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Chronic Alcohol Consumption Induced Tibial Bone Loss and Resulted in Osteopenia in Growing Young Male Rats*

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To determine the deleterious effects of chronic alcohol consumption on bone especially in adolescents or young adults, 8 week-old Sprague Dawley male rats were fed with Lieber-Decarli ethanol liquid diet, containing 36% of energy as ethanol, *ad libitum* (ethanol group) or isocaloric normal liquid diet (control group) for 7 weeks. Body weight was significantly lower in ethanol group than that in control group after 1 week of feeding to the end. Liver weight and the ratio of liver or kidney weight to body weight in ethanol group were significantly increased when compared to those in control group. Ethanol group showed significantly lower serum protein and albumin levels ($p<0.05$), higher total cholesterol and HDL-cholesterol levels ($p<0.05$), and AST, ALT and BUN activities than control group, but serum triglyceride, Ca and phosphate levels were not different. Ethanol group had significantly lower tibial trabecular bone area and serum osteocalcin level than control group ($p<0.05$), but urinary Ca and NTx (cross-linked N-telopeptide of type I collagen) concentrations and serum testosterone and parathyroid hormone levels were not different. In conclusion, chronic alcohol consumption in growing young male rats may result in osteopenia through the reduction of bone formation as well as liver malfunction.

Key words : Chronic alcohol consumption, Osteoporosis, Trabecular bone, Osteocalcin

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INTRODUCTION

Until recently, osteoporosis in men was perceived as an insignificant problem and often was not diagnosed. But increasing longevity is likely to lead to future increases in the incidence of osteoporotic fractures in men as well as in women. Most osteoporotic men have several factors that contribute to the disease. One-half to two-thirds of men with osteoporosis have secondary osteoporosis.[1)] The most important factors include alcohol abuse, glucocorticoid excess and hypogonadism.[2)]

Chronic alcohol consumption can interfere with bone growth and replacement of bone tissue, resulting in decreased bone density and strength and increased fracture.[3-5)] It is known that alcohol-induced osteopenia in humans seems to be associated with multifactors, including hormonal changes, hepatic malfunction, and malnutrition.[6)] Alcohol has been shown to reduce bone formation in healthy humans and animals[7,8)] and decrease the proliferation of cultured osteoblastic cells.[9)] Indices of bone resorption may be increased, decreased, or unchanged.[7,10-13)] These skeletal changes, however, were reported to be independent of liver damage or calciotropic hormone levels.[14)]

According to the report on National Health and Nutritional Survey (NHNS) in Korea, the drinking population in adult males aged over 20 was increased from 74.8% in 1998 to 80.7% in 2003, and it is surprising that 6.0% of males aged 20-59 were drinking almost everyday in 1988 and 9.3% in 2003.[15,16)] It is also a serious problem that 40-45% of college male students were drinking more than once a week,[17,18)] and about 10% were drinking over 3 times a week,[19)] besides, 44.9% in males aged 20-29 usually skipped breakfast.[20)] Frequent alcohol consumption with lack of nutrient intake and bad eating behavior for a long time even in young age can result in many health problems and accelerate aging.

The chronic consumption of alcohol in adolescence

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and young adulthood has significant negative effects on bone growth that remains partially uncompensated in adulthood,[21] results in serious multiple health problems as well as early-onset of osteoporosis. Our purpose in this study was to determine the deleterious effects of chronic alcohol consumption on bone especially in adolescents or young adults, using a rat model fed with Lieber-Decarli ethanol liquid diet or isocaloric control liquid diet.

MATERIALS AND METHODS

1. Experimental Design, Animal and Diet

Sixteen male Sprague-Dawley rats, 8 weeks of age, were maintained under standard conditions (22-25 ℃, 12 hrs light/dark cycle). All animals were acclimatized to standard rat chow (Samyang, Korea) and water *ad libitum* for 1 week and then randomly allocated to control or ethanol group, 8 rats per each. Lieber-Decarli ethanol diet mixture and control diet mixture for rats were purchased from Dyets Inc. (Bethlehem, USA), and then liquid diet containing 1 kcal/ml by mixing with ethanol and water was made up just before feeding (Table 1). Lieber-Decarli ethanol liquid diet (#710260) contained 36% of energy from ethanol (5%, w/v). Control group was pair-fed with an isocaloric amount of control liquid diet (#710027) on the following day. Ethanol was introduced into the diet gradually starting from 0% (w/v) and increasing to a final 5% (w/v) over the first week. Rats were on those feeding regimens for 6 more weeks. At 3 days before sacrifice, 24-hr urine samples were collected from all rats using the metabolic cage. Rats were weighted and fasted overnight and then sacrificed by decapitation.

Table 1. Composition of experimental diets

(g/L)

	Control	Ethanol
Casein	41.4	41.4
DL-Methionine	0.3	0.3
L-Cystine	0.5	0.5
Cellulose	10.0	10.0
Maltose dextrin	115.2	25.6
Corn oil	8.5	8.5
Olive oil	28.4	28.4
Safflower oil	2.7	2.7
Mineral mix	8.75	8.75
Vitamin mix	2.5	2.5
Choline bitartrate	0.53	0.53
Xanthan Gum	3.0	3.0
Ethanol(95% EtOH)	-	67.3 ml
Water	Make up to 1L	

2. Sample Preparation

Blood was taken from the neck vessels and let stand for 30 min at room temperature. Serum was obtained by centrifugation at 1000 x *g for 20 min and stored at -70 ℃ in aliquots until analyses*. Liver, kidney, heart, lung, spleen and testis were removed and weighed quickly. The left tibia was dissected free of soft tissue, and then it was trimmed to expose the metaphysis and fixed in 4% formalin solution.

3. Histomorphometry

Bone histomorphometry was carried out as follows: After the fixation of tibia, 30 ㎛ cross-section of tibia was made and decalcified in 10% nitric acid for 6 hrs and then they were dehydrated with alcohol and embedded in xylene and paraffin, and cut into 4 ㎛ thick sections. Each section was stained with hematoxylin and eosin. The trabeculae area in cancellous bone of proximal tibia metaphysis was measured by quantitative image analysis system (Wild Leitz, Germany).

4. Biochemical Analysis

Serum total protein, albumin, blood urea nitrogen (BUN), total cholesterol, high density lipoprotein (HDL)-cholesterol, total triglyceride, calcium and phosphorus levels, and activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured with Technicon autoanalyzer (Technicon Inc., USA).

The level of osteocalcin (bone gla-protein) in serum was determined by a competitive radioimmunoassay using a commercial kit (Brahams, Germany). The osteocalcin molecules in serum competed with radioactive-labeled osteocalcin in the tracer (38-49^{125}I) for binding sites on the highly specific antibody (polyclonal, sheep) bound to the tubes. After the samples were incubated, non-bound tracer components were decanted and subsequently removed by washing. The residual radioactivity in the coated tubes was then measured using a γ-counter.

Serum parathyroid hormone or testosterone level was measured by immunoradiometric assay employing ^{125}I-labled affinity-purified polyclonal anti-PTH (1-34) antibody or testosterone antibody using a commercial kit

(DPC, USA) following the suggested protocol.

Concentrations of calcium and creatinine in urine were measured using a kit (Youngdong Pharm. Co., Korea). Cross-linked N-telopeptide of type I collagen (NTx) in urine was determined by ELISA method using a commercial kit (Brahams, Germany).

5. Statistical Analysis

Data were expressed as means ± SD. Statistical significance of difference between the means of two groups was determined by student's t-test using SAS program.

RESULTS

1. Body Weight Growth and Organ Weights

Body weight in ethanol group was significantly lower than that in control group after 1 week of feeding (Fig. 1), and the final body weight at 7 weeks of experiment was 401.7±26.7 g in ethanol group, which was significantly lower ($p<0.05$) than 440.4±13.8 g in control group (Table 2).

Liver weight ($p<0.05$) and its relative weight to body weight ($p<0.001$) were significantly higher in ethanol group than those in control group. Liver weight and its ratio to body weight in ethanol group were 10.2±0.4 g and 2.35±0.08%, respectively, while 9.5±0.5 g and 2.01±0.11% in control group. Relative weight of kidney to body weight (0.67±0.05%) was also significantly higher ($p<0.05$) than that in control group (0.62±0.05%).

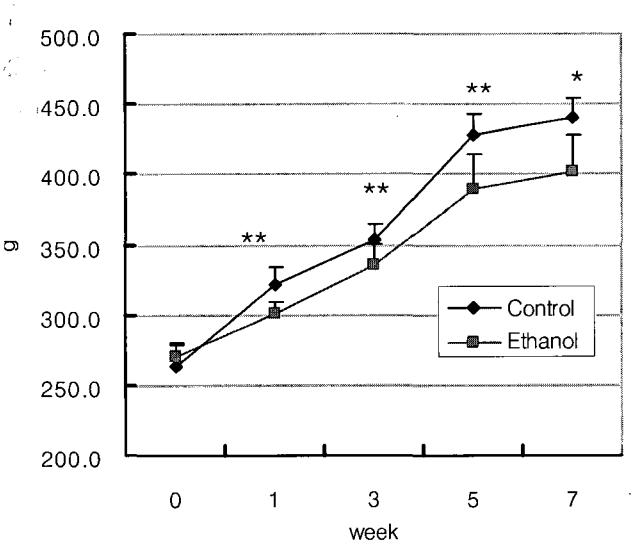


Fig. 1 Body weight changes during 7 weeks of experiment
Values are mean±SD of 8 rats each group.
Significantly different compared to control by t-test at *p<0.05 or **p<0.01

Table 2. Body weight and organ weights in rats during experimental periods

	Control (n=8)	Ethanol (n=8)
Body Weight (g)		
Initial	263.0±16.2	270.8± 8.8
1 week	321.7±12.4	300.8± 8.8**
Final	440.4±13.8	401.7±26.7*
Organ weight (g)		
Liver	9.5±0.5	10.2±0.4*
Heart	1.4±0.2	1.4±0.1
Lung	2.2±0.2	2.5±0.5
Kidney	2.9±0.2	2.9±0.2
Spleen	0.9±0.1	0.9±0.1
Testis	4.1±0.2	4.1±0.2
Relative organ weight (%)		
Liver/BW	2.01±0.11	2.35±0.08***
Heart/BW	0.31±0.04	0.32±0.03
Lung/BW	0.47±0.05	0.58±0.12
Kidney/BW	0.62±0.05	0.67±0.05*
Spleen/BW	0.18±0.02	0.20±0.02
Testis/BW	0.87±0.06	0.94±0.04

Values are mean±SD of 8 rats each group.
Significantly different compared to control by t-test at *p<0.05, **p<0.01 or ***p<0.001

The other organ weights, such as heart, lung, spleen and testis, were not different between the two groups (Table 2).

2. Serum Lipid Profile and other General Parameters

Hepatic synthetic function was evaluated by serum protein and albumin levels, and hepatocellular activity was evaluated by serum AST, ALT and bilirubin. Kidney function was judged by serum BUN level. Ethanol group

Table 3. Biochemical parameters in serum

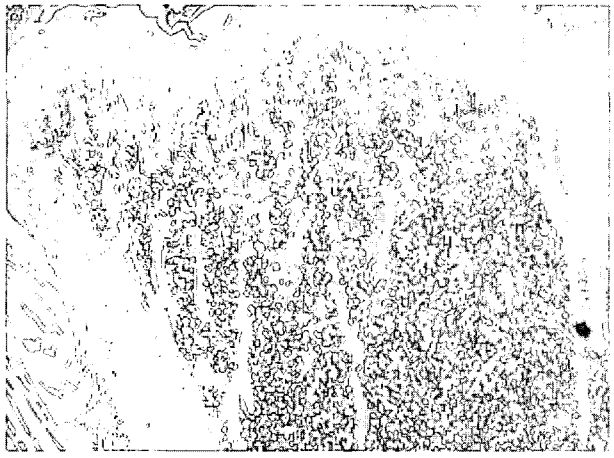
	Control (n=8)	Ethanol (n=8)
Protein *(g/dl)*	7.6±0.4	7.1±0.5*
Albumin *(g/dl)*	4.34±0.13	4.02±0.26*
AST *(U/L)*	377.4±54.0	479.7±76.0*
ALT *(U/L)*	59.0±16.2	132.2±53.5**
BUN *(mg/dl)*	14.5±0.8	21.9±2.3***
Total bililubin *(mg/dl)*	0.65±0.10	0.63±0.07
Glucose *(mg/dl)*	103.0±14.7	108.0±10.9
Triglyceride *(mg/dl)*	64.6±9.5	69.3±14.9
Total cholesterol *(mg/dl)*	54.3±8.9	72.0±12.4*
HDL-cholesterol *(mg/dl)*	24.3±3.3	31.0±4.3*

Values are mean±SD of 8 rats each group.
Significantly different compared to control by t-test at *p<0.05, **p<0.01 or ***p<0.001

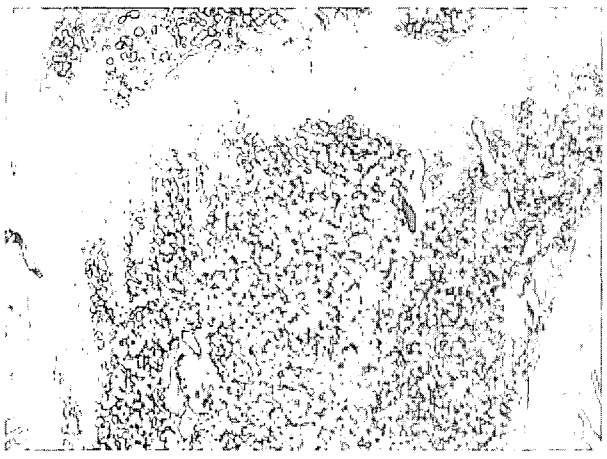
showed significantly lower serum protein and albumin levels ($p<0.05$), and higher total cholesterol and HDL-cholesterol levels ($p<0.05$) than control group, but serum triglyceride level was not different between the two groups (Table 2). Serum total cholesterol level was 72.0±12.4 mg/dl in ethanol group, while 54.3±8.9 mg/dl in control group. AST, ALT and BUN activities in serum were significantly higher in ethanol group compared to control group (Table 3).

3. Tibial Bone Histomorphometry

Fig. 2 shows the trabecular bone distribution in proximal tibia metaphysis stained by Hematoxylin Eosin. Ethanol group had significantly less trabecular bone in cancellous bone than control group, though control group had not normal distribution compared to chow diet-fed rats (not shown). The ratio of trabecular area within the reference area was calculated by image analyzer and then plotted in Fig. 3. Ethanol group had 12.47±3.24% of tibial trabecular bone area, which was significantly lower ($p<0.05$) than 17.05±2.93% in control group.



(A) Control



(B) Ethanol

Fig. 2 Photographs of longitudinal sections of the proximal tibia
Trabecular bone development in the proximal tibia metaphysis was observed under a microscope after H&E staining. (A) Control (B) Ethanol group

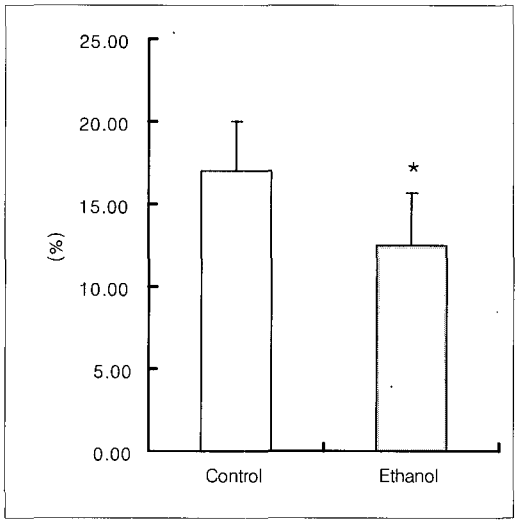


Fig. 3 The ratio of trabecular area in proximal metaphysis of tibia
Trabecular bone area within the reference area in proximal tibial metaphysis was calculated by a computer system attached to image analyzer
Values are mean±SD of 8 rats each group.
*Significantly different compared to control by t-test at $p<0.05$

4. Bone Metabolic Biomarkers and Hormones

Serum calcium and phosphorus levels were not different between ethanol group and control group (Table 4). Serum osteocalcin level was significantly decreased ($p<0.05$) in ethanol group compared to control group: serum osteocalcin level was 2.07±0.70 ng/ml in ethanol group and 2.51±0.91 ng/ml in control group. Urinary

Table 4. Biomarkers and hormone levels related to bone metabolism

	Control (n=8)	Ethanol (n=8)
Serum		
Ca (mg/dl)	10.5±0.7	10.2±0.6
P (mg/dl)	7.0±1.0	7.4±0.8
ALP (U/L)	133.6±13.8	141.2±40.6
Osteocalcin (ng/ml)	2.51±0.91	2.07±0.70*
Testosterone (ng/ml)	1.36±1.12	1.51±0.98
PTH(pg/ml)	1.97±0.92	1.40±0.81
Urine		
Ca/creatinine (mg/mg)	0.11±0.04	0.11±0.04
NTx/creatinine (nmol/mmol)	32.07±7.72	29.44±9.57

Values are mean±SD of 8 rats each group.
* Significantly different compared to control by t-test at $p<0.05$

calcium and NTx (Cross-linked N-telopeptide of type I collagen) levels were not significantly different between the two groups (Table 4).

Serum testosterone and parathyroid hormone levels were not different between the two groups (Table 4).

DISCUSSION

Studies have indicated that 10 to 74% of patients with nontraumatic osteonecrosis of the femoral head are associated with alcoholism,[22,23] and alcohol intake delays fracture healing associated with decreased bone density and mineral content.[24] But, the precise effects of alcohol on the skeleton are not known because it is difficult to distinguish the specific effects of ethanol from comorbidity factors such as poor nutritional status, intestinal malabsorption, smoking and abnormal liver function. In experimental studies using animals, giving alcohol as liquid diet is generally preferred due to easy preparation and dose calibration, and especially the pair-feeding of Lieber-Decarli liquid diet has been used for alcohol studies to exclude the effects of differences or deficiencies of calorie or nutrients intakes.

Changes in bone quality and quantity have been extensively studied with histomorphometric analysis, bone density measurement and peripheral quantitative computed tomography (pQCT). Also, several different urinary and serum markers for monitoring bone resorption and formation have been developed during the last decades. The most commonly used bone formation markers for rat serum are traditional alkaline phosphatase and osteocalcin.[25] Recently, a new urinary marker N-telopeptide (NTx) of type I collagen was developed for the detection of bone resorption. Disadvantages of urinary markers are large variations and a need for adjustment with creatinine concentration.[16]

In the present study, chronic alcohol consumption over 7 weeks significantly ($p<0.05$) reduced the trabecular area in tibia, resulting in a significant osteopenia, and the pair-fed control rats also showed mild osteopenia (Fig. 2, 3), which may be because pair-fed control rats were calorie restricted. Rats consumed ethanol liquid diet 50-70% as much as normal diet when they were fed *ad libitum* in the preliminary study of our lab. Dyer *et al.*[9] also observed that normal liquid diet fed rats for 6 weeks showed higher bone volume in tibia than ethanol liquid diet fed rats, but less than chow diet fed rats, *ad libitum*. In many studies, chronic alcohol consumption in growing male rats decreases bone strength, bone density, bone length and cancellous bone volume.[20,27-29] Turner *et al.*[4] reported that alcohol feeding to rats resulted in a dose-dependent decrease in cancellous bone volume compared with baseline values or controls. Sampson and Spears[21] reported that animals continuing to drink from early growth phases did not catch-up on bone development, and it was conceivable that when individuals stopped drinking, new bone would be formed at normal rats, but the bone growth that had been suppressed during the alcohol abuse was not recovered.

However, the mechanism for the changes due to alcohol remains unclear. In many cell culture studies, ethanol was reported to increase indices of bone resorption in isolated osteoclasts,[30] and decrease indices of differentiation and proliferation in osteoblast-like cells,[9,31,32] but did not have toxic effect on mature osteoblasts.[33] Recently, Dai *et al.*[13] reported ethanol induced IL-6 promoter in bone marrow stromal cells, either directly or indirectly. IL-6, in turn, promoted colony-forming unit granulocyte-macrophage (CFU-GM) formation and receptor activator of nuclear factor κB ligand (RANKL) expression, resulting in osteoclastogenesis and bone resorption.

Vitamin K-dependent protein, osteocalcin, which is synthesized by osteoblasts, is usually measured as a marker of osteoblast function. Most investigators reported a decrease in serum osteocalcin levels both in human[8,34] and rats,[35] that were correlated with liver function enzymes, ethanol dose, femur ash weight, bone strength and bone stiffness.[35,36] Others reported that osteocalcin and alkaline phosphatase and type-1 collagen gene expression in tibial metaphysis were not reduced by 4 weeks of ethanol consumption in adolescent male rats.[28] In the present study, serum osteocalin level was significantly reduced in ethanol-fed rats compared to that in control, but urinary Ca or NTx level, a biomarker of bone resorption, was not different. It is possible that the detrimental effects of ethanol consumption on these bone related biomarkers as well as tibial trabecular bone area should be mitigated because control group showed subnormal status of bone development due to the lack of diet feeding. It will be better that another control group fed *ad libitum* is added in the future study.

In some studies, alcohol intake showed both beneficial and hindering effects on the skeleton, depending on the dose and frequency. Moderate alcohol intake in elderly women was positively associated with spine bone mineral density,[37] and moderate alcohol intake to rats for 6 months without any detrimental effect to liver function increased tibial proximal metaphysis and bone

mechanical strength as compared to the control group, while the excess alcohol intake showed either the same or decreased proximal metaphysis and bone stiffness.[38]

On the other hand, it was reported that serum bilirubin level, and SGOT and alkaline phosphatase activities were all positively correlated with alcohol intake in humans.[38] In the present study, liver function seemed to be damaged by alcohol intake from the results of serum analysis. Lowered serum total protein and albumin levels, and increased AST and ALT activities in ethanol diet fed rats reflect the significant liver malfunction, and the increased liver weight in ethanol group could be possibly resulted from fatty liver. Chronic alcohol intake in growing rats also suppressed the overall growth, as shown in the delayed body weight gain in ethanol group (Fig. 1), which were consistent with the results in other studies.[21,28]

Additionally, concentrations of serum hormones associated with bone cell growth and differentiation are altered frequently. Serum parathyroid hormone (PTH) and testosterone levels were determined, but they were not affected by ethanol consumption in the present study. PTH is one of the principle regulators of calcium homeostasis in humans. In bone, PTH stimulates the release of calcium and phosphate, and in kidney, it stimulates the reabsorption of calcium and inhibits the reabsorption of phosphate. Extracellular calcium concentration is the most important physiological regulator of PTH secretion. Most investigators have reported that chronic alcohol abuse showed contrasting results, with PTH level being increased,[34,40] normal[41] or reduced.[42] In some studies, alcohol consumption antagonized hypercalcemic effects of PTH in rats and dogs.[35,43] Thus, reduced circulating levels of PTH and/or end organ resistance to the hormone may contribute to alcohol-induced bone loss. In the present study, serum PTH level tended to be lowered by ethanol consumption, although it was not significant. It is considered that PTH level is changed tentatively or acutely by alcohol consumption, or lack of Ca intake with alcohol consumption.

It was reported that acute and chronic ethanol consumption in peripubertal and adult males suppresses the hypothalamic-pituitary-gonadal function, resulting in low serum testosterone.[44,45] Whereas direct inhibition of testosterone steroidogenesis has been implicated,[46] the central effects on hypothalamic luteinizing hormone (LH) releasing hormone and pituitary LH have also been demonstrated in the adult male rats,[47] and in peripubertal male rats.[46] But, others reported that serum testosterone and luteinizing hormone (LH) levels were unaffected by 4-weeks of ethanol liquid diet consumption.[28]

Taken together, 7 weeks of alcohol consumption in growing young male rats resulted in severe osteopenia through the reduction of bone formation with delayed total body growth, and liver malfunction. These results showed that very severe damage of bone could occur in young alcohol abuser with bad eating behavior and smoking as well as old alcohol abuser. Therefore, interventions directed toward reducing or eliminating alcohol intake by modifying habitual drinking behaviors in adolescents or young adults should be needed to protect against the negative effects of alcohol on the growing skeleton.

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