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Detrimental Effects of Alcohol on Bone Growth

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1. Introduction

Heavy drinking during adolescence may have immediate as well as long-term detrimental consequences to bone health. The growing skeleton is especially prone to fracture and alcohol may exacerbate fracture risk. Furthermore, a disproportionate amount of peak bone mass is acquired during adolescence. Alcohol, by decreasing bone formation, may decrease peak bone mass, predisposing the skeleton to early onset osteoporosis. Although it is well known that heavy drinking can have detrimental skeletal effects in adults (Turner 2000), few studies have focused specifically on the skeletal consequences of underage drinking in human subjects, in part, due to the difficulty in performing alcohol intervention studies in underage drinkers. As a result, the significance of alcohol consumption during this interval of rapid bone accretion on skeletal health is largely unknown. Thus, relevant animal models are critical for identifying the effects and mechanisms of action of alcohol on bone metabolism during bone growth. This chapter will focus on the detrimental effects of alcohol on the maturing skeleton using the laboratory rat as a model. We will also present evidence that these effects are mediated, at least in part, by alcohol-induced alterations in energy homeostasis.

2. Underage drinking

2.1 Magnitude of problem

Underage alcohol consumption is a major public health concern, especially in industrialized nations. The 2009 National Survey on Drug Use and Health reported that 10.4 million Americans between the ages of 12 and 17 had consumed alcohol during the month preceding the survey. Nearly 7 million of these teens reported engaging in binge drinking (5+ drinks on the same occasion) and 2.1 million classified themselves as heavy drinkers (5+ drinks per occasion on more than 5 days within the last month) (Department of Health and Human Services 2010). Rates of alcohol use, including binge and heavy drinking, have declined slightly since 2002; however alcohol consumption still occurs regularly in over 27% of American teenagers (Department of Health and Human Services 2010). High rates of alcohol consumption in youth were also reported in a 2008 survey of Australian secondary school students (White & Smith 2009). The European School Survey Project on Alcohol and Other Drugs, a survey of adolescent students in 35 European countries, reports even higher rates of alcohol use for European adolescents. The 2007 survey reported that within the month preceding the survey, 61% of students drank, 43% drank heavily, and 18% had been intoxicated (Hibell et al. 2009). However, none of these surveys provide insight regarding...
the effects of underage drinking on bone growth or, if an injury were to occur, on bone repair following the injury. Globally, risky alcohol use among adolescents is on the rise (World Health Organization 2011). Although most under-age drinkers do not become alcohol-dependent, they are at increased risk for a variety of injuries and disorders. Liver cirrhosis, epilepsy, various forms of cancer, cardiovascular disease, and diabetes are just a few of the disorders that have been causally linked to alcohol consumption (Department of Health and Human Services 2010). Furthermore, alcohol use increases the chances of both intentional and unintentional injuries to bone due to violence and accidents.

2.2 Bone growth and maturation during adolescence

The attainment of peak bone mass occurs sometime during the third decade of life (Recker et al. 1992; Lin et al. 2003) but adolescence is a key time period in determining peak bone mass. Bone accrued during the 2 years surrounding the pubertal growth spurt accounts for approximately 25% of peak bone mass (Kontulainen et al. 2007). Roughly 90% of bone mass is achieved by late adolescence (Henry et al. 2004; Whiting et al. 2004). Modifiable factors such as diet are important determinants of peak bone mass (Eisman 1999; Bergmann et al. 2010; Ohlsson et al. 2011) and these effects may be compounded during the pubertal growth spurt. The introduction of factors inhibiting bone accrual during adolescence could lower peak bone mass and lead to decreased bone strength. A low peak bone mass, combined with age-related bone loss, has been shown to increase the likelihood of early onset osteoporosis, and the associated risk of fracture (Cooper et al. 2006; Xu et al. 2011). Reducing fracture risk in elderly osteoporotic populations is important, but 75% of the 6.8 million fractures occurring annually in the United States are not caused by osteoporosis. In fact, the group that accounts for the highest overall fracture rate is adolescent males (Goulding 2007). Heavy alcohol consumption may contribute to the high rate of fractures in this group.

3. Effects of alcohol on bone metabolism in growing animals

3.1 Animal models for investigating the effects and underlying mechanisms of action of alcohol on the maturing skeleton

Due to size and cost considerations, rats and mice are generally the preferred animals for investigating the actions of alcohol on bone metabolism. The reader is directed to our review of the strengths and weaknesses of rodents as animal models for osteoporosis (Iwaniec & Turner 2008). In brief, rodents are similar to humans in that bone grows by a combination of endochondral ossification and periosteal bone formation. Similarly, following the pubertal growth spurt, endochondral ossification slows in magnitude and ultimately ceases (Martin et al. 2003), while periosteal bone formation continues at a slow rate throughout the remainder of life. Humans, rats and mice undergo age-related bone loss, but it is unclear whether the mechanisms for the bone loss are the same across species. Once formed, bone in humans is continuously remodeled. By repairing fatigue damage to bone, bone remodeling serves to maintain bone quality. Bone remodeling in rats is largely limited to endocortical and cancellous bone surfaces. Mice have very high rates of cancellous bone turnover but it is uncertain whether the close temporal and spatial integration of bone formation and resorption that characterizes bone remodeling in humans and rats occurs in mice. Haversion remodeling, the process by which cortical bone is remodeled in humans, is generally absent in small animals such as rats and mice. In spite of
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differences from humans, rodents, especially rats, have proven extremely valuable as preclinical animal models for osteoporosis. Regarding alcohol, not only has the rat accurately modeled the skeletal effects of chronic alcohol abuse in adults, some of the changes in bone and mineral homeostasis originally reported in the rat were subsequently shown to occur in human alcoholics (Turner et al. 1987; Turner et al. 1988).

There is no single pattern of alcohol consumption by underage drinkers. Drinking patterns range from occasional to regular, to binge. Based on dose-response and time-course studies in rats, the effects of alcohol on bone metabolism depend upon peak blood alcohol concentration and duration of exposure (Turner et al. 1998; Turner et al. 2001). As a consequence, no single animal model can replicate all of the actions of alcohol.

To model chronic alcohol consumption, alcohol can be delivered to animals in drinking water, as a component of a liquid diet or as a component of total intragastric nutrition (Lieber et al. 1989; French 2001). Addition of alcohol to drinking water is the simplest method but has significant disadvantages. Because of aversion to alcohol, high concentrations decrease fluid intake which may result in dehydration (Lieber et al. 1989). In addition, it is difficult to equalize macro and micronutrient levels among treatment groups. In particular, the controls receive all of their energy and nutrients from a standard rodent chow diet. This contrasts with the alcohol fed animals who receive their energy from both alcohol and diet, and other nutrients from the chow diet only. Lieber and colleagues, recognizing the limitations of delivering alcohol in drinking water, developed a liquid diet in which alcohol replaced carbohydrates isocalorically (Lieber et al. 1989). We have found this diet to be very useful for investigating the effects of alcohol on bone metabolism.

Total intragastric nutrition, while invasive and very labor intensive, is an alternative method which allows even better control of total nutrition. This method provides the investigator with complete control of the duration of exposure to alcohol. Thus, total intragastric nutrition is especially beneficial for delivering very high amounts of alcohol to induce a specific pathological response. However, because of exquisite sensitivity of the rodent skeleton to the metabolic effects of alcohol consumption, it is rarely necessary to employ alcohol levels that are high enough to require intragastric delivery.

Binge drinking is typically modeled by oral gavage or by intraperitoneal injection. Alcohol can also be delivered by intermittent intragastric infusion. We have experience using the first two methods. Although intraperitoneal injection is a convenient method to deliver alcohol for short duration studies (Turner et al. 1998), we have found that intraperitoneal injection of alcohol over multiple days does not reproduce the response obtained following oral administration. Daily delivery of alcohol (~1.2 g/kg) by either gavage or intraperitoneal injection resulted in a peak blood alcohol level of ~0.1 %. This dose rate had no significant effects on body weight gain, uterine weight or bone parameters in animals where alcohol was delivered by gavage (unpublished data). In contrast, alcohol delivered by intraperitoneal injection injection resulted in decreased cortical bone mass and drastic reductions in bone formation and mRNA levels for bone matrix proteins (Turner et al. 1998). We conclude from this and similar experiments that multiple intraperitoneal injections have severe effects on the skeleton that do not model the normal physiological response to alcohol.

Consistent with our data, Sampson et al. reported no detrimental effects on bone in growing rats using a model for binge drinking in which alcohol was administered by gavage on two consecutive days a week (Sampson et al. 1999). Based on the observation that longer exposure to relatively low blood levels of alcohol has greater effects on bone metabolism
than brief exposure to high blood levels of alcohol, we have focused on skeletal response of growing rats to chronic alcohol consumption.

3.2 Effects of alcohol on the skeleton in growing rats
Long duration studies in growing rats have shown that chronic alcohol consumption decreases peak bone mass. For example, administration of alcohol (38% caloric intake) to post-pubertal Long Evans hooded rats for 10 months resulted in a decrease in tibia length, an increase in the size of the marrow cavity and a decrease in cancellous bone mass (Turner et al. 1988). The latter is important because reduced cancellous bone mass plays a key role in the etiology of osteoporotic fractures. Studies designed to evaluate bone growth have shown that alcohol inhibits the rate of bone elongation as well as addition of bone onto periosteal and endocortical endocortical surfaces of rapidly growing male rats (Figure 1) (Turner et al. 1987). These reductions in bone growth contribute to a decrease in bone mass. Similar changes were observed by Sampson and colleagues in growing female Sprague Dawley rats fed alcohol (Sampson et al. 1996; Hogan et al. 1997; Sampson et al. 1997; Sampson & Spears 1999).

Fig. 1. Representative microcomputed tomography images of tibiae from rats fed control or alcohol diets. In this study, 4-week-old male rats were fed a liquid diet containing alcohol for 4 months. Except for isocaloric replacement of ethanol with maltose dextran, the controls were fed the same diet ad libitum. Chronic alcohol consumption (35% caloric intake) during post pubertal growth reduced peak bone mass as illustrated above for the tibia.

Bone formation is the product of osteoblast number and osteoblast activity. In growing rats, high levels of alcohol consumption were consistently found to decrease the extent of bone surface covered by active osteoblasts. The effect of alcohol on indices of osteoblast activity is less consistent, ranging from no effect to a moderate decrease. Alcohol results in a dose-
associated decrease in osteoclast-lined bone surface (Turner et al. 2001). Thus, in addition to inhibiting bone formation, alcohol appears to inhibit bone resorption. Typical of low bone turnover forms of osteoporosis, bone loss in alcohol-fed rats is relatively slow (Hogan et al. 2001; Turner et al. 2001), a finding consistent with the slow rate of bone loss observed in adult chronic alcohol abusers (Odvina et al. 1995; Pumarino et al. 1996).

Chronic consumption of high levels of alcohol during growth reduces peak bone mass by inhibiting bone acquisition. However, there is conflicting evidence as to whether alcohol also impacts the extent of mineralization of bone matrix. Some, but not all, studies suggest that heavy drinking results in under-mineralization of bone matrix (Schnitzler & Solomon 1984; Turner et al. 1987; Diamond et al. 1989; Bikle et al. 1993; Schnitzler et al. 1994). To investigate this issue more fully, we determined the effects of alcohol consumption on bone formed following osteoinduction by demineralized bone matrix. In this model, ectopic bone is induced to form at extraskeletal sites in an animal by subcutaneous implantation of demineralized bone matrix. Used clinically in orthopedic practice to augment bone formation during fracture repair, osteoinduction is an ideal method to investigate the effect of alcohol on mineralization because experiments can be designed in which bone is not present until introduction of alcohol into the diet. In our studies, described elsewhere in detail (Trevisiol et al. 2007), subcutaneously implanted demineralized allogeneic bone matrix cylinders were used to model osteoinduction. Demineralized allogeneic bone matrix cylinders, prepared from femurs and tibiae of rats fed a normal diet, were implanted into sexually mature male rats adapted to alcohol (ethanol contributed 35% of caloric intake) or control liquid diets. Food intake in the control rats was restricted to match food intake of alcohol-fed animals. The implants were recovered 6 weeks later and analyzed by histology, microcomputed tomography and chemical analysis. Histological evaluation revealed a robust osteoinductive response, resulting in mature bone formation, in implants in rats fed the control diet. Alcohol consumption affected architecture of the implants but not volumetric density or mineral composition. Specifically, alcohol consumption resulted in significant decreases in demineralized allogeneic bone matrix-induced bone volume, bone volume/mg original cylinder weight, connectivity density, trabecular number and thickness, ash weight and % ash weight. There were, however, no changes in mineral (ash) density nor in the relative amounts of calcium, magnesium, iron, selenium and zinc (µg/mg ash), indicating that alcohol consumption reduced the amount of new bone formation but did not reduce mineral content of bone.

Osteoinduction is a key component in fracture repair. The decrease in osteoinduction observed in the rats described above and in subsequent studies (Iwaniec et al. 2008) suggest that alcohol may impair fracture healing. Chronic exposure to dietary alcohol inhibits healing in a variety of models involving injury to bone (Chakkalakal et al. 2005; Wahl et al. 2005). However, it is not clear whether alcohol consumption has a clinically relevant effect on fracture healing in either humans or animal models. Addressing this important question should be a priority of future animal and human research.

4. Alcohol metabolism

It is not known whether the detrimental skeletal effects of alcohol on bone metabolism are due to the parent compound or a metabolite. The metabolism of ethanol occurs predominantly in the liver where ethanol is metabolized to acetaldehyde, a highly toxic metabolite, which in turn is rapidly metabolized to acetate. In addition to being released into
circulation from the liver, it is conceivable that acetaldehyde is produced in skeletal tissue. Such a mechanism could lead to local levels of acetaldehyde that exceed circulating levels. However, based on the inability of cultured osteoblasts to reduce ethanol levels in culture media, it seems unlikely that osteoblasts produce acetaldehyde (Maran et al. 2001). This observation does not preclude the possibility that other cells within the local skeletal environment metabolize alcohol or that circulating levels of acetaldehyde are sufficient to have direct actions on bone cells. Further investigation is required to establish the contribution of acetaldehyde to the detrimental effects of alcohol on bone.

5. Does consuming alcohol have irreversible toxic effects on bone cells?

The question as to whether alcohol leads to irreversible toxic effects on the skeleton is important but has not been fully resolved. Alcohol consumption results in a dose-dependent decrease in bone formation that is paralleled by reductions in osteoblast-lined bone surface and osteoblast precursor pool in bone marrow (Dyer et al. 1998; Rosa et al. 2008). Reduced bone formation is preceded by lower mRNA levels for bone matrix proteins (Turner et al. 2001). These and similar data are often interpreted as evidence of toxicity. In support of toxicity, there is incomplete catch-up growth following cessation of alcohol feeding in growing rats (Sampson & Spears 1999). Similarly, bone mass does not return to normal when alcohol feeding is discontinued in skeletally mature rats (Sibonga et al. 2007). Taken together, these findings suggest that alcohol has irreversible toxic effects on the osteoblast. This conclusion appears to be supported by in vitro studies reporting that direct exposure to alcohol decreases proliferation of cultured osteoblasts and inhibits their synthesis of bone matrix proteins (Giuliani et al. 1999; Vignesh et al. 2006). However, the concentrations of alcohol necessary to achieve the detrimental effects in cell culture described above are generally very high, suggesting that mature osteoblasts are quite resistant to direct toxic effects of alcohol (Maran et al. 2001). In this regard, no direct toxicity was detected in dose-response studies performed on cultured human osteoblasts. Specifically, concentrations of ethanol that would be incompatible with human life had no effect on osteoblast number, proliferation or expression of genes for bone matrix proteins.

In contrast to the high concentrations of ethanol (50mM or greater) required to have direct inhibitory effects on osteoblasts in cell culture, much lower levels of alcohol reduce bone formation in vivo. This finding suggests that the inhibitory effects of alcohol on bone formation are primarily indirect (Turner et al. 2001). To further evaluate whether alcohol has irreversible indirect toxic effects on osteoblasts, we performed studies in which we administered parathyroid hormone to rats that had been fed alcohol (Turner et al. 2001). The bone anabolic effects of intermittent parathyroid hormone have been studied in humans and laboratory animals and currently, parathyroid hormone is the only bone anabolic therapy approved by the Federal Drug Administration for the treatment of postmenopausal osteoporosis. Parathyroid hormone is effective in increasing bone mass in most but not all subjects. Thus, lifestyle factors such as alcohol consumption, may inhibit the skeletal response to parathyroid hormone. However, studies to date in rats suggest that parathyroid hormone and alcohol have opposite but independent effects on bone formation. In other words, alcohol lowered the basal rate of bone formation compared to animals fed a normal diet and parathyroid hormone increased bone formation by the same magnitude in animals fed normal and alcohol diets (Turner et al. 2001; Sibonga et al. 2007; Iwaniec et al. 2008;
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Howe et al. 2011). Additionally, administration of parathyroid hormone reversed bone loss in alcohol-fed rats (Sibonga et al. 2007). Taken together, these findings do not support an irreversible toxic effect of alcohol on bone cells. Indeed, they suggest that bone formation returns to normal following removal of alcohol from the diet, but a pharmacological intervention may be required to restore the bone that had been lost.

6. Mechanisms of action of alcohol on the growing skeleton

6.1 Alcohol is an endocrine disruptor

Bone metabolism is under tight endocrine control and it is well established that excessive alcohol consumption disrupts numerous endocrine functions. For example, alcohol consumption has been reported to alter the levels and skeletal responses to estrogen, vitamin D and parathyroid hormone (Dumitrescu & Shields 2005; Ronis et al. 2007; Sibonga et al. 2007). Each of these hormones play a key role in bone metabolism. As previously reviewed (Turner 2000; Turner & Sibonga 2001), disturbances in signaling by these hormones may contribute to the skeletal response to alcohol in adults. Less investigated, however, are the effects of alcohol on pituitary- (e.g., growth hormone) and adipocyte-derived (e.g., leptin) hormones. Disruption of signalling of hormones that function to integrate growth and energy metabolism by alcohol has not been intensively studied, but may be especially important to the effects of underage drinking on bone growth and maturation. As discussed below, alcohol alters local production and/or circulating levels of bone regulating hormones, proinflammatory cytokines (TNF-α) and adipokines related to energy intake and expenditure. In addition, there is evidence that alcohol results in end-organ resistance to two of the key mediators of energy homeostasis, growth hormone and leptin.

6.2 Impact of alcohol on energy metabolism

6.2.1 Energy metabolism

Bone growth during adolescence is tightly coupled to energy availability (Devlin et al. 2010). Regulation of energy metabolism involves the integration of signals from the digestive system, pancreas, liver, adipose tissue, hypothalamus and pituitary. The messengers that signal energy status and induce physiological adaptations consist of hormones, adipokines, cytokines, growth factors and neuronal networks. Alcohol consumption influences food intake and energy balance by altering the production and target organ response to these signals (Leibowitz 2005; Pravdova & Fickova 2006). As a consequence, we hypothesize that alcohol disrupts the tight coupling between energy availability and bone growth, maturation and turnover.

6.2.2 Effect of alcohol on energy intake

Alcohol has profound, dose-dependent effects on energy intake. Low concentrations of alcohol in the diet (0.5% and 3% caloric intake) were shown to enhance food consumption in rats (Turner et al. 2001; Turner & Iwaniec 2010). In contrast, higher alcohol concentrations generally suppress energy intake. Heavy alcohol consumption reduces bone formation in growing rats compared to pair-fed controls. However, pair-feeding underestimates the detrimental skeletal effects of alcohol consumption because self-selected caloric restriction in alcohol-fed rats also has detrimental effects on bone homeostasis (Maddalozzo et al. 2009).
6.2.3 Effect of alcohol on energy expenditure

Total energy expenditure reflects the sum of basal metabolic rate and energy consumed performing physical activity. The effect of alcohol consumption on total energy expenditure appears to be context dependent. In short-duration studies, pair-feeding control rats to animals fed a diet containing alcohol usually equalizes weight gain among treatment groups, but not necessarily body composition (see below). Also, in a 10-month-long study, slightly more energy was required to achieve the same weight gain in rats fed a diet containing ethanol (38% caloric intake) than the rats fed the control diet (Turner et al. 1988). In a 4-month-long study investigating the skeletal response to physical activity, treadmill-exercised rats fed a diet in which alcohol contributed 35% of caloric intake gained weight in parallel with exercised animals fed the control diet. Furthermore, exercise decreased weight gain compared to pair-fed non-exercising controls. Thus alcohol did not influence the increased energy requirements associated with a higher rate of physical activity. Overall, these findings suggest that alcohol does not have a major influence on overall energy expenditure.

There are, however, situations where alcohol consumption does influence energy metabolism. Specifically, changes in hormonal regulators of energy homeostasis may alter the relationship between alcohol consumption and energy expenditure. For example, estrogen acts physiologically to reduce energy intake and increases expenditure. In ovariectomized rats, estrogen deficiency results in increased weight gain which is due to a combination of hyperphagia and reduced energy expenditure. Similar to estrogen, alcohol increased energy expenditure in ovariectomized rats. As a consequence, pair-fed ovariectomized rats consuming a control diet gain more weight than animals fed the alcohol containing diet (Kidder & Turner 1998).

6.2.4 Body composition

Body composition was altered in sexually mature male rats fed alcohol (35% caloric intake) for 3 months. The alcohol-fed animals had less peripheral fat and a lower whole body bone mineral content compared to age-matched controls (Maddalozzo et al. 2009). In spite of an overall reduction in fat mass, bone marrow adiposity was increased in the rats fed alcohol (Maddalozzo et al. 2009). Similar to rodents, reduced peripheral fat and increased bone marrow adiposity is associated with chronic alcohol abuse in men (Liangpunsakul et al. 2010). In contrast, in a recent study investigating the role of estrogen in the skeletal response to alcohol we noted that heavy (35% caloric intake) alcohol consumption in slowly growing ovariectomized rats reduced overall body weight gain but increased white adipose tissue mass. Thus, there may be gender differences in the effects of alcohol on energy homeostasis. The role of peripheral and bone marrow fat in regulation of bone metabolism appears complex and has generated a great deal of interest in recent years. As discussed below, alcohol-induced changes in peripheral and bone marrow fat depots may play an indirect but nevertheless important role in mediating the detrimental effects of alcohol consumption on the adolescent skeleton.

7. Alcohol disrupts the actions of key hormones that regulate energy metabolism

7.1 Leptin signaling is required for normal bone growth

As mentioned above, skeletal growth is tightly coupled to energy balance via complex and incompletely understood mechanisms. Leptin, the protein product of the ob gene, is
produced by adipocytes and functions as a messenger in a feedback loop between adipose tissue and the hypothalamus. As such, leptin contributes to the regulation of energy intake and expenditure (Figure 2). Leptin also acts on other organs, including bone. Leptin-deficient \textit{ob/ob} mice are morbidly obese and develop multiple pathologies associated with metabolic syndrome. Additionally, \textit{ob/ob} mice have notable skeletal abnormalities. Initially, the \textit{ob/ob} mouse was described as having high bone mass, but subsequent studies revealed a complex skeletal phenotype; compared to wild type (WT) mice, \textit{ob/ob} mice have a low total and cortical bone but increased vertebral cancellous bone (Ducy et al. 2000; Bartell et al. 2011). The decrease in cortical bone in \textit{ob/ob} mice is in part due to a decrease in bone length.

![Fig. 2. Regulation of energy metabolism by leptin. Adipose tissue-derived leptin acts on the hypothalamus to increase energy expenditure and decrease appetite. These metabolic changes contribute to a negative feedback loop antagonizing further fat accumulation. Chronic alcohol consumption disrupts energy homeostasis by causing hypophagia, increased energy expenditure, and hypoleptinemia. Peripheral (serum) leptin functions physiologically to couple systemic growth (including bone growth) to energy availability. We hypothesize that chronic alcohol consumption during adolescence, by decreasing serum leptin levels, reduces bone growth which in turn contributes to the reduced peak bone mass observed in growing rats fed a diet containing alcohol.]

To evaluate the effect of hypothalamic signaling as a mediator of the skeletal response to leptin, we performed a study in which growing \textit{ob/ob} mice were injected in the hypothalamus with either adeno-associated virus-leptin (rAAV-lep) or a control vector coding for green fluorescent protein (rAAV-GFP). Treatment with rAAV-lep restored the \textit{ob/ob} skeletal phenotype to WT by increasing femoral length and total bone volume, and decreasing femoral and vertebral cancellous bone volume. As a consequence, at 15 weeks post-rAAV-lep injection the \textit{ob/ob} mice no longer differed from WT mice (Iwaniec et al. 2007). In recent unpublished studies we have shown that daily subcutaneous administration of leptin increases the longitudinal rate of bone growth in \textit{ob/ob} mice as well as bone formation on cortical and cancellous bone surfaces. Taken together, these results suggest that leptin functions as an essential factor for normal bone growth and turnover.
7.2 Reduced leptin signaling as a mechanism for the detrimental effects of alcohol on bone growth

Alcohol alters serum leptin levels in humans and animals (Nicolas et al. 2001; Santolaria et al. 2003; Calissendorff et al. 2004; Otaka et al. 2007; Maddalozzo et al. 2009). Decreased leptin levels are often associated with chronic alcohol consumption in humans, even after accounting for alcohol-induced reductions in fat mass (Nicolas et al. 2001; Calissendorff et al. 2004; Otaka et al. 2007). However, the change in leptin levels depends upon the level and pattern of alcohol consumption. In our model of chronic adolescent drinking, there is a substantial decrease in serum leptin levels (Figure 3). Additionally, there are studies suggesting that alcohol impairs leptin signaling by inducing target organ resistance to the hormone (Gordeladze et al. 2002).

Fig. 3. Evidence for decreased leptin levels in serum of alcohol-fed rats compared to ad libium-fed (Ad lib) controls. Four-week-old male rats were fed alcohol containing (35%caloric intake) or control diets for 3 months. Data are mean ± SE, *P<0.05.

Leptin, in addition to having central actions mediated through the hypothalamus, has the potential to act directly on target organs, including bone (Burguera et al. 2001; Reseland et al. 2001; Gordeladze et al. 2002; Thomas 2004). The putative hypothalamic-mediated and direct pathways of leptin action on bone metabolism have been reviewed by Hamrick (Hamrick et al. 2004; Hamrick & Ferrari 2008). By transplanting bone marrow cells from leptin receptor-deficient db/db mice into WT mice, we have shown that the physiological actions of leptin on bone turnover are primarily due to peripheral leptin signaling (Unpublished data).

Leptin deficiency results in skeletal abnormalities that, in many ways, are similar to effects of chronic alcohol abuse. Specifically, leptin deficiency and alcohol consumption in growing rodents each result in decreases in longitudinal bone growth, radial bone growth, and cancellous bone turnover (Turner 2000; Iwaniec et al. 2007). Also, leptin deficiency and alcohol consumption in growing rodents each result in elevated bone marrow adiposity (Steppan et al. 2000; Hamrick et al. 2004; Hamrick et al. 2005; Hamrick & Ferrari 2008). Thus, one mechanism by which alcohol consumption may decrease bone acquisition during adolescence is by reducing leptin levels. Further studies are required to determine whether
normalization of leptin levels in alcohol-fed growing rats corrects the detrimental effects of alcohol on bone growth, architecture and turnover.

8. Osteoblasts and adipocytes

As discussed, we have shown that alcohol consumption increases bone marrow adiposity and decreases both bone formation and peak bone mass in a rat model for chronic alcohol abuse (Maddalozzo et al. 2009). Adipocytes and osteoblasts are derived from bone marrow mesenchymal stromal cells (Vaaninen 2005; Gimble et al. 2006) (Figure 4). An inverse association between bone mass and bone marrow adiposity is commonly observed (Pei & Tontonoz 2004; Morita et al. 2006) and, although yet to be firmly established, several lines of evidence suggest that there is a cause and effect relationship. A deficiency in PPAR\(\gamma\), a key mediator of adipocyte differentiation, reduced fat and enhanced osteogenesis (Akune et al. 2004), suggesting that suppression of adipogenesis leads to increased bone formation. Based primarily on cell culture studies, some investigators have concluded that increased adipocyte differentiation inevitably occurs at the expense of osteoblast differentiation. If correct, the increase in bone marrow fat in alcohol-fed rats may play a causative role in the decrease in bone formation. In support of this idea, alcohol increased PPAR\(\gamma\) expression, increased adipocyte differentiation and decreased osteoblast differentiation in an immortalized mesenchymal stem cell line (Wezeman & Gong 2004). It should be mentioned, however, that a close inverse association between bone marrow fat and bone formation is not always apparent (Menagh et al. 2010; Turner & Iwaniec 2011). This has led us to suggest that changes in osteoblast differentiation are not inevitably coupled to changes in adipocyte differentiation. Instead, we have proposed that some regulatory factors have opposite effects on osteoblast and adipocyte differentiation but others have actions that are limited to one or the other cell lineage.

Fig. 4. Osteoblasts and adipocytes are derived from bone marrow stromal cells. They produce factors that act locally to influence bone growth and turnover. Whereas leptin and IGF-I enhance bone formation, adiponectin and TNF-\(\alpha\) inhibit osteoblast differentiation.

There is an alternative, non-mutually exclusive mechanism by which peripheral and/or bone marrow fat can influence bone metabolism. More than 50 adipocyte-derived adipokines, growth factors, and proinflammatory cytokines have been identified. Several of these factors, including leptin, adiponectin, tumor necrosis factor-alpha (TNF-\(\alpha\)) and insulin-like growth factor-I (IGF-I) are known to have direct effects on bone cells. Adipocytes produce IGF-I in
response to growth hormone and are reported to be an important source of systemic IGF-I (Vikman et al. 1991). Although bone marrow adipocytes produce a spectrum of factors with differing effects on bone formation, most investigators believe that the factors which inhibit bone formation generally predominate. This belief is based on the observation that increased marrow fat is typically associated with osteopenia. If this interpretation is correct, chronic consumption of alcohol would tend to perpetuate a continued cycle where increased bone marrow fat would lead to additional fat accumulation and additional bone loss.

| Genes Related to Lipid Synthesis and Storage | Change  |
|---------------------------------------------|---------|
| Sortilin                                    | +4.7 fold |
| Very-long-chain Acyl-CoA dehydrogenase      | +4.5 fold |
| Glycerol-3-phosphate acyltransferase        | +6.7 fold |
| Lipoprotein lipase                          | +7.9 fold |
| Non specific lipid transfer protein         | +2.9 fold |
| Fatty acid synthase                         | +3.8 fold |
| Lysophospholipase                           | +5.6 fold |
| sn-glycerol-3-phosphate acyltransferase    | +4.7 fold |
| Non-specific lipid transfer protein         | +2.9 fold |
| Phosphatidate phosphohydrolase type 2       | +4.9 fold |
| Phospholipase C                             | +3.6 fold |
| Phosphocholine cytidyltransferase           | +5.3 fold |
| Branched chain α-keto acid dehydrogenase E1| +14.4 fold |

| Genes Related to Lipolysis and β-Oxidation  |       |
|---------------------------------------------|-------|
| 3-Oxoacyl-CoA thiolase                      | -3.2 fold |
| DcoH                                        | -5.3 fold |
| Ryudocan (Syndecan-4)                       | -4.8 fold |
| D-β-hydroxybutyrate dehydrogenase           | -3.2 fold |

| Genes Related to Adipocyte Differentiation, Function and/or IGF-I Signaling | Change  |
|---------------------------------------------------------------------------|---------|
| Glucocorticoid receptor                                                  +2.5 fold |
| Glucocorticoid regulated kinase                                          +7.0 fold |
| 11-β-hydroxysteroid dehydrogenase, type 2                                +4.2 fold |
| Stat5b                                                                    -2.6 fold |
| Alpha-1β adrenergic receptor                                             +3.6 fold |
| GPAT                                                                      +4.7 fold |
| PI3K                                                                      -3.0 fold |
| IP3                                                                       +3.2 fold |
| Natriuretic factor precursor                                             +6.1 fold |
| ApoE                                                                      -11.6 fold |
| Basic fibroblast growth factor (FGF)                                     +3.4 fold |
| FGF-receptor activating protein                                          +6.1 fold |
| 12-lipoxygenase                                                          -8.2 fold |
| β-nerve growth factor                                                    +2.7 fold |
| Galanin                                                                  -2.7 fold |
| VGAT                                                                     -3.0 fold |
| GABA-A receptor delta                                                    -2.7 fold |

Table 1. Alcohol increases expression of genes associated with adipogenesis and lipid synthesis and storage.
In order to identify key metabolic pathways impacted by alcohol on bone marrow fat, we analyzed the effects of chronic alcohol consumption on global gene expression (Affymetrix and Research Genetics rat chips) in the distal femur metaphysis (bone and marrow). In these studies, RNA isolated from individual animals (n=3/group) was analyzed. Alcohol significantly increased expression of key genes associated with fat storage and decreased expression of genes associated with lypolysis (Table 1). In addition, alcohol significantly increased expression of genes associated with adipogenesis. Many of the latter genes are differentially regulated by TNF-α and IGF-I. Importantly, we have verified that alcohol results in rapid increases and decreases in TNF-α and IGF-I gene expression, respectively (Turner et al. 1998). These results are consistent with the hypothesis that alcohol alters energy metabolism in bone marrow in a manner that promotes adipocyte formation and deposition of fat in the bone marrow, potentially at the expense of osteoblast formation. The results do not, however, provide a specific mechanism for the changes.

9. Growth hormone signaling is required for normal bone growth and remodeling

Growth hormone is the most important regulator of postnatal growth and has actions that overlap with leptin. Growth hormone plays multiple important direct and indirect roles in coupling energy expenditure to growth, including the growth of bone. Growth hormone deficiency in humans and animals is associated with decreased bone growth and osteopenia (Nilsson et al. 1995; Kasukawa et al. 2004). Osteoblasts and chondrocytes have receptors for growth hormone and the hormone elicits rapid effects on these cells in culture (Ohlsson et al. 1998). As discussed below, there is evidence that alcohol disrupts growth hormone signaling.

9.1 Alcohol results in skeletal resistance to growth hormone

Hypophysectomy prevented body weight gain and this effect was reversed by growth hormone treatment in rats regardless of dietary alcohol. Compared to normal rats, hypophysectomized rats had less cancellous bone, and lower rates of longitudinal growth and bone formation but higher bone marrow adiposity (Figure 5). Short duration (8 d) treatment of hypophysectomized rats with growth hormone increased cancellous bone formation and longitudinal growth rates, and decreased bone marrow adiposity. Alcohol consumption, however, blunted the effects of growth hormone on bone elongation, cancellous bone formation and bone marrow adiposity. These findings suggest that alcohol induces skeletal resistance to growth hormone at the level of the growth hormone receptor. There is precedence for skeletal resistance to growth hormone in rats. Skeletal resistance to growth hormone was reported in rat models for disuse (Bikle et al. 1995; Kostenuik et al. 1999; Sakata et al. 2003; Sakata et al. 2004) and senescence (Ren et al. 1999). In regard to the former, alcohol has been shown to accentuate the detrimental skeletal effects of disuse (Hefferan et al. 2003). Further studies, however, are required to determine whether the putative resistance to growth hormone is due to reduced receptor number or impaired post-receptor signaling.

Osteoblasts, chondrocytes, and adipocytes have receptors for growth hormone, as do their stromal cell precursors, and the hormone elicits rapid effects on these cells in culture (Ohlsson et al. 1998). Growth hormone increases stromal cell number and differentiation of
stromal cells to osteoblasts. However, growth hormone suppresses adipocyte differentiation. As discussed below, alcohol-induced reductions in growth hormone signaling could be responsible for the observed changes in the balance between adipocyte and osteoblast differentiation.

Fig. 5. Effects of hypophysectomy (panels A and B, proximal tibia, 36X objective) and alcohol consumption (panels C and D, lumbar vertebra, 20X objective) on bone marrow adiposity in growing male rats. Severe growth hormone deficiency induced by hypophysectomy resulted in very rapid (within days) increase in bone marrow adiposity in growing rats. An increase in adiposity is also observed in adolescent rats following 6 weeks of consuming alcohol. As described in the text, the increase in bone marrow fat in rats fed a diet containing alcohol is associated with skeletal resistance to growth hormone.

Hypophysectomized animals are deficient in several hormones known to influence bone metabolism; including growth hormone, leptin, sex steroids, and adrenal and thyroid hormones. Thus, the profound skeletal changes resulting from hypophysectomy need not be exclusively due to growth hormone deficiency. However, we have shown that growth hormone replacement is sufficient to increase bone formation and decrease bone marrow adiposity to pituitary-intact control values. In contrast, administration of thyroxine, cortisol or 17β-estradiol to hypophysectomized rats was ineffective in normalizing either bone formation or bone marrow adiposity (Menagh et al. 2010). Also, short duration treatment with growth hormone did not restore white adipose tissue mass or serum leptin levels to
normal. These findings suggest that the profound changes in leptin levels following hypophysectomy are not essential for the actions of growth hormone on bone growth, turnover or adiposity. Thus, growth hormone appears to be sufficient to increase bone formation and reduce bone marrow adiposity in hypophysectomized rats. However, pharmacological replacement with growth hormone may obscure the role of leptin deficiency as a regulator of bone metabolism. Physiologically, low leptin levels are associated with impaired growth hormone signaling. Thus, it is likely that leptin and growth hormone have overlapping effects on bone growth and turnover.

9.2 Alcohol impairs IGF-I signaling in bone
IGF-I mediates most, if not all, of the actions of growth hormone on bone and multiple lines of evidence indicate that IGF-I is essential for normal bone growth and remodeling. The activity of IGFs depends upon specific receptors, whose numbers are regulated, on target cells (Brown-Borg 2003). IGF-I, in addition to stimulating osteoblast differentiation, acts as an autocrine growth and survival factor for osteoblasts and may be essential for these cells to maintain their fully differentiated phenotype. Studies in mice and humans have confirmed the important actions of IGF-I on bone metabolism. IGF-I knockout mice are severely osteopenic and have reduced bone formation, despite the ability to produce growth hormone (Stabnov et al. 2002). The liver is the principal source of circulating IGFs. However, IGF-I is produced locally by osteoblasts, adipocytes and cartilage cells. The relative importance of locally produced versus systemic IGF-I on bone metabolism is under investigation. Compelling data support a role for locally produced IGF-I in regulation of bone metabolism. Targeted over-expression of IGF-I in mouse osteoblasts resulted in increased bone formation (Zhao et al. 2000), and osteoblast-derived IGF-I is required for the bone anabolic response to parathyroid hormone (Bikle et al. 2002; Wang et al. 2007). We have shown that parathyroid hormone increases bone formation in hypophysectomized rats. This response is accompanied by an increase in skeletal IGF-I mRNA levels with no rise in circulating IGF-I, illustrating the important role of locally produced IGF-I. However, equally compelling data support a role for circulating IGF-I in the regulation of bone metabolism. IGFs circulate bound to binding proteins which either potentiate or antagonize IGF-I activity in specific tissues, and the circulating levels of these binding proteins are regulated by a variety of factors (Jones & Clemmons 1995) Liver IGF-I-deficient (Lid) mice and acid labile subunit (a key component in the IGF-I serum transport complex) knockout (Alsko) mice exhibited relatively normal growth and development, despite having 75% and 65% reductions in serum IGF-I levels, respectively. The double knockout mice (LA), however, exhibited growth inhibitions and osteopenia that were reversed by IGF-I treatment (Yakar et al. 2002). More recent findings using a variety of model systems support a regulatory role for systemic IGF-I on bone metabolism (Yakar et al. 2002; Mohan et al. 2003; Wang et al. 2004; Mohan & Baylink 2005). Taken together, the above findings suggest that locally produced as well as circulating IGF-I are both important to skeletal growth and remodeling and are likely to have overlapping but not identical actions. There is mounting evidence that the skeletal effects of growth hormone, via IGF-I signaling, are impaired by alcohol consumption. Chronic alcohol abuse results in decreased serum IGF levels, reduced mRNA levels for IGF-I in liver and altered hepatic synthesis of IGF binding proteins (Turner et al. 1998; Lang et al. 2000). Locally, alcohol decreases IGF-I gene expression in bone (Turner et al. 1998). Alcohol may also reduce IGF receptor number in
target cells (Lang et al. 2000). Thus, the numerous ways that alcohol could disrupt IGF-I signaling fall into two general non-mutually exclusive classes of action: 1) decreased IGF-I bioavailability and 2) target organ resistance to GH. Exposure to alcohol rapidly (within 8 hours) decreases mRNA levels for IGF-I in bone tissue (Figure 6). This decline precedes decreases in mRNA expression for bone matrix proteins and subsequent bone matrix synthesis (Turner et al. 1998). Thus, decreased production of IGF-I may play a causative role in mediating the inhibitory effects of alcohol on bone formation. Osteoblasts generate IGF-I and the growth factor is deposited into bone matrix where it is retained until released by osteoclast-mediated bone resorption. IGF-I located in bone matrix is thought to be osteoblast-derived but its origin has not been rigorously investigated. IGF-I in bone matrix, irrespective of origin, helps couple bone formation to bone resorption during bone remodeling: IGF-I released from the matrix during bone resorption acts in concert with other matrix-derived growth factors (e.g., TGF-β) to induce renewed bone formation (Centrella et al. 1991; Mohan & Baylink 1991). Additionally, IGF-I incorporated into bone matrix plays a role in mediating bone healing when released following a fracture (Okazaki et al. 2003).

Fig. 6. mRNA levels of IGF-I in distal femur are reduced within 8 h of administration of alcohol (1 g/kg) and return to normal by 24 hours. Values are man ± SE, n=4-5/group. *p <0.05 compared to time 0.

9.3 Parathyroid hormone may reverse alcohol-induced inhibition of bone formation by increasing IGF-I gene expression in skeletal tissues
The molecular mechanisms that mediate the bone anabolic response to parathyroid hormone are incompletely understood but appear to require IGF-I signaling. As mentioned, animals with low circulating levels of IGF-I have deficient bone formation (Yakar et al. 2005). Also, bone in IGF-I knockout mice is insensitive to parathyroid hormone, suggesting that IGF-I is essential for the bone anabolic effects of the hormone (Miyakoshi et al. 2001;
Bikle et al. 2002). Acute administration of alcohol decreases IGF-I mRNA levels in liver and bone (Lang et al. 1998). Alcohol also reduces the circulating level of IGF-I. Thus, alcohol abuse could decrease the skeletal response to parathyroid hormone by reducing systemic and/or locally produced IGF-I or, alternatively, by inducing a target organ resistance to IGF-I signaling (Wang et al. 2007). Parathyroid hormone increases mRNA levels for IGF-I in skeletal tissue in rats (Watson et al. 1995) and the hormone has been shown to be effective in increasing cancellous bone formation in severely growth hormone-deficient hypophysectomized rats (Schmidt et al. 1995). Since hypophysectomized rats have very low serum IGF-I levels, locally generated IGF-I may be sufficient for the bone anabolic effects of parathyroid hormone (Fielder et al. 1996). However, other studies suggest that systemic IGF-I is critically important for the bone anabolic response to the hormone (Yakar et al. 2006). Thus, parathyroid hormone-induced IGF-I in bone cells may compensate for the reduced circulating levels of the growth factor in alcohol-fed rats. Regardless of the relative importance of locally generated versus systemically derived IGF-I, parathyroid hormone increases cancellous bone formation in alcohol-fed rats without the requirement for restoring normal serum IGF-I levels. Taken together, these findings suggest that alcohol consumption results in a defect in growth hormone signaling that leads to impaired production of IGF-I by bone cells. Parathyroid hormone reverses this defect by its ability to increase IGF-I expression in bone cells by a growth hormone-independent mechanism. These findings provide a mechanistic explanation for the observed ability of parathyroid hormone to maintain normal bone formation in alcohol-fed rats and to reverse bone loss in a rat model for chronic alcohol abuse, whether or not alcohol is removed from the diet (Turner et al. 2001; Sibonga et al. 2007; Iwaniec et al. 2008; Howe et al. 2011).

10. Alcohol may disturb estrogen signaling

Although the skeletal changes in alcohol-fed rats are similar to those observed in growth hormone-deficient and leptin-deficient mice and rats, disruption of growth hormone and leptin signaling may represent only two of numerous mechanisms by which alcohol negatively impacts the growing skeleton. Disruption of estrogen signaling is another potential mechanism. We have already discussed estrogen as an important regulator of energy metabolism. In addition, estrogen is an important regulator of bone growth where the hormone plays an essential role in the sexual dimorphism of the skeleton (Turner et al. 1994).

The possible mechanisms by which alcohol could influence the skeletal response to estrogen on bone have been reviewed (Turner & Sibonga 2001). More recent in vitro studies suggest that very high concentrations of alcohol increase estrogen receptor levels but disrupt normal estrogen receptor signaling in cultured osteosarcoma cells (Chen et al. 2006; Chen et al. 2009). It is not clear, however, whether the much lower levels of alcohol exposure experienced by most adolescent drinkers would have this effect. Also, alcohol consumption results in similar skeletal abnormalities in male and female growing rats. In contrast, whereas estrogen receptor blockade by the potent estrogen receptor antagonist ICI 182,780 largely recapitulates the skeletal response to ovariectomy, the antagonist had no effect on bone growth and turnover in growing male rats (Sibonga et al. 1998; Turner et al. 2000). Therefore, alterations of estrogen receptor signaling may contribute to but are unlikely to be the major cause for the detrimental effects of alcohol consumption on the growing skeleton.
11. Summary

The skeletal changes in growing rats consuming alcohol are similar to the skeletal changes observed in growth hormone-deficient rats; decreased bone elongation, decreased cortical and cancellous bone mass, decreased bone formation and resorption, and increased bone marrow adiposity. Studies performed in hypophysectomized rats suggest that growth
hormone is essential to maintain a mature osteoblast phenotype and suppress excessive adipogenesis. We have also shown that alcohol impairs the action of growth hormone to increase bone growth and turnover, and decrease bone marrow adiposity in hypophysectomized rats. These results are consistent with skeletal resistance to growth hormone as a contributing mechanism for the detrimental skeletal effects of chronic alcohol consumption. Other studies suggest that heavy drinking decreases energy intake and in some situations can increase energy expenditure. It is likely that reduced leptin signaling is, at least in part, responsible for changes in energy homeostasis. Reduced leptin levels may also contribute to the alcohol-induced inhibition of bone growth and turnover, and increase in bone marrow adiposity. Thus impairments in growth hormone and leptin signaling may act in consort to mediate the reduced peak bone mass in rats fed a diet containing alcohol. Although our work to date has focused on leptin and growth hormone, it does not preclude an important role for other factors, such as estrogen, that facilitate the coupling of bone growth to energy metabolism. As depicted in our working model (Figure 7), alcohol consumption may also decrease the deposition of growth factors into bone matrix prior to its mineralization. Thus, heavy underage drinking, in addition to an immediate increase in the likelihood of a fracture, may have serious long-term consequences to bone health. By decreasing peak bone mass, heavy alcohol consumption may increase the risk for premature osteoporosis and by decreasing the incorporation of skeletal growth factors into bone during formation, alcohol may increase the risk for impaired healing should a fracture occur.

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