

Effects of ethanol on the ultrastructure of the hamster femur

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Summary. Several previous studies have indicated that chronic ingestion of ethanol exerts harmful effects on bones. However, few data are available concerning the effects of ethanol on the ultrastructure of bone. To further elucidate the effects of ethanol on bone, we studied the morphology of femur in golden hamsters after long-term treatment with ethanol. Six-week-old male hamsters were divided into 4 groups. Ethanol-treated animals were given ethanol at a concentration of 7% with food and water freely available, whereas the pair-fed animals (weight-matched to ethanol hamsters) had tap water available as the only drinking fluid. The femur weight, blood ethanol and serum calcium concentrations were determined after 3 and 5 months. The bone mineral density (BMD) of the whole body was measured before and after the experiment. Femurs of both sides were dissected and processed for morphometric measurement, light microscopy, scanning and transmission electron microscopy. In the ethanol-treated hamsters, BMD of the whole body and the weight of femur tended to decrease when compared with those of the controls. Light microscopy and scanning electron microscopy showed that the trabecula in the distal end of the femur from ethanol-treated hamsters were thinner than those of the controls. We also observed the disrupted swollen mitochondria of the femoral osteoblasts and osteocytes in the ethanol-treated hamsters. No significant difference in serum calcium levels and femoral osteoclasts was found. These results indicate that long-term treatment with ethanol results in disruption of femoral osteoblasts and reduction of bone mass in trabecular bone.

Key words: Ethanol, Femur, Golden hamster, Ultrastructure

Introduction

Alcohol is consumed widely by young and old alike in our society. Although excessive acute or chronic ethanol drinking represents a serious hazard to health, alcohol is still the second most widely used psychoactive substance in the world, after caffeine (Samson and Harris, 1992). Chronic consumption of ethanol has been known to be a significant contributing factor to osteoporosis and bone loss. Bone samples from alcoholic patients have a low trabecular bone volume and normal or reduced osteoid (Arlot et al., 1983). Alcoholics demonstrate a significant increase in the incidence of bone fractures, especially in the femoral neck as compared to age- and sex-matched control populations (Bikle et al., 1985; Spencer et al., 1986; Laitinen and Välimäki, 1991; Sampson et al., 1998). The mechanism for ethanol-induced bone diseases seems to be complex: a direct effect of ethanol on bone cells and an indirect or modulating effect through calcium regulating hormones such as vitamin D metabolites, parathyroid hormone (PTH), and calcitonin (Bikle et al., 1985; Laitinen and Välimäki, 1991; Sampson, 1997; Sampson et al., 1998). Chronic alcoholics or chronic ethanol-treated laboratory animals have a PTH level that is frequently reported to be either elevated or at the upper end of normal (Williams et al., 1978; Feitelberg et al., 1987; Bikle et al., 1993). Previously, we found morphologically that short-term treatment with ethanol suppressed the hamster parathyroid activity and stimulated the thyroid C-cell, and long-term treatment stimulated the parathyroid function (Chen et al., 1999, 2000). Previous studies have shown that ethanol decreases trabecular bone volume and impairs bone formation in rats (Baran et al., 1980; Crilly et al., 1988; Peng et al., 1988; Diamond et al., 1989; Sampson et al., 1998). In addition, long-term treatment with ethanol results in decreased osteoblast number (Baran et al., 1980; Crilly et al., 1988) and increased mineralization lag time (Baran et al., 1980; Diamond et al., 1989). Cheung et al. (1995) reported that ethanol had an immediate direct effect on bone cells *in vitro*, resulting in increased resorption by osteoclasts. Most of these studies were conducted by

light microscopy and morphometrical method. Few data are available concerning the effects of ethanol on the ultrastructure of bone. The current study is concerned with the morphological, especially the ultrastructural changes in hamster femur after long-term treatment with ethanol to determine whether ethanol directly affects the osteoblast, osteocyte, and osteoclast.

Materials and methods

Six-week-old male golden hamsters with an average body weight of 92 g were randomly divided into 4 groups of 15 animals each. Hamsters were housed in stainless steel cages that were equipped with depressions where food pellets (CE-2, Clea Japan Inc.) were placed. Experimental animals were given tap water and 7% ethanol solutions provided in calibrated glass bottles fitted with ball bearing tubes to limit spillage. The ethanol solution intake by a hamster averaged 11.2 ml per day. Control hamsters had tap water available as the only drinking fluid. Three and 5 months later, the blood was taken from heart under sodium pentobarbital anesthesia. The blood ethanol concentrations were determined by gas chromatography, and the serum calcium concentrations were measured using a Corning calcium analyzer 940. The bone mineral content (BMC) and the bone mineral density (BMD) of the whole body were measured by Dual Energy X-ray Absorptiometry (DXA) using a Toyo Medic QDR type 2000 before and after the experiment. The femurs of both sides were dissected and processed for morphometric measurement, light microscopy and scanning and transmission electron microscopy.

A total of 5 animals in each group were used for SEM observation. The distal femurs were trimmed in the sagittal plane to expose the epiphyseal and metaphyseal trabecular bone and treated for about 4 hours with 5%

sodium hypochlorite (commercial bleach). The bones were dehydrated in acetone and critical-point dried, mounted on stubs, and coated with gold/palladium using a sputter coater. The bones were examined in a Hitachi S-3500 N scanning electron microscope.

A total of 5 animals in each group were used for morphometric measurement. The femurs were dissected and cleaned of soft tissue. The femur length was measured from the tip of the greater trochanter to the distal surface of the lateromedial condyles. The dry weight of the femur was measured on a precision balance. A total of 5 animals in each group were used for LM and TEM observation. The femurs were cut in half longitudinally with a razor blade. The distal metaphysis was minced quickly and fixed in freshly prepared 2.5% glutaraldehyde with 0.2M cacodylate buffer at pH 7.2 overnight and then decalcified with 10% EDTA-Na₂ solution. The tissues were postfixed in 1% OsO₄ in 0.2M cacodylate buffer (pH 7.2), dehydrated through ascending concentrations of acetone, and embedded in Epon 812. Semi-thin sections were stained with Azur II and observed with a light microscope. Ultrathin sections were prepared on a Porter-Blum MT-1 ultramicrotome. After being stained with uranyl acetate and lead salts, the sections were examined under a Hitachi H-800 electron microscope.

All data are presented as mean±SEM. Statistical analysis was done using StatView J-4.5 (Abacus Concepts). Group mean values were compared by one-way analysis of variance (ANOVA) and Fisher's PLSD test for multiple comparisons as the *post hoc* test. A *p* value < 0.05 was considered statistically significant.

Results

Table 1 shows the blood ethanol concentration, serum calcium concentration and BMC, BMD of whole

Table 1. Blood ethanol (mg/ml), serum calcium concentration (mg/100 ml) and whole body BMC (gram), whole body BMD (mg/cm²) before (B) and after (A) experiment.

TIME	PROTOCOL	ETHANOL	CALCIUM	BMC (B)	BMC (A)	BMD (B)	BMD (A)
3 m	Control	<0.1	10.88±0.13	1.956±0.198	3.457±0.122	95.8±3.6	117.5±4.4
	Experiment	0.33±0.06*	11.00±0.19	1.970±0.194	3.420±0.104	96.2±4.8	110.8±3.5
5 m	Control	<0.1	10.93±0.11	2.001±0.200	3.643±0.251	95.7±5.3	121.3±3.4
	Experiment	0.33±0.06*	10.77±0.13	2.003±0.199	3.509±0.249	94.3±2.9	111.2±3.3

Values are shown in mean±SEM. *: *p*<0.05.

Table 2. Comparison of body weight (gram), femur weight (mg) and femur length (mm) before (B) and after (A) experiment.

TIME	PROTOCOL	BODY WEIGHT (B)	BODY WEIGHT (A)	FEMUR WEIGHT	FEMUR LENGTH
3 m	Control	91.5±10.3	145.7±18.9	214.4±5.4	27.00±0.84
	Experiment	92.6±9.8	146.1±12.4	207.4±6.1*	27.07±0.71
5 m	Control	93.3±11.5	158.6±21.8	223.3±7.3	27.70±0.90
	Experiment	91.9±10.6	152.9±14.4	211.2±7.5*	27.66±0.95

Values are shown in mean±SEM. *: *p*<0.05.

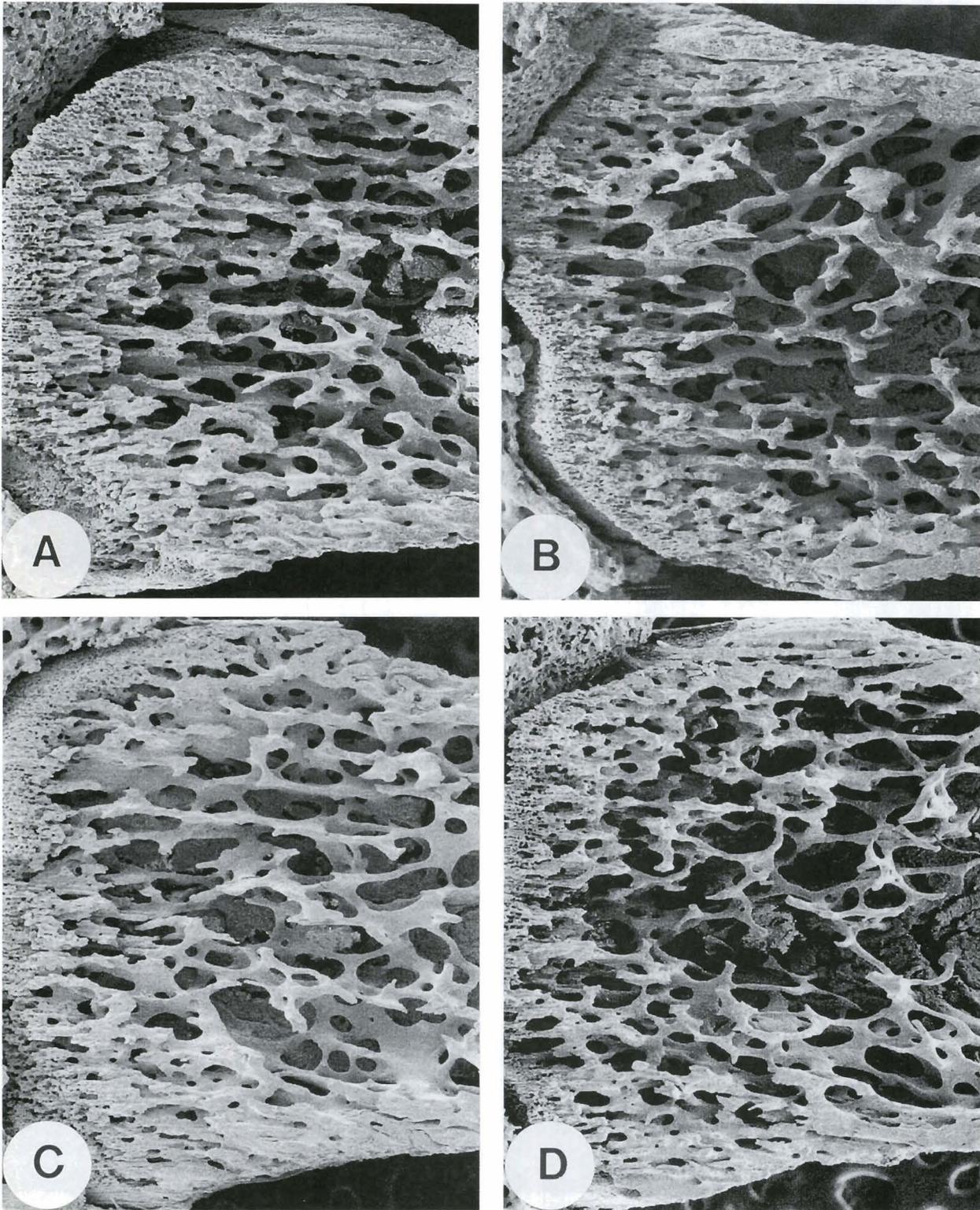


Fig. 1. Scanning electron micrographs of the distal metaphysis of hamster femurs. A. 3-month-control hamster. B. 3-month-ethanol-treated hamster. C. 5-month-control hamster. D. 5-month-ethanol-treated hamster. The amount of trabecular bone is reduced in ethanol-treated hamster. x 32

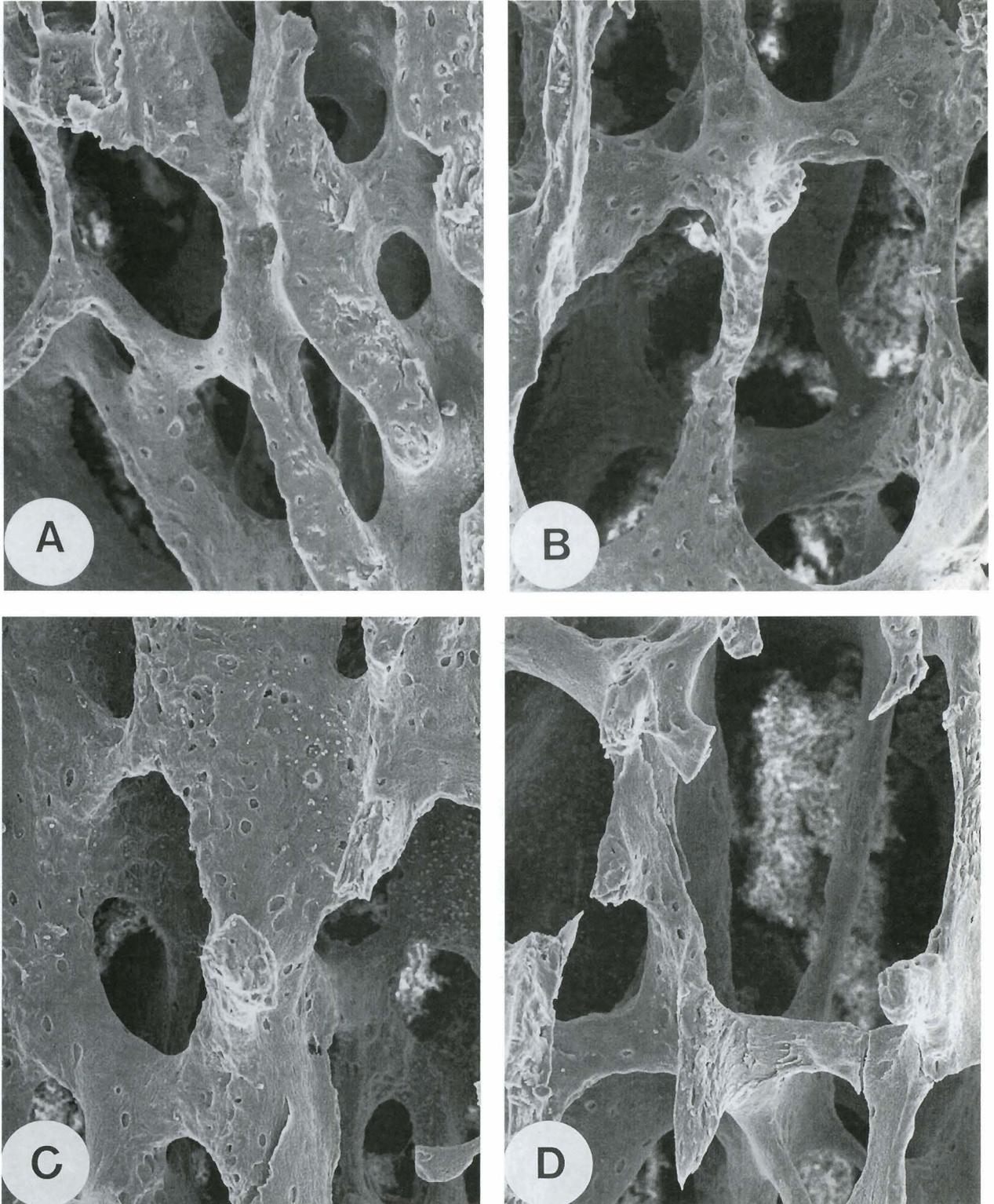
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Fig. 2. Scanning electron micrographs of the distal metaphysis of hamster femurs. **A.** 3-month-control hamster. **B.** 3-month-ethanol-treated hamster. **C.** 5-month-control hamster. **D.** 5-month-ethanol-treated hamster. The trabecula are thin and discontinuous in B and D, while the trabecula are thick and continuous in A and C. x 200

body. The blood ethanol concentration of animals after 3- and 5-months treatment with ethanol was significantly high ($p < 0.05$) compared with that of the respective control animals. There was no significant difference between the control and ethanol-treated groups regarding the serum calcium concentration and BMC. The BDM of whole body seemed to be decreased in 3- and 5-month-treated animals when compared with that of the controls. The body weight, femoral weight and femoral length are shown in Table 2. There was no significant difference between control and ethanol-treated groups concerning body weight and femoral length. The dry femoral weight of animals after 3- and 5-months treatment with ethanol was significantly decreased ($p < 0.05$) as compared with that of the respective control animals. The trabecular bone was markedly reduced in ethanol-treated animals under light microscope.

In SEM images from the distal part of the femurs, the amount of trabecular bone was clearly reduced in the metaphyseal region of 3- and 5-month-treated hamsters when compared with the controls (Figs. 1, 2). The trabeculae of ethanol-treated animals were thin, and discontinuous, while the controls were thick and

continuous (Figs. 1, 2). There was no significant difference in the amount of cortical bone between control and ethanol-treated animals.

Femoral osteoblasts, osteocytes, and osteoclasts were observed in TEM. In the control animals, the osteoblasts had abundant cisternae of granular endoplasmic reticulum and prominent well-developed Golgi complexes. Mitochondria were large, with well-developed cristae (Fig. 3A). Most mitochondria appeared healthy and intact (Fig. 3A). In 3- and 5-month-treated hamsters, the osteoblasts contained abundant granular endoplasmic reticulum. Mitochondria demonstrated pronounced vacuolization and some were uniformly swollen and disrupted (Fig. 3B). Osteocytes in control animals were smaller and had a narrow rim of cytoplasm with only a few mitochondria and very few cisternae of granular endoplasmic reticulum. Mitochondria appeared normal, with apparent cristae. Osteocytes were situated in the lacunae within bone and were connected to adjacent cells by numerous long cytoplasmic projections which traveled in canaliculi through mineralized matrix (Fig. 4A). The osteocytes of 3- and 5-month-treated animals showed a little flattened

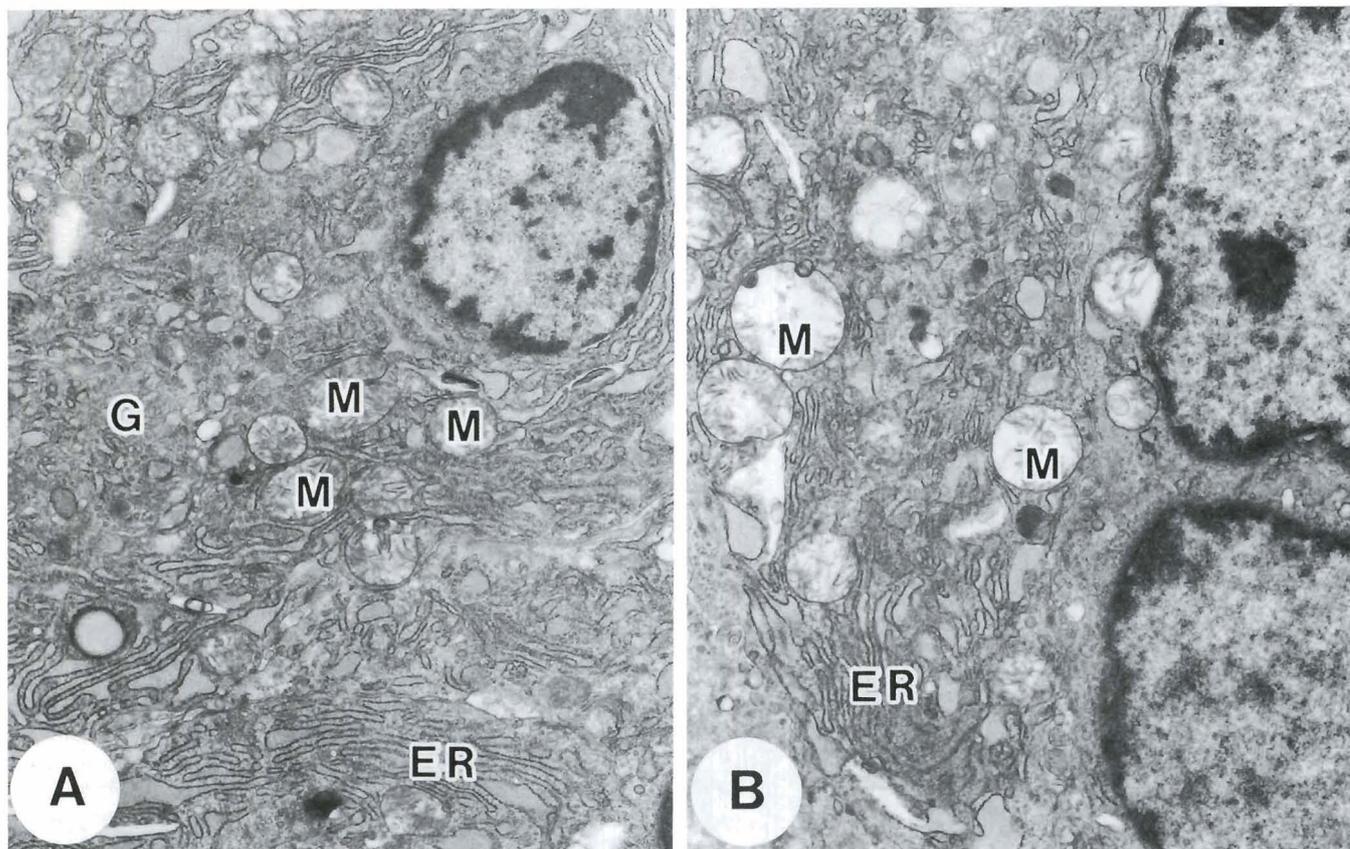


Fig. 3. Transmission electron micrographs of osteoblasts in femur. **A.** 5-month-control hamster. Abundant cisternae of the granular endoplasmic reticulum (ER) and well-developed Golgi complex (G) are observed in the cytoplasm. Mitochondria (M) appear normal. **B.** 5-month-ethanol-treated hamster. The cisternae of the granular endoplasmic reticulum (ER) are prominent. Mitochondria (M) are swollen and disrupted. $\times 8,800$

appearance. Some mitochondria were swollen, disrupted and demonstrated vacuolization (Fig. 4B). The morphology of femoral osteoclasts in both control and ethanol-treated groups was similar. Osteoclasts were generally multinucleated with a ruffled border closely applied to the resorbing surface of bone. Numerous mitochondria with a normal profile were present in the cytoplasm. There were no swollen mitochondria in osteoclasts of either control or ethanol-treated animals.

Discussion

Our results showed that long-term treatment with ethanol produced ultrastructural changes in hamster femurs. These changes were associated with reduced bone mass and osteoporosis. Previous studies indicated that chronic consumption of ethanol greatly reduced values in bone morphology, such as length and volume,

as well as weight, density, and morphometric parameters in laboratory animal models (Baran et al., 1980; Arlot et al., 1983; Peng et al., 1988; Diamond et al., 1989; Sampson et al., 1998). The pathogenesis of ethanol-induced bone mass loss and osteoporosis seems to be complex. More direct studies of osteoblastic function indicated that ethanol significantly reduced the number of osteoblasts, and directly inhibited the osteoblastic cell proliferation and activity (Baran et al., 1980; Crilly et al., 1988; Klein, 1997). Overall, ethanol appears to suppress osteoblast function resulting in decreased bone formation. Long-term treatment with ethanol also influenced the osteocytes, inducing the morphological changes of rat osteocytes (Lorenz and Janicke-Lorenz, 1983). Effect of long-term treatment with ethanol on bone resorption is even more unclear. Long-term treatment with ethanol caused an increase (Baran et al., 1980; Bikle et al., 1993), a decrease (Mobarhan et al.,

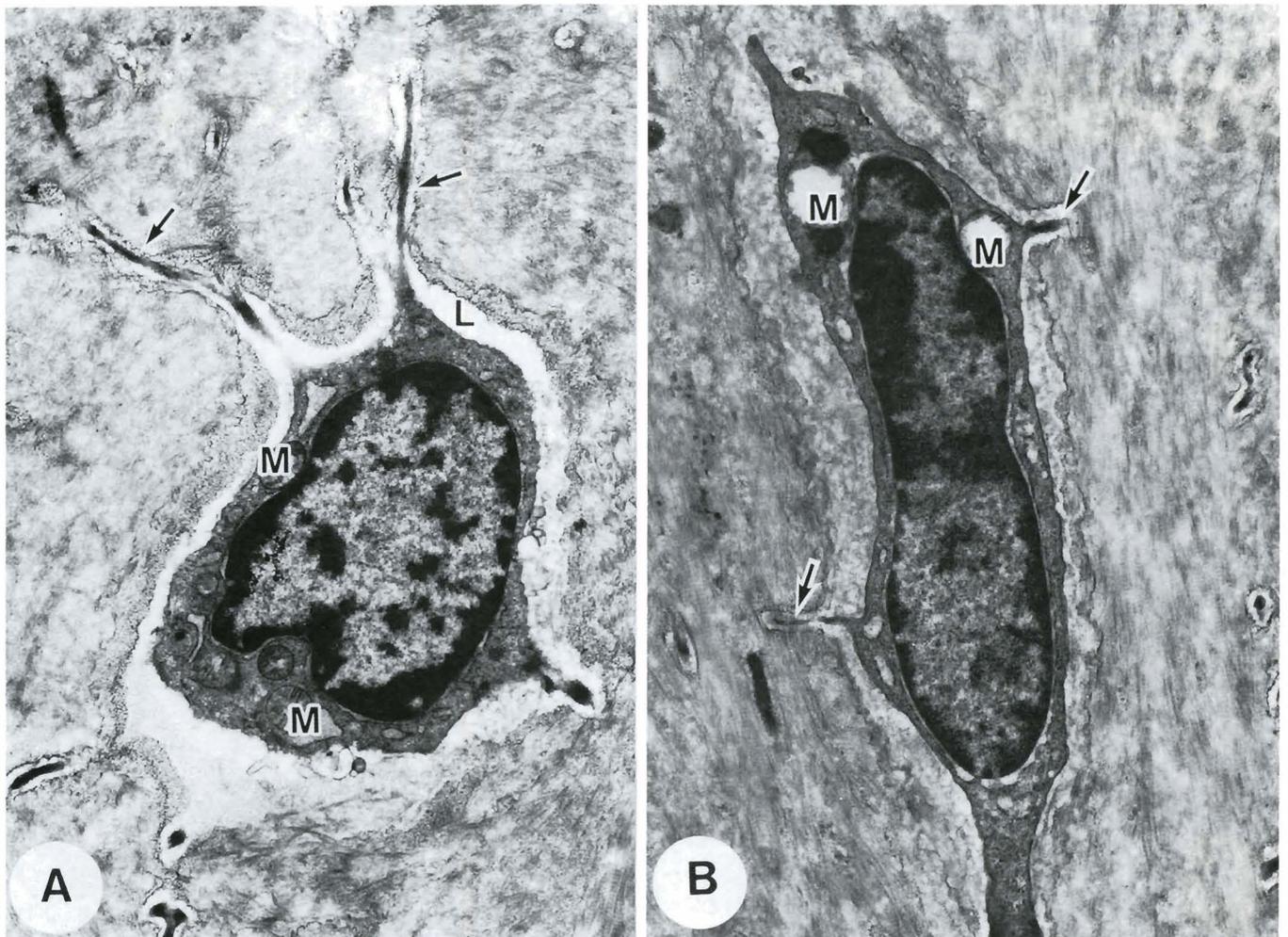


Fig. 4. Transmission electron micrographs of osteocytes in femur. **A.** 5-month-control hamster. Osteocyte, situated in a lacuna (L), extends several long processes (arrows). The cytoplasm contains a few normal mitochondria (M). **B.** 5-month-ethanol-treated hamster. Osteocyte is seen a little flattened. Some mitochondria (M) are swollen and disrupted. Arrows: cell processes. x 12,000

1984), or no change in bone resorption (Crilly et al., 1988; Diamond et al., 1989).

Recently, we found morphologically that long-term treatment with ethanol stimulated the parathyroid function, with no significant changes of thyroid C-cell (Chen et al., 1999, 2000). We consider that long-term consumption of ethanol stimulates the synthesis and secretion of PTH. PTH is a principal hormone responsible for calcium homeostasis in mammals through its action on target cells in bone and kidney. PTH stimulates osteoclast-mediated bone resorption. It is conceivable that long-term consumption of ethanol leads to bone loss and osteoporosis partly mediated by the action of PTH. Considering the morphological changes of osteoblast, osteocyte, and osteoclast, it is suggested that long-term consumption of ethanol has a direct toxic effect on bone and an indirect effect mediated by PTH and/or other bone regulating hormones.

As to the ultrastructural mechanism, it is well documented that mitochondria are sensitive indicators of cellular pathology. Under certain circumstances, mitochondria respond to cellular insult, resulting in the formation of swollen mitochondria and giant mitochondria. Several previous studies indicated that ethanol induced morphological and functional changes of mitochondria in hepatocytes and pancreatic exocrine cells (Schilling and Reitz, 1980; Tandler et al., 1996; Puzziferri et al., 2000). Findings include markedly enlarged and structurally disrupted mitochondria in liver and pancreatic exocrine cells, alteration in several enzymatic activities and contents of cytochrome in liver mitochondria, severe disruption of mitochondrial functions resulting in a notable fall of oxidative phosphorylation (Schilling and Reitz, 1980; Tandler et al., 1996; Puzziferri et al., 2000). In the present study, we observed osteoblasts, osteocytes, and osteoclasts in femur from control and ethanol-treated hamsters in the TEM. The swollen mitochondria were observed in osteoblasts and osteocytes of 3- and 5-month-treated animals. Osteoblasts manufacture bone matrix and their mitochondria are intimately involved in the calcification process, although the exact mechanisms remain unknown. Long-term treatment with ethanol may have adversely affected the mitochondria of osteoblasts in hamsters of our study, thus impairing their ability to manufacture bone. We did not find any changes in the mitochondria of osteoclasts of 3- and 5-month-treated animals. It was supposed that ethanol might have a preferential effect on mitochondria of certain cells.

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