	4 mo	8 mo
Wahl *et al*, 2006	Mouse (C57BL/6)	M	36% of calories	Liquid diet	3 mo	12 mo
Wezeman *et al*, 2003	Rat (Sprague-Dawley)	M/F	36% of calories	Liquid diet	4 wk	30 d

3- Material & Methods

3.1 Animals

A total of 34 late adolescent/young adult (mean age 6.7 years) male rhesus macaques (*Macaca mulatta*) were used. The monkeys were born and reared in captivity at the Oregon National Primate Research Center at Oregon Health and Sciences University in Beaverton, OR. They were housed individually in quadrant cages under constant temperature (20-22 ºC), humidity (65%), and a 12-h light cycle (light 0700-1900 hrs) in a room allowing visual, auditory, and olfactory contact with other monkeys. Weights were recorded weekly throughout the duration of the study. Animals used in this study were pooled from 3 cohorts of monkeys. The first cohort was sacrificed in May of 2010 (n=5 control, 8 ethanol), the second in March of 2012 (n=4 control, 8 ethanol), and the third in July of 2012 (n=4 control, 5 ethanol). All of the animals from each cohort were subjected to the same study design (see below).

3.2 Experimental Design

3.2.1 Induction phase:

The experimental design is described in detail in Grant et al (2008) [255]. Briefly, animals were initially trained to self-administer food, water, and ethanol using an operant panel mounted to their cage over a period of 2 to 3 weeks. Previously alcohol-naïve monkeys were then conditioned to drink increasing doses of ethanol (4% w/v) in a step-wise fashion over 4 consecutive 30-day periods for a total of 120 days. During the first 30 days the animals drank only water. During the next 30-day period, animals drank a predetermined volume of ethanol solution corresponding to 0.5 g/kg/d ethanol, followed by volumes of ethanol corresponding to 1.0 and 1.5 g/kg/d during the 3rd and 4th 30-day periods, respectively. Monkeys were given only the option to drink ethanol until the required dose was reached, at which point animals were then only allowed to drink water. This step-wise increase in alcohol induction was done to circumvent establishment of ethanol taste-aversion.

3.2.2 Voluntary drinking phase:

Following the 120-day induction period, monkeys were given simultaneous access to both water and ethanol (4% w/v) and allowed to voluntarily self-administer ethanol and/or water for 22 h/d (1100-0900 hrs each day), 7 d/wk for 12 months.

Control animals (n=13) were allowed to self-administer a maltose-dextrin solution that was isocaloric to the ethanol solution consumed. Drinking data for all animals was recorded daily by measuring the change in the masses of water and the ethanol/maltose-dextrin solution.

The 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 sacrifice, the first through sixth lumbar vertebrae, the distal femora, and the tibiae/fibulae from each animal were harvested and placed directly into 70% ethanol, and stored at 4°C until analysis.

3.3 Dual-Energy X-Ray Absorptiometry

Bone mineral content (BMC, g/cm), bone area (cm^2) and BMD (g/cm^2) in the lumbar vertebrae (LV1-4) and tibiae/fibulae (the tibiae and fibulae were analyzed together) were determined *post mortem* using a dual-energy X-ray absorptiometry scanner (DXA, Hologic Discovery A, Waltham, MA, USA) and Hologic APEX System Software, Version 3.1.1. Tibial/fibular and vertebral scans were conducted in the posterior-anterior view. The tibiae/fibulae and LV1-4 were scanned using the small animal regional high-resolution option. Quality control check was performed against the Anthropomorphic Spine Phantom and Small Animal Step Phantom provided by the manufacturer. All scans were performed and analyzed by the same individual. The coefficient of variation evaluating test-retest reliability for DXA scans in our laboratory is 1.0% for BMC, bone area, and BMD.

3.4 Micro Computed Tomography

Micro computed tomography (µCT) was performed for nondestructive 3-dimensional analysis of cancellous and cortical bone microarchitecture [256] using a Scanco µCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland).

The 3rd lumbar vertebral body (LV3) and right distal femur of each monkey were scanned at a voxel size of 36x36x36 µm. In the LV3, the entire region of secondary spongiosa between the cranial and caudal growth plates was evaluated (Figure 3.1). In the distal femur, 60 slices (2.2 mm) of bone at a distance of 4 slices immediately proximal to the caudal growth plate (or growth plate remnant) in the

metaphysis and the entire region of secondary spongiosa at a distance of 4 slices immediately distal to the caudal growth plate (or growth plate remnant) in the epiphysis were evaluated (Figure 3.1). In addition to the distal femur and LV3, the right distal third of the tibia of each animal was scanned (at a voxel size of 30x30x30 µm). In each monkey, 66 slices (2.0 mm) of bone were evaluated in the metaphysis at a distance of 165 slices (5.0 mm) proximal to the caudal growth plate, and 33 slices (1.0 mm) of bone were analyzed in the tibial diaphysis at 1/3 of the length of the tibia measured from the distal tip of the medial malleolus to the intercondylar eminence (Figure 3.1); thus, the cortical bone region assessed by µCT corresponds to the histological sampling site evaluated (see below).

Cancellous bone (lumbar vertebra, distal femur, and distal tibia) was analyzed at a threshold of 175, and cortical bone (tibial diaphysis) was analyzed at a threshold of 245. Threshold values were determined empirically based on a gray scale of 0-1000. Cancellous bone measurements included: (1) the ratio of cancellous bone volume to tissue volume (%), (2) connectivity density (an indicator of the connectivity of trabeculae within a given area of bone [257]), (3) structural model index (measures whether trabeculae are more plate-like or rod-like [258]), (4) trabecular thickness (the thickness of individual trabeculae, µm), (5) trabecular number (the number of trabeculae intersected by a computer-generated line drawn through the bone, 1/mm), and (6) trabecular separation (the spacing between trabeculae, µm). Cortical bone measurements included: (1) cross-sectional tissue volume (cortical and marrow volume, mm^3), (2) cortical volume (mm^3), (3) marrow volume (mm^3), (4) cortical thickness (mm), (5) maximum moment of inertia (an indicator of bone mechanical strength in bending, mm^4, I_{max}), (6) minimum moment of inertia (an indicator of bone mechanical strength in bending, mm^4, I_{min}), and (7) the polar moment of inertia (an indicator of bone mechanical strength in torsion, mm^4, I_{polar})

3.5 Quantitative Bone Histomorphometry

Tibial length was measured as the distance from the proximal tip of the intercondylar eminence to the distal tip of the medial malleolus. Cross-sections of the tibial diaphysis (50-µm thick) were cut at 1/3 of the length from distal end of the tibia using an IsoMet® Low Speed Saw (Buehler, Lake Bluff, IL, USA). Sections were

ground on a roughened glass surface (using 220 grit aluminum oxide powder) to an approximate thickness of 25 µm and assessed for (1) bone area (mm^2) and (2) the number of open and closed osteons per bone area (See figure 3.2). Incomplete osteons were defined as open osteons (with or without label) that were visually, obviously much larger than competed (closed) osteons. Fluorochrome-based measurements of bone formation in cortical bone included: (1) the number of labeled osteons/bone area (intracortical osteons that were single or double labeled, see figure 3.2), (2) mineralizing perimeter/bone area (bone perimeter covered with double plus half single fluorochrome label normalized to bone area, %), and (3) mineral apposition rate (the distance between two fluorochrome markers that comprise a double label in an osteon divided by the 14-day interval between label administration, µm/day).

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, USA). Bone area and the number of complete and incomplete osteons were assessed using a 2x objective, and fluorochrome-based measurements were assessed using a 4x objective.

3.6 Serum Measures of Bone Resorption

Serum samples were collected from all monkeys at necropsy, and were assessed for carboxy-terminal collagen crosslinks (CTX) using Serum CrossLaps ELISA (Immunodiagnostic Systems Inc, Fountain Hills, AZ, USA)

3.7 Blood Alcohol Concentrations

Blood samples were collected every 2-5 days from the saphenous vein of monkeys in the alcohol group just before the lights were turned off (between 1800 and 1900 hours). Blood samples were sealed in airtight vials containing 0.5 ml of distilled water and 0.02 mL of 10% isopropanol (internal standard), and stored at $-4^{O}C$ until assayed using gas chromatography (Hewlett-Packard 5890 Series II, Avondale, PA, equipped with a headspace autosampler, flame ionization detector, and a Hewlett Packard 3392A integrator).

3.8 Statistical Measures

All data are expressed as mean ± SE. Data were analyzed with a 2-tailed *t* test using R (R Development Core Team, Vienna, Austria). A *p*-value of ≤ 0.05 was considered significant.

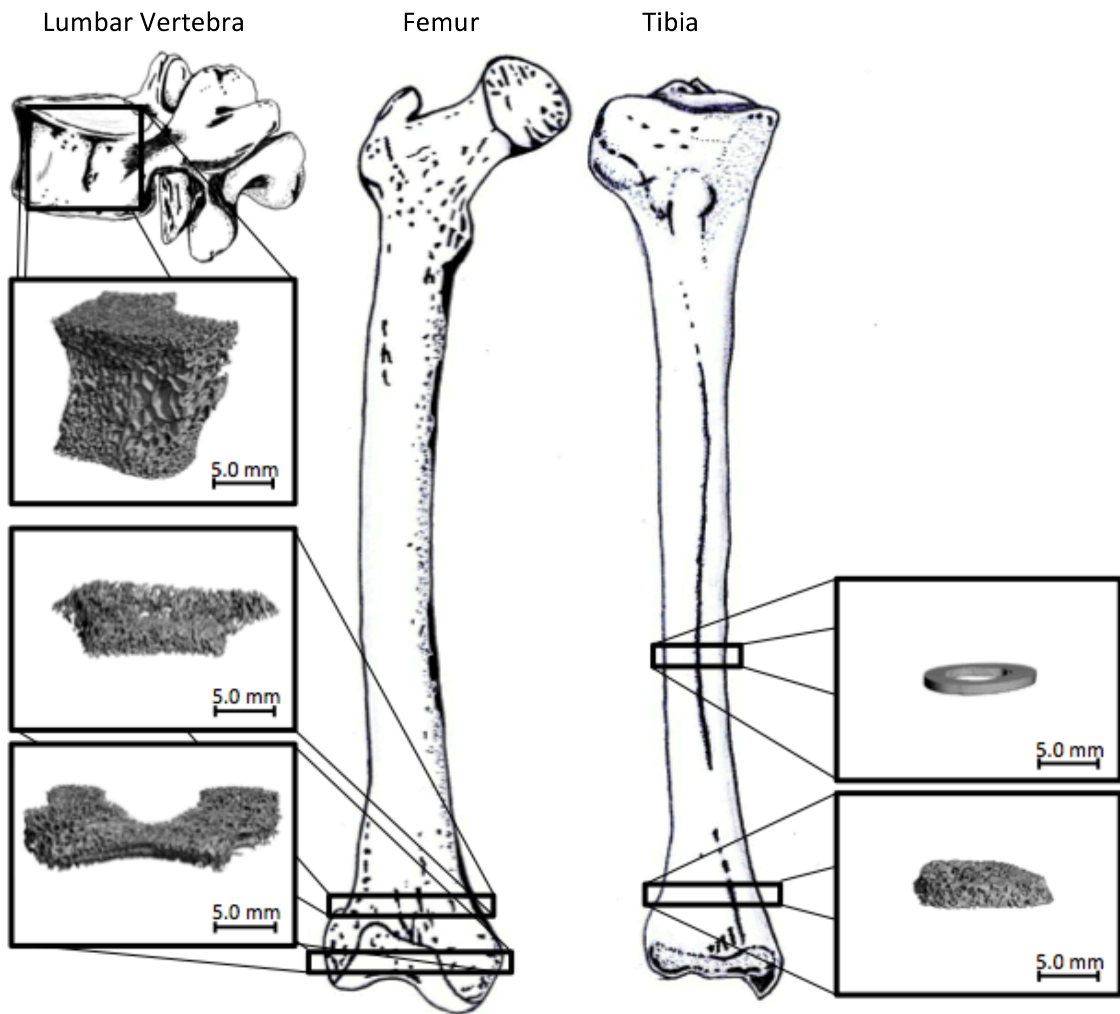

Figure 3.1 Volumes of interest in the lumbar vertebra, femur, and tibia,

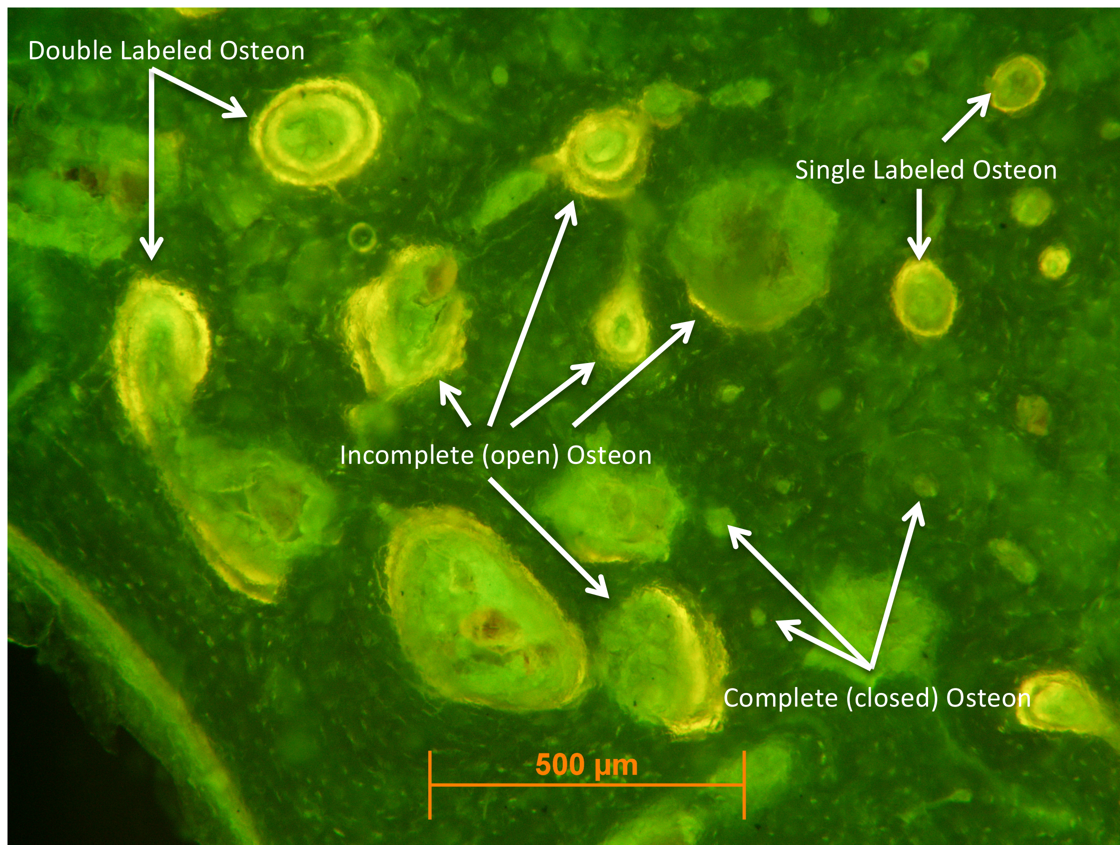

Figure 3.2 Examples of incomplete (open) and complete (closed) osteons.

4- Results

Study population characteristics (age, weight, average alcohol consumption, and average blood alcohol concentration) in control and ethanol groups are shown in Table 4.1. Significant differences were not detected in mean age or in terminal body weight or between control and ethanol groups. Animals in the alcohol group drank an average of 2.8 g ethanol per kg body weight per day over the 12-month duration of the study resulting in blood alcohol concentrations ranging from 20 to 140 mg/dL and averaging 76.7 mg/dL. By design, animals in the control group did not consume any ethanol.

The effects of alcohol consumption on bone mass and bone microarchitecture in the lumbar vertebra are summarized in Table 4.2. Significant differences in lumbar vertebral bone area, BMC, or BMD were not detected between control and alcohol groups. Moreover, no difference was observed for any cancellous microarchitectural parameter measured in the lumbar vertebra (bone volume/tissue volume ratio, connectivity-density, structural model index, trabecular number, trabecular thickness, or trabecular separation).

The effects of alcohol consumption on cancellous bone microarchitecture in the distal femur are summarized in Table 4.3. Significant differences between control and alcohol groups were not detected for any cancellous microarchitectural parameter measured in the distal femur metaphysis or epiphysis (bone volume/tissue volume ratio, connectivity-density, structural model index, trabecular number, trabecular thickness, or trabecular separation).

The effects of alcohol consumption on bone size, bone mass, and bone microarchitecture in the tibia are summarized in Table 4.4. Significant differences between control and alcohol groups were not detected for tibia length or for tibia/fibula bone area, BMC, or BMD. Additionally, significant differences between control and alcohol groups were not observed for any cancellous microarchitectural parameter measured in the distal tibia metaphysis (bone volume/tissue volume ratio, connectivity-density, structural model index, trabecular number, trabecular thickness, or trabecular separation) or in any measured parameters of cortical bone architecture in the tibia

diaphysis (cross-sectional volume, cortical volume, marrow volume, cortical thickness, I_{max}, I_{min}, or I_{polar}).

Quantitative intracortical bone histomorphometry results are shown in Figures 4.1-4.3. The numbers of labeled osteons per bone area were significantly lower in the alcohol group than the control group (Figures 4.1 and 4.2). Additionally, the density of incomplete osteons was significantly lower in alcohol-consuming monkeys relative to controls (Figure 4.2). Significant differences in mineral apposition rate were not detected with treatment (Figure 4.3).

Serum measures of biochemical markers of bone turnover are shown in Figure 4.4. Serum CTX was significantly higher in the alcohol group compared to the control group (Figure 4.4).

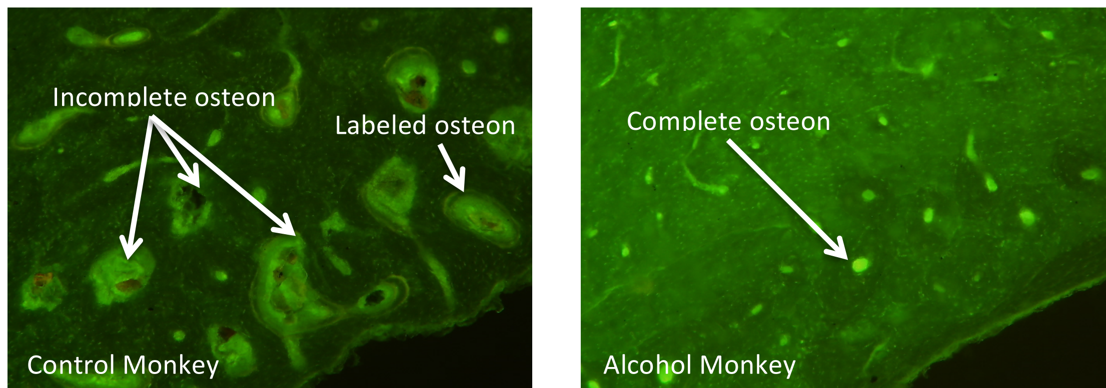

Figure 4.1 Representative histological sections of the tibial diaphysis from control (left) and alcohol (right) animals.

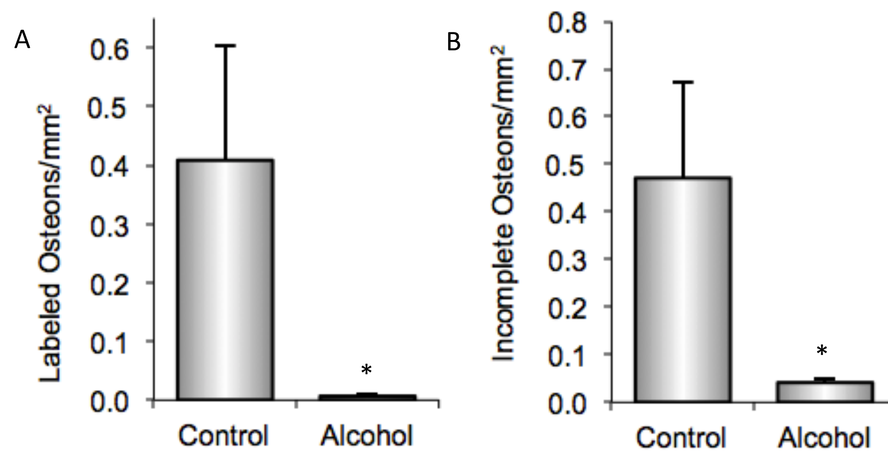

Figure 4.2 (A) Number of labeled osteons per square millimeter of bone in control and alcohol monkeys. (B) Number of incomplete osteons per square millimeter of bone in control and alcohol monkeys. Data are mean ± SE. * $P<0.05$ compared to control.

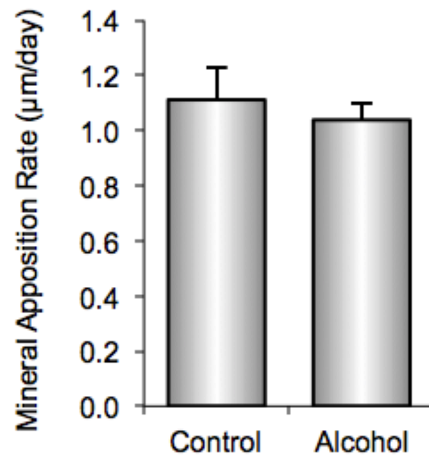

Figure 4.3 Mineral apposition rate in control and alcohol monkeys. Data are mean ± SE.

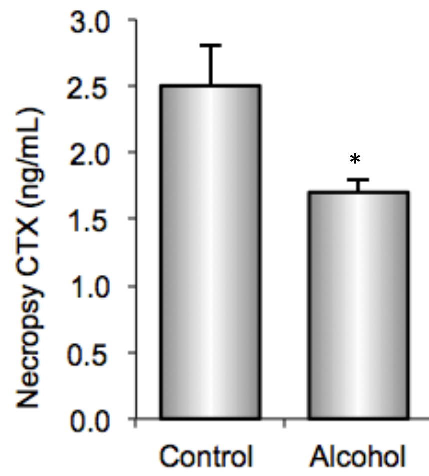

Figure 4.4 Serum CTX levels in alcohol and control monkeys at necropsy. Data are mean ± SE. * P<0.05 compared to control.

Table 4.1 Study Population

Endpoint	Control	Alcohol	*t* test, *p*
Age (years)	6.7 ± 0.3	6.7 ± 0.2	0.95
Body Weight (kg)	8.5 ± 0.5	8.7 ± 0.2	0.64
1st 6 mo avg ethanol intake (g/kg)		2.5 ± 0.2	
2nd 6 mo avg ethanol intake (g/kg)		3.0 ± 0.1	
12 mo avg ethanol intake (g/kg)		2.8 ± 0.1	
1st 6 mo avg BAC (mg/dL)		67.7 ± 7.8	
2nd 6 mo avg BAC (mg/dL)		81.6 ± 9.2	
12 mo avg BAC (mg/dL)		76.7 ± 8.8	

Data are mean ± SE, BAC = Blood Alcohol Concentrations

Table 4.2 Effects of Alcohol on Bone in the Lumbar Vertebrae

Endpoint	Control	Alcohol	*t* test, *p*
Lumbar Vertebra 1-4			
Bone Area (cm^2)	18.8 ± 1.3	17.3 ± 1.3	0.83
BMC (g)	5.11 ± 0.55	5.35 ± 0.50	0.75
BMD (g/cm^2)	1.54 ± 0.13	1.44 ± 0.16	0.82
3rd Lumbar Vertebra (Cancellous Bone)			
Bone Volume/Tissue Volume (%)	21.4 ± 0.6	19.9 ± 1	0.19
Connectivity-density ($1/mm^2$)	9.96 ± 0.68	10.24 ± 0.53	0.74
Structural Model Index	0.98 ± 0.09	1.15 ± 0.08	0.16
Trabecular Number (1/mm)	1.5 ± 0.0	1.5 ± 0.0	0.97
Trabecular Thickness (μm)	148 ± 3	140 ± 4	0.11
Trabecular Separation (μm)	631 ± 17	635 ± 15	0.87

Data are mean ± SE, BMC = Bone Mineral Content, BMD = Bone Mineral Density

Table 4.3 Effects of Alcohol on Bone in the Femur

Endpoint	Control	Alcohol	*t* test, *p*
Distal Femur Epiphysis (cancellous bone)			
Bone Volume/Tissue Volume (%)	29.7 ± 1.2	29.9 ± 1.1	0.95
Connectivity-density (1/mm^2)	7.53 ± 0.91	7.73 ± 1.10	0.89
Structural Model Index	0.09 ± 0.15	0.04 ± 0.12	0.80
Trabecular Number (1/mm)	1.7 ± 0.1	1.7 ± 0.1	0.91
Trabecular Thickness (µm)	193 ± 10	187 ± 6	0.63
Trabecular Separation (µm)	569 ± 34	564 ± 26	0.91
Distal Femur Metaphysis (cancellous bone)			
Bone Volume/Tissue Volume (%)	18.8 ± 1.3	17.3 ± 1.3	0.83
Connectivity-density (1/mm^2)	5.11 ± 0.55	5.35 ± 0.50	0.75
Structural Model Index	1.54 ± 0.13	1.44 ± 0.16	0.82
Trabecular Number (1/mm)	1.4 ± 0.1	1.5 ± 0.1	0.80
Trabecular Thickness (µm)	164 ± 9	160 ± 5	0.65
Trabecular Separation (µm)	710 ± 31	710 ± 32	0.99

Data are mean ± SE

Table 4.4 Effects of Alcohol on Bone in the Tibia

Endpoint	Control	Alcohol	*t* test, *p*
Tibia Length (mm)	168 ± 2	172 ± 1	0.15
Tibia/fibula			
Bone Area (cm^2)	28.1 ± 0.7	27.2 ± 0.5	0.33
BMC (g)	11.0 ± 0.6	10.1 ± 0.4	0.22
BMD (g/cm^2)	0.39 ± 0.01	0.37 ± 0.01	0.21
Tibia Diaphysis (cortical bone)			
Cross-Sectional Volume (mm^3)	78.0 ± 3.2	78.2 ± 2.4	0.96
Cortical Volume (mm^3)	52.5 ± 2.5	51.7 ± 1.3	0.78
Marrow Volume (mm^3)	25.5 ± 1.9	26.5 ± 1.9	0.71
Cortical Thickness (mm)	2.10 ± 0.86	2.07 ± 0.63	0.82
I_{max} (mm^4)	564 ± 55	559 ± 31	0.94
I_{min} (mm^4)	330 ± 23	328 ± 20	0.94
I_{polar} (mm^4)	894 ± 77	887 ± 50	0.94
Distal Tibia Metaphysis (cancellous bone)			
Bone Volume/Tissue Volume (%)	20.7 ± 1.5	20.8 ± 1.4	0.92
Connectivity-density ($1/mm^2$)	6.45 ± 0.64	6.02 ± 0.48	0.60
Structural Model Index	1.13 ± 0.12	1.03 ± 0.12	0.54
Trabecular Number (1/mm)	1.6 ± 0.1	1.5 ± 0.1	0.56
Trabecular Thickness (μm)	163 ± 6	165 ± 6	0.81
Trabecular Separation (μm)	629 ± 35	645 ± 21	0.70

Data are mean ± SE, BMC = Bone Mineral Content, BMD = Bone Mineral Density

5- Discussion

In this study we examined the effects of 12 months of voluntary self-administration of alcohol on bone mass, microarchitecture, and turnover in male rhesus monkeys. To the best of our knowledge, this is the first study to examine the effects of alcohol abuse on indices of intracortical bone remodeling in an animal model. An average consumption of 2.8 ± 0.1 g/kg/d ethanol, resulting in 12-month average blood alcohol concentrations of 76.7±9 mg/dL (0.07%), had no effect on bone mass or bone microarchitecture; however, alcohol consuming animals exhibited markedly lower histological indices of intracortical bone resorption and formation, as well as a significantly lower biochemical marker of global bone resorption. Therefore, our data indicates that 12 months of alcohol abuse significantly depresses intracortical bone remodeling without having significant effects on bone mass or microarchitecture.

Our findings are consistent with the general consensus that alcohol abuse reduces biochemical markers bone formation in humans [58, 71, 73, 74, 90-93]. Even moderate alcohol consumption has been reported to significantly decrease serum indicators of bone formation [65, 67]. However, biochemical indices of bone remodeling are not specific to either cancellous or cortical bone. Histomorphometric analysis of bone biopsies allows for accurate assessment of both cortical and cancellous bone formation. Analyses of human transiliac biopsies have associated alcohol abuse with reduced histological indices of cancellous bone formation [76, 86-89]; however, to the best of our knowledge no study in humans has investigated the effects of alcohol on bone remodeling at any skeletal site other than the iliac crest, and only one study in humans has reported the effects of alcohol on indices of intracortical bone remodeling [89]. In a study by Schnitzler et al (2010), analysis of transiliac biopsies from 16 male alcoholic patients with chronic pancreatitis showed that alcohol abuse was associated with lower endocortical osteoid surface, mineral apposition rate, and bone formation rate and a greater amount of endocortical eroded surface; however, no effects of alcohol were observed on osteonal osteoid thickness or surface, eroded surface, mineral apposition rate, or osteonal bone formation rate [89]. These results are not consistent with the findings of our study. Although we cannot be certain that the inconsistency is not due to species differences, other explanations seem more likely; for example, the influence of the presence of multiple comorbidities including type II

diabetes and chronic pancreatitis in these patients could help explain these differences. Pancreatitis is associated with nutrient malabsorption, low serum vitamin D levels, and alcohol-independent effects on bone mass in humans [259]. The monkeys in the present study did not suffer from notable comorbidities. Moreover, the location of the cortical bone analyzed might explain incongruence between results, as cortical bone at the iliac crest is not subjected to the same amount of mechanical loading as cortical bone at the tibial diaphysis.

Reports regarding the effects of alcohol consumption on bone resorption are less consistent, with studies in human alcoholics reporting lower [86], higher [87, 90, 92, 94], or no difference [76] in resorption. In our study, we observed marked reductions in the number of incomplete osteons in tibial diaphyseal cortical bone of alcohol-consuming animals, suggesting that intracortical bone resorption is significantly decreased with alcohol. This conclusion is supported by our finding of lower serum biomarkers of resorption (CTX) in alcohol-consuming animals. Since bone formation is generally coupled to bone resorption, it is possible that these alcohol-induced reductions in intracortical bone resorption might contribute to the decreased intracortical bone formation observed in our study.

Numerous observational and case-control studies in alcoholics have associated chronic alcohol abuse with lower BMD and BMC [57, 71-78], as well as altered parameters of cancellous bone microarchitecture at the iliac crest [89]. However, in many of these studies subjects had numerous alcoholism-related comorbidities that could have alcohol-independent skeletal effects (such as liver or kidney disease, smoking tobacco, or other comorbidities). Laitinen et al (1993) reported that an average consumption of 186±85 g/d (~13 drinks/d) in women (mean age 38±6 years) without cirrhosis had no effect on BMD [58]. This finding is consistent with our results and suggests that in an otherwise healthy individual, heavy alcohol consumption does not necessarily affect bone mass or bone microarchitecture.

Rodent models of chronic alcohol abuse have also associated heavy alcohol consumption with reduced bone mass [96-98, 101, 103, 104, 136, 153] and abnormal cortical and cancellous bone microarchitecture [96, 97, 103, 140]. However, most

studies in rodents have been done in rapidly growing, skeletally immature animals. The monkeys in this study are at or near skeletal maturity, thus they model young adult humans. Hence, the negative effects of alcohol administration on bone mass and architecture observed in studies in rodents occur at least in part through different physiological processes (reduced gain of bone rather than loss of bone). Skeletally mature rats also exhibit lower bone mass and altered bone architecture with administration of alcohol-containing liquid diets compared to animals consuming liquid diets *ad libitum* [100, 135, 144]. However, when skeletally mature control rats are pair-fed to alcohol-consuming rats, differences in bone mass and microarchitecture between alcohol and control rats are much smaller compared to rats that are *ad libitum* fed [144], and significant differences do not appear until after a longer duration of time [144]. Moreover, even when control rats are pair fed, animals in the alcohol group sometimes have smaller body weights compared to controls [135, 144]. In the present study, no significant difference was observed in the body weight of monkeys in the control group compared to the alcohol group.

The cellular and molecular mechanisms underlying the effects of alcohol on bone are not fully understood, and it is likely that alcohol acts on bone through the combination of a number of mechanisms, both direct and indirect (see Maurel et al (2012) for a more in depth review of the mechanisms of alcohol on bone [260]). There is evidence from cell culture studies that high concentrations of ethanol can directly affect osteoblasts, osteoclasts, and osteocytes. Furthermore, alcohol has been shown to act as an endocrine disruptor, altering levels of and skeletal response to sex hormones (e.g. estrogen and testosterone), vitamin D, and parathyroid hormone, as well as influencing the skeletal effects of pituitary (e.g. growth hormone) and adipocyte (e.g. leptin) derived hormones. Given the striking effects of alcohol on intracortical bone remodeling observed in the present study, further investigation into the cellular mechanisms of these effects is warranted in future studies.

Our study has several limitations of note. First, our study design lacks the ability to determine a molecular mechanism through which alcohol may reduce bone turnover. Second, although reduced bone remodeling during the period when animals are reaching skeletal maturity would suggest reduced bone quality (and possible impaired

peak bone mass accrual), which would indicate increased fracture risk, our study did not directly investigate the effects of alcohol abuse on the mechanical properties of bone. Our study does however have several notable strengths. The pattern of alcohol abuse exhibited by the monkeys in this study recapitulates patterns of alcohol abuse in humans. As reported in cynomolgus monkeys under the same alcohol treatment protocol [255], given 22 h/d open access to ethanol (4% w/v) and water, monkeys frequently engaged in episodes of spree (or binge) drinking during which the animals would "gulp" rather than "sip" alcohol. Also, blood alcohol concentrations were highly variable within each animal, with intra-individual variations of 0 to 300 mg/dL on different days (data not shown). The patterns of alcohol consumption and blood alcohol concentrations in the monkeys within this study are remarkably similar to drinking patterns and blood alcohol concentrations of alcoholic men given 24 h/d free access to ethanol for 10-60 consecutive days in a hospital ward [261-263]. Finally, our study allows for the assessment of the effects of alcohol on intracortical bone remodeling in a weight bearing skeletal site.

6- Conclusions

The results of the present study indicate that 12 months of voluntary alcohol abuse in male rhesus monkeys does not have significant effects on bone mass or bone microarchitecture; however, this pattern and duration of alcohol abuse did result in significantly reduced intracortical bone turnover. Reduced bone turnover could contribute to deficient repair of microfractures accrued during activities of daily living and lead to reduced bone mechanical properties independent of bone mass or microarchitecture, which could contribute to increased bone fracture risk. Further studies are required to evaluate the effects of alcohol on bone quality in cortical bone.

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