

Fracture Healing and Bone Mass in Rats Fed on Liquid Diet Containing Ethanol

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Background: Studies in animal models for alcohol abuse have suggested that ethanol inhibits bone growth, decreases bone formation, and increases fracture risk.

Methods: Experimental tibia fracture healing in rats fed a liquid diet containing 7.2% ethanol for 8 weeks was investigated with histological and osteodensitometric studies with respect to the control group. After 4 weeks of vitamin A- and sucrose-enriched milk containing 7.2% ethanol feeding, we created closed tibia fractures, which were then fixed with intramedullary nails, in 10 rats. After a follow-up time of 4 weeks, the rats were killed for examination. The same procedure was performed in another 10 rats, which were fed on the same diet (isocaloric modified liquid diet) but without ethanol and used as the control group. A histological scoring system was developed for fracture healing.

Results: Histological evaluation of fracture region revealed an average fracture healing score of 1.9 in the ethanol-fed group versus 2.6 in the control group ($p = 0.014$). In the test group, dual-energy x-ray absorptiometry measurements in the fracture region showed a mean bone mineral density of 0.11 ± 0.03 g/cm², whereas it was 0.130 ± 0.051 g/cm² in the control group ($p = 0.000$). The mean bone mineral content in the fracture region was 0.103 ± 0.08 g/cm² in the test group versus 0.128 ± 0.06 g/cm² in the control group ($p = 0.000$). A significant correlation was found among histological scores, bone mineral density ($r = 0.62$, $p = 0.04$), and bone mineral content ($r = 0.63$, $p = 0.04$).

Conclusions: This study showed that rats fed on a diet mixed with ethanol have a histologically delayed fracture healing associated with decreased bone density and mineral content. Besides the negative effects of ethanol on bone metabolism, it also interferes with the fracture-healing process.

Key Words: Ethanol, Fracture Healing, Bone Mineral Density, Bone Mineral Content, Rat.

LONG-TERM ALCOHOL CONSUMPTION can interfere with bone growth and replacement of bone tissue (i.e., remodeling), resulting in decreased bone density and an increased risk of fracture (Kristensson et al., 1980; Maran et al., 2001; Nyquist et al., 1999; Sampson et al., 1998; Turner, 2000). However, pathogenesis of alcohol-associated bone disease and the effect of chronic alcoholism on fracture healing remain unclear. Alcohol-induced osteopenia seems to be multifactorial, including hormonal changes, liver cirrhosis, and malnutrition (Santolaria et al., 2000).

Histological evidence for decreased bone formation is supported by consistent findings of reduced serum osteocalcin, a protein secreted by osteoblasts and a biochemical marker of bone formation. A decrease in osteocalcin level after ethanol intake suggests that ethanol may have a direct toxic effect on osteoblast activity and proliferation (Bikle et al., 1993; Chavassieux et al., 1993; Gonzalez-Calvin et al., 1993; Medras and Jankowska, 2000; Pepersack et al., 1992).

A decline in both the number and function of the osteoblasts results in decreased bone volume and strength. The amount of bone mineral is parallel to the strength of healing bone tissue. Dual-energy x-ray absorptiometry (DEXA) is a useful method in evaluating both bone mass and strength of fracture healing (Aro et al., 1989; Markel and Chao, 1993). In this study, we evaluated the effects of ethanol on fracture healing and bone mass by using histological and DEXA techniques in rats fed a liquid diet containing 7.2% ethanol for 8 weeks.

METHODS

Adult male Wistar rats, weighing 218 to 280 g at the beginning of the experiment, were used. They were placed in a quiet, temperature ($21 \pm 2^\circ\text{C}$) and humidity ($60 \pm 5\%$)-controlled room in which a 12-hr light:dark cycle was maintained. They were individually housed and were submitted to forced alcoholization as previously described (Uzbay and Kayaalp, 1995). Briefly, all rats were fed by a modified liquid diet (MLD) for 7 days. The composition of the MLD was cow's milk, 925 ml; vitamin A, 5000 IU; and sucrose, 17 g. The MLD had 4.187 kJ/liter. At the end of 7 days, MLD with 2.4% ethanol (v/v; ethanol 95.6%, Tekel, Turkish State Monopoly, Izmir) was administered to group 1 (ethanol-treated rats, $n = 10$) for 3 days. Then, the ethanol concentration was increased to 4.8% for 3 days, and finally the rats completed the rest of the 8 weeks with a diet containing 7.2% ethanol. When the ethanol concentration was increased, sucrose was reduced to maintain the isocaloric of the diet. Group 2 (pair-fed control rats, $n = 10$) was fed an isocaloric MLD without ethanol throughout the experiment. MLD was prepared daily and presented at the same time of

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the day (1100 hr). The liquid diet was offered in special glass bottles to prevent spillage. Water was also freely accessed in different bottles. At the end of 4-week period, closed tibial fractures were developed and fixed intramedullarily. All rats were maintained on the liquid diet regimen for 8 weeks. The daily weights of the rats and the amounts of diet intake were recorded. All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health (National Research Council, 1985) and were approved by the Committee on Animal Research at İnönü University, Malatya, Turkey.

Surgical Technique

In this experimental study, a modified method of developing closed tibial fracture on rats, described by An et al. (1994), was used. In experimental fracture healing, it is important to obtain a standard fracture. We developed fractures with the same mechanism after gaining experience on more than 20 rats killed for other research in our laboratory. The rats in the test and control groups were anesthetized intraperitoneally with chloral hydrate 36 mg/ml (1 ml/100 g body weight). After local preparation of the skin, a 1-mm Kirschner wire was inserted percutaneously just proximal to the tibial tuberosity into the intramedullary canal, and a tibial fracture was created at the junction of the middle and distal third of the tibia with manual bending until the bone broke. After reduction of the fracture, the Kirschner wire was proceeded up to the ankle level. All fractures were stable at the end of the operation, and rotation was checked by comparing the alignment of the foot and the thigh. After surgery, the animals were given buprenorphine 0.2 mg/kg body weight at 12-hr intervals for 2 days. After 8 weeks, the rats were killed, and the broken tibias were preserved in 10% formaldehyde solution.

Osteodensitometric and Pathologic Investigation

The bones were evaluated with the small-animal software program of the Norland XR-36 bone densitometry equipment (Norland Medical Systems, Inc., White Plains, NY). After the densitometric evaluation of the samples in groups, statistical analyses were performed. After decalcification in formic acid and staining with hematoxylin and eosin, the bones were evaluated histologically. A scoring system was developed for fracture healing. This system included zero points for nonunion, one point for healing with complete fibrous callus, two points for some calcification with fibrous tissue dominance, and three points for completely calcified callus or new bone formation dominance (Figs. 1-3).

Statistics

Statistical analyses were performed with version 10.0 of SPSS for Windows (SPSS Inc., Chicago, IL). Weight, bone mineral density (BMD), and bone mineral content (BMC) values of each group were expressed as mean \pm SD and were compared by use of the Mann-Whitney *U* test for detecting differences between independent groups. In the evaluation of correlation, Spearman's correlation coefficient was estimated. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

The animals were in excellent health throughout the treatment. None of them developed infection. Neither group showed any difference in physical activity. At the beginning of the experiment, there was no difference between the weights of the test and control group rats ($p = 0.364$). At the time of killing, all animals were approximately the same weight (control and ethanol-fed rats; $p = 0.384$; Table 1). During the experiment, rats ingested 7.2% ethanol, 16.0 to 20.4 g/kg/day (mean, 17 g/kg/day, corresponding to 4.5 g/day; Fig. 4). The histological study



Fig. 1. Healing with fibrous callus: the entire section shows only fibrous tissue proliferation with new capillaries (hematoxylin and eosin, $\times 40$). Although none of control group rats showed this kind of healing, in ethanol-treated rats healing with fibrous callus was observed.



Fig. 2. Healing with fibrous tissue dominance and some chondrocytes together with matrix calcification (hematoxylin and eosin, $\times 40$). In both groups, this kind of healing was seen, although in the ethanol-treated group it was more obvious.

showed that the average score of fracture healing was 1.9 in ethanol-fed rats and 2.6 in control rats. This difference was statistically significant ($p = 0.014$; Fig. 5). The DEXA measurements of the fracture region demonstrated that tibial BMD ranged from 0.106 to 0.117 g/cm² (mean, 0.111 \pm 0.035 g/cm²) in ethanol-fed group and from 0.120 to 0.137 g/cm² (mean, 0.130 \pm 0.051 g/cm²) in the control group. The difference between the groups was statistically significant ($p = 0.000$; Fig. 6). Measurements with DEXA demonstrated that BMC in the fracture region was 0.089 to 0.116 g/cm³ (mean, 0.103 \pm 0.083 g/cm³) in the test group and 0.120 to 0.139 g/cm³ (mean, 0.128 \pm 0.060 g/cm³) in the control group (Fig. 7). The difference between the groups was also statistically significant ($p = 0.000$). A significant correlation was found among histological scores, BMD ($r = 0.64$, $p = 0.04$), and BMC ($r = 0.63$, $p = 0.04$).

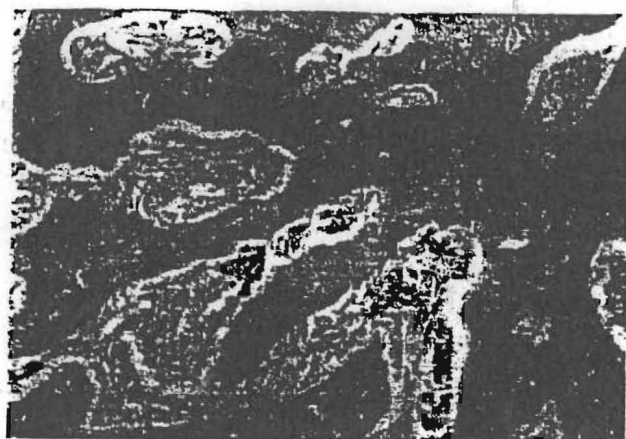


Fig. 3. Healing with new bone formation within bone lamellar and residual chondrocytes with mineralized matrix (hematoxylin and eosin, $\times 40$). Such healing was significant in control group rats without ethanol feeding.

Table 1. Body Weight and Weight Increase in Ethanol-Fed Rats and Controls During an 8 Week Fracture-Healing Study

Variable	Ethanol-fed (g)	Control (g)	P-Value
Initial weight	250.2 \pm 17.4	244 \pm 17.4	0.364
Last weight	277.5 \pm 15.7	272 \pm 16.6	0.384
Weight increase in 8 weeks	28 \pm 6.35	27.3 \pm 5.22	0.940

Data are presented as mean \pm SD.

DISCUSSION

The precise effects of alcohol on the human skeleton are not known because it is difficult to distinguish the specific effects of ethanol from comorbidity factors such as poor nutritional status, deficiency of electrolytes, malabsorption related to chronic pancreatitis, cigarette smoking, and abnormal liver function. In addition, it is often difficult to interpret the human studies because of wide variations of the patient population in age and duration and patterns of alcohol abuse (Turner, 2000). Although the effects of alcohol consumption on bone have been studied extensively in animal models, there is no ideal animal model that matches the human situation completely. Rats have been used for the study of the mechanisms by which alcohol affects bone mass, volume, and strength (Hogan et al., 2001; Kusy et al., 1989; Peng et al., 1982; Sampson, 1998; Tarkoff and Hoffman, 2000). In addition, rats are reported to be the most frequently used animals for studies of fracture healing (An et al., 1994; Janicke-Lorenz and Lorenz, 1984). In rats, chronic ingestion of alcohol causes tolerance development. To obtain this tolerance, alcohol may be given by inhalation, nasogastric tube, or liquid diet. In experimental studies, giving alcohol as liquid diet is generally preferred due to easy preparation and dose calibration. In the nutrition of mammals, milk contains almost all of the essential factors except vitamin A. In our study, an MLD reinforced with vitamin A and sucrose, described by Uzbay and Kayaalp (1995), was used. With this diet, the ethanol consumed by the rats ranged from 12.6 to 17.6 g/kg/day, and an average

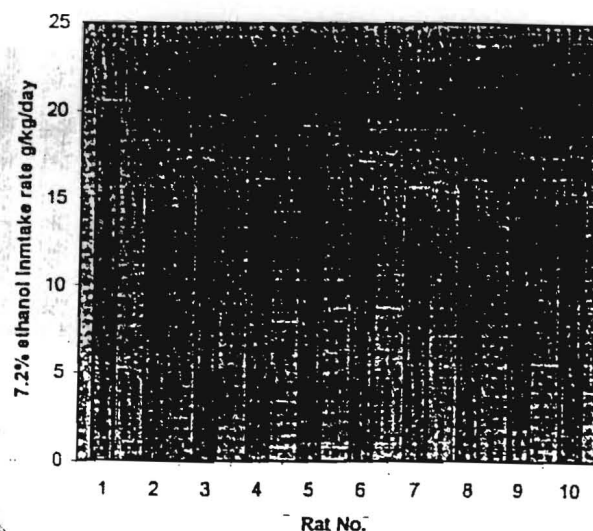


Fig. 4. The graph shows the daily amount of 7.2% ethanol intake (range 16.0–20.4 g/kg; mean, 17 g/kg/day, corresponding to 4.5 g/day).

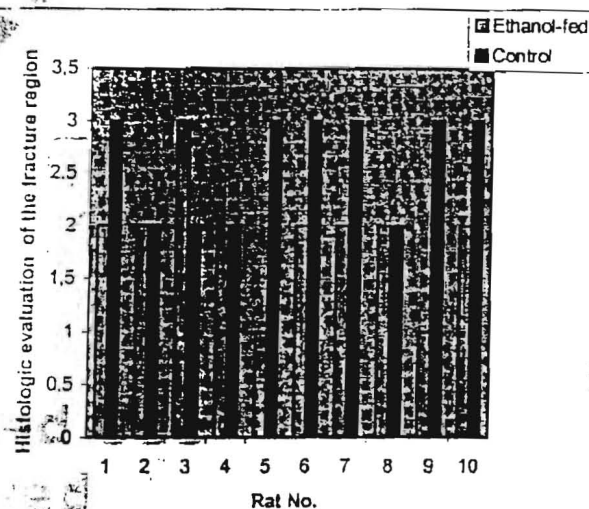


Fig. 5. The graph shows the histological scores of the fracture region. The average score of fracture healing was 1.9 in the ethanol-fed group and 2.6 in the control group ($p = 0.014$, Mann-Whitney U test). Although no nonunion was noted in the histological evaluation of fracture healing in relation to the level of repair process, it was observed that fracture healing in rats fed a liquid diet containing 7.2% ethanol progressed more slowly.

301 mg/dl blood alcohol level was obtained at the end of 4 weeks.

The dose dependency of ethanol-induced derangements of bone and mineral metabolism is unknown. In male chronic alcoholics, a daily consumption of ethanol exceeding 120 g/day (corresponding to 1.7 g/kg/day) has been reported to have a negative effect on BMD and biochemical markers of bone metabolism (Maran et al., 2001; Nyquist et al., 1996). However, it is difficult to demonstrate

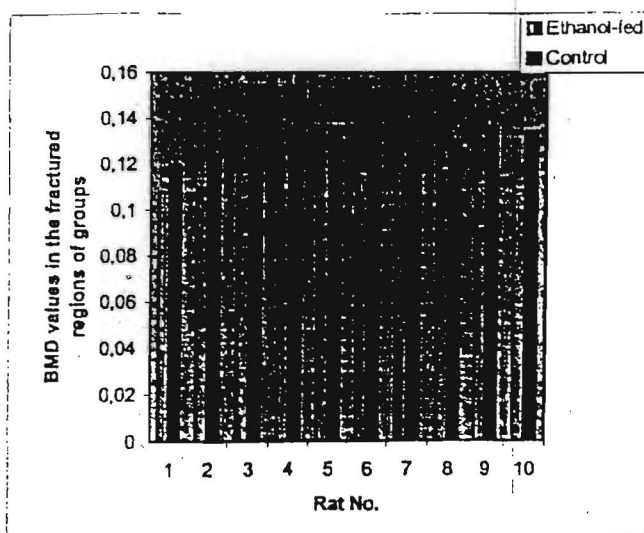


Fig. 6. The comparison of bone mineral density (BMD) values of the fracture region in both of the groups. Mean BMD was 0.111 ± 0.035 g/cm² in ethanol-fed rats and was 0.130 ± 0.051 g/cm² in the control group (mean \pm SD, $n = 10$). Differences between BMD values were significant ($p = 0.000$, Mann-Whitney U test).

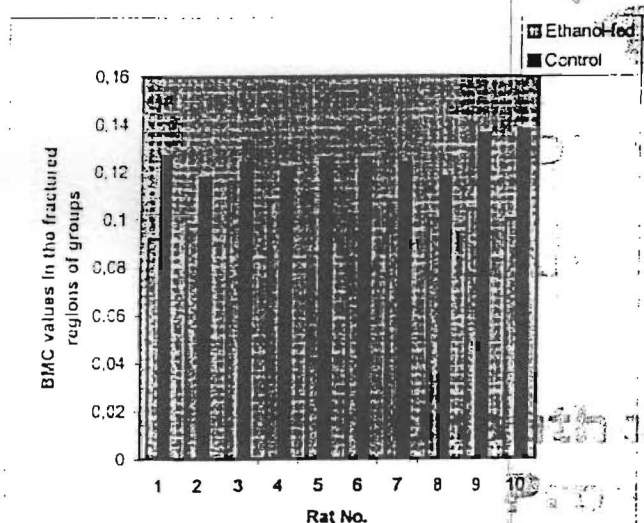


Fig. 7. The comparison of bone mineral content (BMC) values in the fractured region in both of the groups. Mean BMC was 0.103 ± 0.083 g/cm² in the test group and 0.128 ± 0.060 g/cm² in the control group (mean \pm SD, $n = 10$). Differences between BMC values were significant ($p = 0.000$, Mann-Whitney U test).

either alcohol-induced bone loss or an increased fracture rate in population-based studies (Hoidrup et al., 1999; Hollbrook and Barret-Cannor, 1993; Perry et al., 1999). Additionally, the level of ethanol consumption in rats cannot be related directly to humans because the rates of metabolism differ between the species. In the experimental study of Nyquist et al. (1999), male rats were fed a liquid diet containing 15% ethanol for 5 weeks. In their study of male rats, the daily intake of ethanol was 3.1 g (SD 0.2, corresponding to 7.2 g/kg/day). They found that this

amount of ethanol concentration in the liquid diet caused a significant reduction in BMD and an increase in healing time for transverse fractures. In another study, which used the Lieber-De Carli diet (Lieber and De Carli, 1989), inclusion of ethanol 9 to 15 g/kg/day and consumption of alcohol that comprised 36% of caloric intake for 3 weeks inhibited bone formation; this delayed bone mineralization and decreased bone strength (Turner et al., 1991). In our study of rats, the daily intake of 7.2% ethanol was 16.0 to 20.4 g/kg (4.5 g/day; corresponding mean, 17 g/kg/day). Using this liquid diet containing 7.2% ethanol in rats, we observed a significant reduction in BMD and BMC values with the DEXA method. We believe that this diet is very beneficial in experimentally evaluating the effects of ethanol on bone.

Although no nonunion was noted in the histological evaluation of fracture healing in relation to the level of repair process, it was observed that fracture healing in rats fed a liquid diet containing 7.2% ethanol progressed more slowly in correlation with BMC and BMD values.

In conclusion, our study showed that rats fed an ethanol mixed diet have histologically delayed fracture healing associated with decreased bone density and mineral content. Besides the negative effects of ethanol on bone metabolism, it also interferes with the fracture healing process. To explain the mechanism of this effect, we believe that more advanced biochemical and molecular research is needed.

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