The effect of chronic alcohol administration on bone mineral content and bone strength in male rats.

P.D.Broulik[1],J.Rosenkrancová,[2]P.Růžička[2],R.Sedláček[2] T.Zíma[3]

[1]Third Medical Clinic,[3]Department of Clinical biochemistry,First Medical Faculty,Charles University ,Prague and [2]Faculty of mechanical Engineering,Department of Mechanics,Prague,Czech republic.

Keywords: Key words:alcohol,bone mineral density,bone strength,male rats

Correspondence:Prof. PD Broulik 3rd Medical Department,General Teaching Hospital U nemocnice l Prague 128 08 Czech republic
E-mails:pbrou@lf1.cuni.cz

Abstract

Alcohol use has been identified as being a risk factor for the development of osteoporosis. Eight male Wistar rats at two months of age were alcohol fed 7.6 g 95% ethanol /1 kg b.w per day to evaluate the effects of long-term administration (three months) of alcohol in drinking water . We have a used dose which is considered to be comparable to a dose of 1 litr of wine or 2.5 litr of 12^{0} beer used in male adults daily. The bones were tested mechanically by a three point bending test in a Mini Bionix (MTA) testing system. The bones from alcohol fed rats were characterized by a reduction in bone density as well as ash, calcium and phosphate content. In alcohol fed rats the reduction in bone mineral density (10%) was reflected in the reduction of mechanical strength of femur (150 ± 5.5 v.s 178 ± 3.2 N/mm^2) which is reduction about 12%. Alcohol significantly altered femoral cortical thickness. Alcohol in our experiment itself does not exert any antiandrogenic effect and it does not produce changes in the weight of seminal vesicles.Liver function test (GGT,ALP,AST,) did not differ between alcohol fed rats and control rats. Alcohol induced bone loss is associated with increased bone resorption and decreased bone formation..

These results document the efficacy of alcohol at dose 7.6 g 95% ethanol/1 kg b.w to cause bone loss and loss of bone mechanical strenght in intact rats. The results of the present study may be interpreted as supporting the hypothesis of alcohol as a risk factor for osteoporosis.

Introduction

Excesive alcohol intake is a well recognized cause of secondary osteoporosis.
Alcohol abuse is associated with increases in both the incidence of fractures and complication in fracture healing (Kanis et al. 2005). Higher intakes appear to be associated with an increased fracture risk and hip fracture risk (Hoidrup et al. 1999,Mukamal 2007, Iwaniec 2008). There are changes in bone structure detected by histomorphometry and there is a decrease in bone mineral content Although the relatioship between alcohol ingestions and bone disease is evident the mechanism by which alcohol induces osteoporosis remain unclear. Younger alcoholic patients without other diseases may suffer from an increased risk to develop low bone mineral density (Malik 2009). It is not clear whether alcohol independently affects bone mass. Bone changes may also be produced by factors commonly associated with ethanol abuse like nutritional deficiensies, liver demage and hypogonadism (Odvina et al. 1995,Kim 2003). Thus etiology of alcohol associated bone disease is multifactorial.Also not all studies show an association of alcohol and fracture risk (Berg 2008). The effects of more moderate intakes are not thought to be deletrious to skeletal health (Orwoll et al. 2000)**.** Studies of postmenopausal women show a positive association between bone mineral density and alcohol intake,but for men and premenopausal women the bone-alcohol relationship remains unclear (Wosje 2007).The purpose of the current study was to evaluate the effects of moderate dose ethanol chronic administration on bone mass and strength in intact rats.In our experiment we have used three point bending test which is useful for measuring the mechanical properties of bones from small animals.

Materials and methods

Two months old male strain rats (Velaz Prague), weighing 205 g were used for the experiments. They were fed on a standard diet containing 23% protein, 1.2% calcium and 0.6% phosphorus with water ad libitum and were kept in an indirectly illuminated room with controlled temperature at 24 ± 2^{o} C. The study was performed in accordance with the Guide for care and use of laboratory animals published by the National Institutes of Health. The animals were randomly divided into two groups of 8 animals each. Two month old male rats drinking daily 7.6 grams of pure ethanol/1kg b.w daily for 3 months which corespond to a consumption of 1 litr wine or 2.5 litr of 12 ^{0} beer in male adults and were compared to rats without ethanol. Controls were pair-fed isocaloric diet which subsitutes calories supplied by ethanol.

In our experiment alcohol was mixed in the drinking water (190 ml 95 % ethanol/1000 ml).. The solution of alcohol in water has been made every day and consumption was measured daily.. We have known the exact amount of daily water consumption so that the amount of water with alcohol daily corresponded to the dose of given drug**.** At the end of the experiment the animals were killed, under ether anesthesia, the blood being drawn by cardiac pucture and the seminal vesicles were removed, cleaned and weighed.

Femurs were isolated, cleaned of soft tissues. Marrow elements were flushed out with cold saline through a needle inserted into marrow cavity. Finally the bone was weighed and placed in an unstoppered glass vial filled with deionized water which was then put into desiccator. The bones were suspended on a fine wire mesh and weighed in air and in water to accuracy of 0.1mg. Volume and density of the tibia were calculated from the mass in air and water by Archimedes principle (Kalu et al. 1994). Although assessing bone density using Archimedes principle is time consuming it measures true rather than apparent density.(Deyhim et al. 2006) The bones were then dried to constant weight and then incinerated for 24 hours at 600 ^{o}C to white ash which was weighed. Bone ashes were then dissolved in hydrochloric acid before determination of calcium and phosphorus content. Bone calcium was measured by the method of (Gitelman 1967) and bone phosphorus according (Kraml 1966).

The activity of plasma Tr-ACP (EC 3.1.3.2) was determined as previously described (Štěpán et al. 1983). The activity of plasma bone alkaline phosphatase (ALP), ALT (alanine aminotransferase), AST (asparate aminotransferase), GGT(gamma glutamyl transferase) were determined by automat Modular Roche .

Seminal vesicles were cleaned and weighed and the weight was expressed in relative values (mg/100 g body weight).

For the bone morphology we used the method presented by (Beall et al. 1984 and Vanderschueren et al. 1992). Standardized roentgenographs of right femur were made using Philips mamo diagnost 3000 X - ray machine at controlled exposures of 26 kV at 5.5 mA. Morphometric measurements were performed directly on the X rays after magnification by fine caliper. On the roentgenographs the external, inner bone diameter and cortical width were measured after magnification with fine caliper at 40 % of the total length starting from the distal end.

The three-point bending test was used to measure the mechanical properties of the femur. Left femurs were also removed and cleaned of tissue and immediately soft wrapped in gauze soaked in isotonic saline and frozen. After thawing the bones tested were hydrated in distilled water at room temperature 22 ^{o}C over a period of 24 hours before experiment. Prior to mechanical testing, all soft tissues were removed and specimens were placed on two rounded bars set 20 mm apart in order to apply the load from medial side of the rat femur (Turner and Burr 1993;Broulik et al. 2005). The MTS Mini Bionix 858.02 biomechanical testing system was used for the three-point bending test. The program, written for the test control in TestWare software, controls cross-head speed and measures important quantities such as load, deflection and time. Bending strength σ_{omax} (MPa =N x mm $^{-2}$) was calculated from the ultimate force F_{max} (N) and section modulus of bone Z_{omin} (mm^3) where $\sigma_{omax} = M_{omax}/Z_{omin} = F_{max}.L/(4 \times Z_{omin})$ where M_{omax} (N x mm) is maximal bending moment and L (mm) is distance between two supporting bars. Ultimate load (force at failure) is the maximum load that a specimen withstands before fracture.

The bones were measured according to the following principle: the bone cross section at the fracture location was resurfaced by grinding, to get a planar surface perpendicular to the femur axis, and marked by the colored felt-tip pen and scanned by flat tabletop scanner at resolution 1200 x 1200 dpi that means pixel size 0.021 x 0.021 mm. The full color digital image obtained from scanner was sent through the process of segmentation. The binary image of the same resolution and size was derived from color image. The binary contains values 0 and 1, 1 meaning that the corresponding pixel belongs to the bone cross-section, 0 meaning the pixel does not belong to the bone. (Růžička et al. 2002)The binary image was converted into the program written in MATLAB (Turner 1993). This program calculates every important dimensions of the bone cross-section these are A, b_{min}, b_{max}, J_{min} and Z_{omin}. A refers to the cross-section area, b_{min} and b_{max} are minimal and maximal dimensions of the bone cross-section, J_{min} is the minimal moment of inertia. σ_{max} expressed as (N/mm^2) is bone strength is an intrinsic property of bone.

Load-deflection curves were recorded at the cross-head of 2 mm/min by load indicator with a measuring range 0 - 500 N .

The means ± 95% confidence intervals were computed and the significance of differences between the means was evaluated by an analysis of variance and Duncan´s test (Duncan 1955).

Results

The results are summarized in **Tab.1**.There were no clinical signs of morbidity in any of the two groups studied. All rats gained weight during the study but groups given alcohol had smaller weight gains and lower final body weight when they were killed than the control animals. However the difference did not reach statistical significance.The animals were paired and and we did not observe decreased food intake in alcohol fed animals. Serum calcium, phosphorus, total protein, amylase were not significantly different in either control and alcohol fed animals. Abnormal liver metabolism was not evident and the ethanol dose had a minimal effect on liver metabolism. Liver function test (gama glutamyl transferase, asparate aminotransferase, alanine aminotransferase) did not differ between alcohol fed rats and control rats. Serum alkaline phosphatase was significantly lower in the ethanol-fed as compared with the control animals. None of the rats in this study had diagnosis of chronic pancreatitis (normal level of plasma amylase). Plasma tartrate resistant acid phosphatase (TrACP-EC 3.1.3.2) levels in ethanol fed rats were significantly higher than those in controls. $p < 0.01$. The increase in plasma tartrate resistant acid phosphatase in ethanol fed rats would be consistent with increase of bone resorption. No significant changes were observed in the weight of the seminal vesicles a highly androgen dependent tissue in alcohol fed animals.

The densities of the femurs, ash weights and calcium and phosphorus content of the femur were significantly reduced in alcohol fed rats , compared with the animals without alcohol.($p < 0.01$) Three point biomechanical testing of femurs from ethanol fed rats revealed significant reduction in cortical strength and energy to failure.

In alcohol fed rats the reduction in bone mineral density (10%) was reflected in the reduction of mechanical strength of femur (158 ± 5.6 v.s 178.9 ± 3.2 N/mm^2) , which is reduction about 12% (table 1).

Femoral length and outer diameter were not significantly different between alcohol fed rats and control rats. However alcohol fed rats had significatly decreased femoral cortical thickness. **Tab 2**.

Discussion

Alcohol causes substantial decreases in femural density and biomechanical properties of femur in intact rats. We have used a dose which is considered to be comparable to a dose of 1litr of wine or 2.5 litr of 12^{0} beer used in male adults daily. The mean values of bone density of the femur were significantly different between the control and alcohol administered rats. Bone density and bone mineral content, however are an indirect measure of bone quality, reflecting the amount and arrangement of the mineral phase but not bone strength itself. In the case of assessing bone density in small animals using Archimedes principle it measures true rather than apparent density.(Deyhim et al.2006)

Measurement of bone mechanical properties is an important tool for evaluating the functional significance of changes in bone mass and or architecture.

Mechanical properties of bone do not depend solely on mineralization, but on organization and microscopic structure of bone material. Loss of bone strength can arise from changes in spatial distribution of bone material without any loss of osseous tissue or change in ash composition.

In our experiment there were reductions in the mechanical strength of the midshaft femur in alcohol fed rats when compared to control rats (Table 1).This may be explained by ethanol induced cortical bone loss (change of cortical width). Histomorphometric studies have shown that in alcohol fed rats there is statistically significant reduction in cortical thickness ($p < 0.05$).Table 2. In alcohol fed rats the reduction in bone mineral density (10%) was reflected in the reduction of mechanical strength of femur (150 ± 5.5 v.s 178 ± 3.2 N/mm^2) which is reduction about 12%.

It is not known if the effects of alcohol on bone cells are direct. It is possible that alcohol alters the activity, production or binding of growth factors produced by the cells. Complete information concerning the mechanism of the skeletal effects of alcohol is still lacking.

In the present study there was no evidence of liver disease or hypogonadismus in any of alcohol fed rats; therefore the confounding effects of these variables on bone mass were excluded. It appears that prolonged alcohol intake is necessary for the negative effects of alcohol to be observed.

Both chronic and acute administration of ethyl alcohol can supress plasma testosterone levels in men and in experimental animals. However we did not find significant changes in the weight of the seminal vescles a highly adrogen dependent tissue after alcohol administration. No significant changes were observed in rats after administration of alcohol. Our ethanol

dose had a minimal effect on liver metabolism. We can assume that ethanol 7.6g 95% ethanol /1kg bw a day was solely responsible for the observed changes in bone metabolism. High bone turnover states may compromise bone strength by reducing bone mass causing focal areas of weakness in trabeculae and preventing complete mineralization of bone structural units (Ott 2001).

The decrease in cortical thickness in alcohol fed rats may result from increased resorption at the endocortical surface or decreased formation at either the endosteal or periosteal surface. Increased TrACP and decreased ALP levels in alcohol fed rats (Table 1).

Our results demonstrate that alcohol causes a loss of bone mass and bone strength, in cortical bone possibly through an increase in bone resorption expressed by increased TrACP . It is known that increased resorption results in a decrease in bone density and is the cause of bone loss in postmenopausal women.

Interpretation of the data obtained in this study is difficult because the mechanism by which alcohol acts on bone remains unclear. However several lines of evidence which exist in the literature allow some speculation about the significance of alcohol effect on bone.

Alcohol intake is a risk factor for osteoporosis (Kanis et al. 2005;Cummings et al. 1995). There was significant inhibition of cell proliferation and extracellular matrix synthesis. In vitro studies have shown that alcohol directly increases bone resorption and inhibits bone formation. (Santori et al. 2008). In addition animal studies have demostrated that alcohol ingestion results in bone loss and inhibition of bone matrix synthesis and mineralization in rats (Bell et al. 1987) Increased bone resorption has been demonstrated in alcoholic subjects (Bickle et al. 1993)). Ethanol in the rat diet especially during the postinjury period was solely responsible for inhibition of bone repair. (Chakkalakal et al. 2005)

While we have shown that alcohol is effective on the bone in the rats model, its potential in humans is still unknown.

After long term treatment in rats with moderate ethanol dose this drug is able to decrease bone mineral density and biomechanical properties of bone suggesting possible efficacy of alcohol increase fracture risk in humans. The relationship between ethanol use and bone mineral mass is not mediated via an effect on androgen action or abnormal liver metabolism.. .In our experiments increased bone resorption and reduction in bone formation in our alcohol fed rats have been demonstrated.

This work was supported by the research projects MSM 0021620806 and LC06044 granted by the Ministry of Education Youth and Sports of Czech republic

References

BEALL PT, MISRA LK, YOUNG RL, SPJUT HJ, EVANS HJ, Le BLAME A: Clomiphene protects against osteoporosis in the mature ovariectomized rat.*Calcif Tissue Int* **36:**123- 125, 1984

BELL NH, TURNER RT, GREENE VS:Demonstration that ethanol inhibits bone matrix Synthesis and mineralization in the rat.In:Cohn DV,Martin TJ,Munier PT eds. Calcium regulation and bone metabolism Amsterdam:*Excerpta Medica* **636:**1987.

BERG KM, KUNINS HV, JACKSON JL, NAHVI S, CHAUDHRY A, HARRIS KA, MALIK R: Association between alcohol consumption and both osteoporotic fracture and bone density. *Am J Med* **121**:406-418, 2008.

BICKLE D, STESIN A, HALLORAN B, STEINBACH L, RECKER R.Alcohol induced bone disease: relationship to age and parathyroid hormone levels.*Alcohol Clin Exp Res* **17:**690-695, 1993.

BROULIK PD, ROSENKRANCOVA J, RUŽIČKA P, SEDLAČEK R.: Effect of alendronate administration on bone mineral density and bone strength in castrated rats. *Horm Metab Res* **37**:414-418, 2005.

CUMMINGS SR, NEVITT MC, BROWNER WS, STONE K, FOX KM, ENSRUND KE, CAULEY J, BLACK D, VOGT TM.Risk factors for hip fracture in white women *N Engl J Med* **3**: 767-773, 1995.

DEYHIM F, GARICA K, LOPEZ E, GONZALES J, INO S, GARCIA M, PATIL BS. Citrus juice modulates bone strength in male senescent rat model of osteoporosis. *Nutrition* **22**:559- 563, 2006.

DUNCAN D: Multiple range and multiple F test.*Biometrics* **11**:1-4, 1955.

GITELMAN H: An improved automated procedure for the determination of calcium in biological specimens.*Anal Biochem* **18**:521-531,1967.

HOIDRUP S, GRONBAEK M, GOTTSCHAU A, LAURITZEN JB, SCHROLL M and the Copenhagen Centre for Prospective Population Studies (1999) :Alcohol intake,beverage preference and risk of hip fracture in men and women. *Am J Epidemiol* **149:**933-1001, 1999.

CHAKKALAKAL DA, NOVAK JR, FRITZ ED, MOLLNER TJ, McVICKER DL, GARVIN KL, McGUIRE MH, DONOHUE FM: Inhibition of bone repair in rat model for chronic and excessive alcohol consumption.*Alcohol* **36**:201-214,2005.

IWANIEC UT, TREVISIOL CH, MADDALOZZO GF, ROSEN CJ, TURNER RT: Effects of low dose parathyroid hormone on bone mass,turnover and ectopic osteoinduction

in rat model for chronic alcohol abuse. *Bone* **42**:695-701, 2008

KALU DN, HARDIN RR, CECKERHAM R: Evaluation of the pathogenesis of skeletal changes in ovariectomized rats. *Endocrinology* **115**:507-511,1994.

KANIS JA, JOHANSSON H, JOHNELL O, ODEN A, DeLAET Ch, EISMAN JA, POLS H, TENENHOUSE A: Alcohol intake as a risk factor for fracture.*Osteoporosis Int* **16**: 737- 742, 2005.

KIM MJ, SHIM MS, KIM MK, LEE Y, SHIN YG, CHUNG CH, KWON SO: Effect of chronic alcohol ingestion on bone mineral density in males without liver cirrhosis. *Korean J Intern Med* **18**:174-180, 2003

KRAML J: A semiautomated determination of phospholipids. *Clin Chim Acta* **13**: 442-448, 1966

MALIK R, GASSER RW, KEMMLER G, MONCAYO R, FINKENSTEDT G, KURZ M, FLEISCHHACKER WW:Low bone mineral density and impaired bone metabolism in young alcoholic patients without liver cirrhosis:A cross sectional study. *Alcohol Clin Exp Res* **33**:375-381, 2008

MUKAMAL KJ, ROBBINS JA, CAULEY JA, KERN LM, SISCOVICK DS: Alcohol consumption,bone density and hip fracture among older adults:the cariovascular Health study. *Osteoporosis Int* **18**:593-602, 2007.

ODVINA VC, SAFI I, WOJTOWICZ CH, BARENGOLTS EI, LATHON P, SKAPARS A, DESAI PN, KUKREJA SC: Effect of heavy alcohol intake in the absence of liver disease on bone mass in black and white men.*J Clin Endorcrinol Metab* **80**:2499-2503, 1995.

ORWOLL ES, BEVAN L, PHIPPS KR: Determinants of bone mineral density in older men *Osteoporos Int* **11**:815-821, 2000.

OTT SM.: Fractures after long term alendronate therapy. *J Clin Endocrinol Metab* **86:**1835-1842, 2001.

RUŽIČKA R, MICHALEK J, ROSENKRANCOVA J, SEDLÁČEK R.: Cross section parameters acquisition from digital images. Prague, Proc. of workshop 2002. Prague CTU, **B**:910-91, 2002.

SANTORI C, CECCANTI M, DIACINTI D, ATTILIA ML, TOPPO L, D ERASMO E, ŔOMAGNOLI E, MASCIA ML, CIPIRANI C, PRASTARO A, CARNEVALE V, MINISOLA S: Skeletal turnover Bone mineral density and fractures in male chronic abusers of alcohol. *J Endocrinol Invest* **31**:321-326, 2008.

ŠTĚPÁN JJ, ŠILINKOVA-MALKOVA E, HAVRANEK T, FORMANKOVA J,

ZICHOVA J, LACHMANOVA J, STRAKOVA M, BROULIK P, PACOVSKY V.: Relationship of plasma tartrate-resistant acid phosphatase to the bone isoenzyme of serum alkaline phosphatase in hyperpathyrodism.*Clin Chim Acta* **133**:189-200, 1983.

TURNER CH, BURR DB.: Basic biomechanical measurements of bone:A tutorial. *Bone* **14**:595-608, 1993

VANDERSCHUEREN DE, VAUTTERCK AMH, SUIKER WJ, WISSER LPC, SCHOT R, BOUILLON R: Bone and mineral metabolism in aged male rats.Short and long term effects of androgen deficiency.*Endocrinology* **130**:2906-2916, 1992.

WOSJE KS, KALKWARF HJ: Bone density in relation to alcohol intake among men and women in the United States. *Osteoporosis Int* **18**:391-400, 2007

.

.

.

Table 1.variables of initial and final body weight,density of femur,femur calcium a phosphate content,femoral bone strength,plasma ALP and ACP,AST,ALT,GGT and AMS in individual groups of animals

(means ± SD) $p < 0.01$ vs. intact animals

	Controls n-8	**Alcohol n-8**
Initial body weight (g)	220 ± 4.2	210 ± 5.0
Final body weight (g)	302 ± 5.2	290 ± 7.0
Femur dry weight (mg)	407 ± 17	357 ± 16•
Femur volume (ul)	379 ± 9	382 ± 15
Density of femur (g/ml)	1.620 ± 0.03	1.480 ± 0.04 •
Femur ash content (g/ml)	0.734 ± 0.07	0.645 ± 0.06 •
Femur calcium (mg/ml)	278 ± 11	245 ± 7 •
Femur phosphate (mg/ml)	123 ± 5	106 ± 7•
Bone strength (N/mm²)	178 ± 3.2	158 ± 5.6 •
Seminal ves. (mg/100 g b.w.)	232 ± 18	229 ± 0.4
ALP (ukat/l)	7.4 ± 0.16	6.4 ±0.58•
Tartrate ACP (ukat/l)	0.44 ± 0.04	0.60 ± 0.10 •
Calcium (mmol/l)	2.4 ± 0.1	2.5 ± 0.2
Phosphate (mmol/l)	1.2 ± 0.1	1.3 ± 0.1
AST (ukat/l)	3.1 ± 1.1	3,6 ± 1.01
ALT (ukat/l)	0.53 ± 0.16	0.71 ± 0.19
Amylasa (ukat/l)	27.9 ± 4.2	22.9 ± 4.07
GGT (ukat/l)	0.04 ± 0.05	0.06 ± 0.05

Table 2. Variables of morphometric measurements on femur in individual groups of animals mm (mean ± SE); $p < 0.01$ vs. intact animals

	Controls **n - 8**	**Alcohol** **n - 8**
Femur length (mm)	39.2 ± 0.2	39.0 ± 0.3
Outer diameter (mm)	4.18 ± 0.06	4.15 ± 0.05
Inner diameter (mm)	2.79 ± 0.08	2.88 ± 0.05 *
Cortical width (mm)	1.39 ± 0.02	1.27 ± 0.05 *