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CHRONIC AND INTERMITTENT EXPOSURE TO ALCOHOL VAPORS: A NEW MODEL OF ALCOHOL-INDUCED OSTEOPENIA IN THE RAT

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ABSTRACT

Background: Different models are used to study the effects of chronic alcohol consumption on bone tissue in the rat. However, the current models take several months to show indices of osteopenia as observed in chronic drinkers. Numerous studies have supported that chronic and intermittent exposure to ethanol vapors has predictive validity as a model of alcohol dependence in humans. However, this model has never been applied to bone research to study its effects on the parameters that define osteopenia. This was the goal of the present study in the rat.

Methods: Male Wistar rats were exposed to ethanol vapor inhalation (E, n=6) or air (controls, C, n=6). Animals were exposed to chronic (11 weeks) and intermittent (14 h a day) ethanol vapor reaching stable BALs (150-250 mg/dl) at the end of the third week of inhalation. After the sacrifice, right and left femur and tibia were dissected free of fat and connective tissue and bone mineral density (BMD) was assessed by dual X-ray absorptiometry. The microarchitecture of the femur was studied using micro-computed tomography.

Results: The BMD of the left and right femurs and the left tibia was lower in the ethanol group compared to the control group. The bone volume fraction (BV/TV) and the bone surface density (BS/TV) were lower in the ethanol group compared to control animals. The trabecular number was lower in the ethanol group while the trabecular spacing was higher.

Conclusion: The decrease of the BMD, BV/TV and Tb.N is in the same range as what is observed in human drinkers and what is reported with other animal alcohol models (Lieber-DeCarli liquid diet, ethanol in the tap water). Therefore this model could be useful to study the effects of chronic alcohol consumption in the bone research field and has the advantage of controlling easily targeted BALs.

Keywords: alcoholism, animal model, bone, ethanol inhalation, osteopenia

INTRODUCTION

Alcohol consumption affects numerous organs and tissues, such as bone. Chronic heavy alcohol consumption is one of the major causes of secondary osteoporosis in men, with hypogonadism and excessive corticosteroid use (Ebeling, 1998; Peris et al., 1995). Heavy alcohol consumption decreases the bone mineral density (BMD) (Alvisa-Negrin et al., 2009; Gonzalez-Calvin et al., 1993) and increases the fracture risk, due to a decrease of the trabecular and the cortical bone (Hogan et al., 1997; Sampson et al., 1997; Turner, 2000).

There are different procedures available to study the effects of alcohol consumption on bone in rodents (Maurel et al., 2012). Intraperitoneal (i.p.) injections and gavage are efficient methods for achieving high blood alcohol levels (BALs) (Callaci et al., 2009) but these techniques are stressful for the animals and may lead to inflammation and necrosis of tissues. It is difficult to administer alcohol with these techniques for a long period of time (several months) (Lieber et al., 1989). Another usual procedure whereby ethanol is provided is the use of a liquid diet allowing researchers to control the percentage of ethanol contained in the diet and also the amount of nutrients (Hogan et al., 1999; Sampson, 1998; Turner et al., 2001). This technique is less stressful for the animals and is used by many researchers as the risk of dehydration among the ethanol-fed rats is limited (D'Souza El-Guindy et al., 2010; Maurel et al., 2012). The drawback of employing this technique is that it does not mimic the human consumption pattern due to the fact that liquid and food are mixed (D'Souza El-Guindy et al., 2010). Other studies use a forced exposure in which only an alcohol solution is available as the only source of fluid, but rats may refrain from drinking, and therefore suffer from dehydration and achieve lower BALs (D'Souza El-Guindy et al., 2010; Lieber et al., 1989). Furthermore, the weight loss resulting may hide the direct effects of ethanol on bone, when the mechanism of action of ethanol on the skeleton is the primary aim of the study.

For four decades a procedure of chronic intoxication by inhalation of ethanol vapors has been successfully used in rats to overcome their innate aversion for alcohol solution and induce both physical and behavioural alcohol dependence. The principle is to maintain constant blood ethanol concentrations for several hours a day in order to induce physical dependence that should develop over days or weeks. Thus this procedure is useful because it allows the experimenter to control both duration and value of the peak BALs while water and food are available *ad libitum*. Importantly, intermittent exposure more closely mimics the binge intake pattern in human alcoholics and, thus, provides a very good model of the development of dependence and compulsive use and relapse after periods of abstinence in human alcoholics (O'Dell et al., 2004). Chronic intermittent exposure to ethanol vapor is thus recognized as the gold standard model in the research on alcohol addiction (Naassila et al., 2000; Simon O'Brien et al., 2011; Valdez et al., 2002). It is therefore useful to test the efficacy of treatments on the excessive motivation to drink alcohol in association with dependence and relapse.

To date and to our knowledge, only one study used this protocol in the bone research field (Torricelli et al., 2007). The researchers investigated the osteoblasts from trabecular bone of rats after 7-week exposure to ethanol vapor, and found that the osteoblast proliferation rate and activity were significantly affected by alcohol exposure (Torricelli et al., 2007). However, no study has assessed the effects of chronic intermittent exposure to ethanol vapor on bone mineral density and bone microarchitecture. This was the objective of the present study in the rat.

MATERIAL AND METHODS

Animals

Twelve male Wistar rats weighing 200-250g upon arrival arrived in the animal care unit from Charles River Laboratory (L'Arbresle, France). Wistar rats is the most popular strain used in the ethanol inhalation procedure and is largely used in bone research. The animals were housed four per cages, in a light-reversed 12-hour light/dark cycle, in a temperature controlled at 21°C ± 0.5°C. The humidity was controlled (55 ± 10%) and the animals had water and food *ad libitum*.

Ethanol Vapor Inhalation

Six rats (E) were housed individually in standard cages, had unlimited access to water and were exposed to ethanol vapor in sealed clear plastic chambers (550 liters) (Naassila et al., 2000; Simon O'Brien et al., 2011). Ethanol vapor was generated by introducing pressured air in a 96% ethanol reservoir (60-80% of hygrometry). Ethanol vapor concentration was gradually increased for 3 weeks in order to reach stable BALs (between 150 and 250 mg/dl). Ethanol was evaporated for 14 hours during a day (on at 7.00 pm, off at 9.00 am) (O'Dell et al., 2004) for 8 weeks (Rimondini et al., 2002). Blood alcohol levels were regularly determined to permit the adjustment of ethanol vapor concentrations using an Analox AM1 Instrument analyzer (Imlab, Lille, France). Rat submitted to this chronic ethanol vapor inhalation (Ethanol group) displayed a physical dependence, shown by the appearance of somatic withdrawal signs, as previously described (O'Dell et al., 2004; Roberts et al., 2000). During the same period, six control animals (C) were placed in a separate sealed plastic chamber and exposed to a continuous flow of air.

Bone density measurements

After the sacrifice (lethal injection of pentobarbital), the right and left tibias and femurs were excised, cleared of fat and connective tissues, placed in paraformaldehyde 4% at 4°C.

The tibia and femur bone mineral content (BMC) and BMD were measured by DXA (Discovery, Hologic, Bedford, Massachussets, USA) using a specific 'small animal body composition' mode. The exam was performed *ex vivo*. In order to mimic the soft tissue present *in vivo*, the bones were placed in a physiological saline solution during the exam. This apparatus is commonly used in small animals, and has been validated in our laboratory (Lespessailles et al., 2009; Maurel et al., 2011a).

Microarchitecture of the trabecular bone

The microarchitecture of the metaphysis of the femoral bone was analyzed by high resolution µCT (Skyscan 1072, Skyscan, Aartselaar, Belgium). We chose this subregion because it is rich in trabecular bone. The characteristics and methods used in our laboratory have previously been described (Bonnet et al., 2005). The parameters analyzed were: the bone volume fraction (BV/TV, %), the trabecular thickness Tb.Th (mm), the trabecular number Tb.N (1/mm), the trabecular spacing Tb.Sp (mm) and the degree of anisotropy DA.

Statistics

Data are presented as mean ± standard deviation (SD) for all bone parameters. The statistical analyses were done using the Statview 1992-98 SAS software. Normal distribution (Gaussian) of the data was assessed with the Shapiro-Wilk test. Student t-tests were used when the distribution followed the normal law. Otherwise, the U Test from Mann & Whitney was used. The statistical degree of significance was set at $p < 0.05$.

RESULTS

At the time of the sacrifice, BALs were around 1.8 g/l in the ethanol group and we did not observe weight loss in the ethanol-exposed group (405±11 and 402±4 g in control and ethanol groups, respectively).

Bone mineral content and bone mineral density

We did not observe any difference for the bone mineral content of the right and left tibias and femurs. However, we found a lower BMD in the ethanol group compared to the control group at the left and right femurs and at the left tibia (Table 1). There was a trend for the right tibia BMD to be lower in the ethanol group (p=0.054).

Microarchitecture of the trabecular metaphysis of the femur

The bone volume fraction (BV/TV) was lower in the ethanol group compared to control animals (Table 2). The trabecular number was lower in the ethanol group (Figure 1). On the opposite, and logically, the trabecular spacing was increased in the ethanol group (Table 2). The trabecular thickness was not different between the groups after the protocol, as for the degree of anisotropy.

DISCUSSION

We designed this *in vivo* study to assess if the intermittent exposure to ethanol vapor can be relevant to study alcohol-induced osteopenia. We observed lower BMD at the tibia and the femurs in the ethanol group, as well as lower trabecular bone parameters, similar to other protocols of ethanol administration.

The BMD was lower in the femurs (-7%) and in the tibia (-5%) from the ethanol group compared to control animals, after intermittent exposure to ethanol vapor for 11 weeks and

targeted BALS of 1.8 g/l. These data are consistent with the data reported in the literature. Bonnet et al. (Bonnet et al., 2006) found a 8% decrease of the femur BMD and a 10% decrease of the tibia BMD following ovariectomy in female rats. Our group displayed a 13% lower BMD at the femur in male Wistar rat following 17 weeks of alcohol treatment (alcohol in the drinking tap water, 35% v/v and separated from the food) (Maurel et al., 2011a). Broulik et al. reported a 9% lower femur density in rats after 3 months of alcohol treatment (alcohol in the drinking tap water, 7.6g/kg) (Broulik et al., 2010). Therefore, this alcohol treatment can induce an osteopenia similar to those observed with a liquid diet or with tap water containing ethanol in animal models.

In humans, the BMD is generally decreased by 1-10%, depending on the region analyzed (Table 3). Laitinen et al. reported 1% lower BMD at the vertebrae (Laitinen et al., 1992). Alvisa-Negrin et al. reported 8% lower BMD in male and female alcoholic subjects (Alvisa-Negrin et al., 2009).

In the present study, microarchitectural parameters were also drastically affected after 11 weeks of exposure to ethanol vapor. The bone volume fraction (BV/TV) was decreased by 37% in the ethanol group compared to the control group and the trabecular number (Tb.N) was decreased by 37% while the trabecular spacing was increased by 82%. These results are consistent with the literature. Our team previously reported decreases of 25% of the BV/TV with a solution containing 35% ethanol v/v in male Wistar rats (Maurel et al., 2011b). Maddalozzo et al. also reported decreases of 22% of the BV/TV, 25% of Tb.N and an increase of 44% of the Tb.Sp, following 3 months of treatment with 35% ethanol liquid diet in 4 week-old male Sprague-Dawley rats (Maddalozzo et al., 2009). In a study by Sampson et al., the BV/TV was decreased by 46%, the Tb.N by 43% and the Tb.Sp was increased by 119% following 8 weeks of ethanol liquid diet (35% of the calories) in 4 week-old female

Sprague-Dawley rats compared to pair-fed controls (Sampson et al., 1997). Our results are close to the changes observed by Sampson et al. (1997) in young female rats. The changes are a little lower but our rats are older and as shown by the study by Sampson (Sampson et al., 1998), the effects of alcohol tend to be lower on some parameters in older animals. In comparison with animal models with similar BALs, the effects of this protocol on BV/TV and Tb.N are close. However, we did not observe an effect of alcohol on trabecular thickness (Tb.Th) with this technique, as for the study by Sampson et al. with similar BAL (Sampson, 1998).

There are very few papers assessing bone microarchitecture after chronic alcohol consumption in men and women (Maurel et al., 2012). In human subjects, with consumption comparable to BAL of 200 mg/dl in animals, the trabecular bone volume analyzed by histomorphometry is lower by 26 to 36% (Table 3). Then the data found in our model are in the same range, and the BV/TV decrease is very close to the data reported by De Vernejoul et al. (De Vernejoul et al., 1983). We observed lower trabecular number (Tb.N), as observed before in post menopausal women and pre menopausal women with osteoporosis (Dufresne et al., 2003; Liu et al., 2010).

This ethanol vapor exposure model allows easily achieving high BALs, the BALs can be controlled easily and bone pathology as osteopenia can be induced similarly to other models. In conclusion, the chronic and intermittent exposure to alcohol vapor seems to be a good model to study the alcohol-induced osteopenia in the rat.

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Figure 1: 3D reconstruction of the trabecular bone of the femur metaphysis in Control (C)

and Ethanol (E) groups, after micro-computed tomography acquisition.
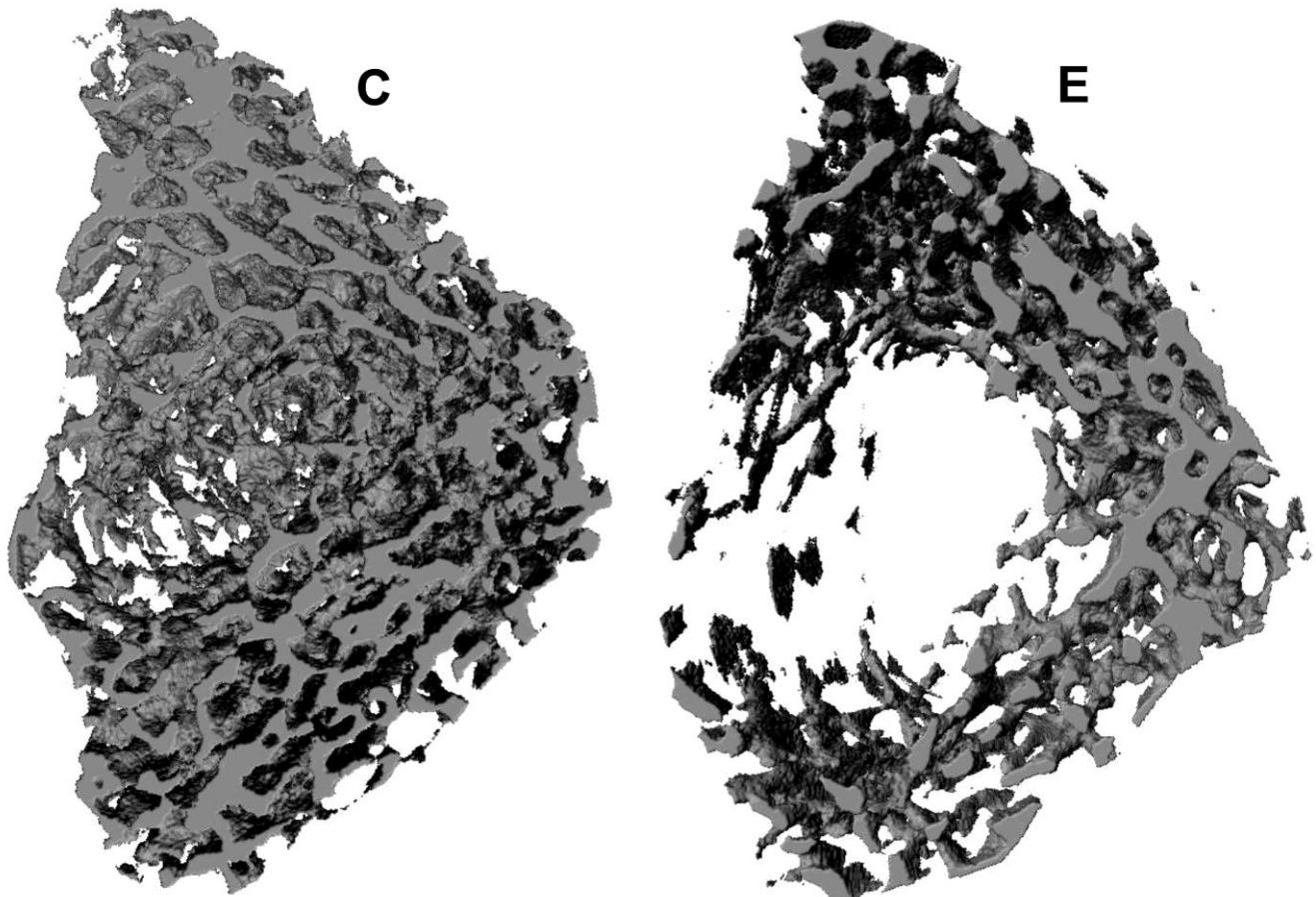
	Controls	Ethanol	p
Left femur BMC (g)	0,53 ± 0,03	0,50 ± 0,03	NS
Left femur BMD (g/cm2)	0,250 ± 0,011	0,234 ± 0,010	0,025
Right femur BMC (g)	0,53 ± 0,03	0,50 ± 0,04	NS
Right femur BMD (g/cm²)	0,255 ± 0,012	0,238 ± 0,009	0,037
Left tibia BMC (g)	0,35 ± 0,03	0,36 ± 0,04	NS
Left tibia BMD (g/cm²)	0,214 ± 0,007	0,204 ± 0,007	0,025
Right tibia BMC (g)	0,36 ± 0,04	0,33 ± 0,05	NS
Right tibia BMD (g/cm²)	0,211 ± 0,012	0,195 ± 0,016	0,054

Table 1

	Controls	Ethanol	p
BV/TV (%)	20,93 ± 4,78	13,17 ± 4,92	0,025
Tb.Th (mm)	0,096 ± 0,002	0,096 ± 0,009	NS
Tb.N (1/mm)	2,18 ± 0,48	1,37 ± 0,50	0,016
Tb.Sp (mm)	0,34 ± 0,13	0,62 ± 0,34	0,037
DA	2,25 ± 0,21	2,23 ± 0,34	NS

Table 2

Model	Alcohol consumption	Age	Sexe	Effects on bone tissue	Study
	~ 204g/ d for 28 years	~50 years	F / M	Subtotal BMD : -8% Leg BMD: -10% (DXA)	*Alvisa- Negrin et al. 2009*
	~180g/ d	35-50 years	M	Trab. Bone vol: -36% Mean trab. Thickness: -18% (histomorphometry)	*De Vernejoul et al. 1983*
	~223g/ d for 6-30 years	31-61 years	M	BMD: -1% (DXA) lower when adjustment for body weight and age	*Laitinen et al. 1992*
	170-210g/ d for 11-15 years	27-50 years	M / F	Low BMD in lumbar spine, femoral neck, total hip: 24% of males (DXA) Intertrochanteric region: low BMD: 5% females	*Malik et al. 2009*
	~170g/ d for 7-26 years	~41 years	M	BV/TV: -26% Tb.Th: -33% Tb.N: NS Tb.Sp: +4% (histomorphometry)	*Schnitzler et al. 2010*

Table 3

Table legends

Table 1: Bone mineral content (BMC) and bone mineral density (BMD) of the right and left femur and tibia, assessed by DXA after the sacrifice.

Table 2: Microarchitectural parameters of the trabecular bone in the femur, assessed *ex vivo* by microCT.

Table 3: Bone mineral density and microarchitecural changes in human studies of alcohol abuse.