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A Potential Experimental Model For the Study of Osteopenia in CCl_4 Liver Cirrhotic Rats*

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In order to search for an experimental model to further investigate the osteopenia associated to liver cirrhosis (LC), this study has been focused on investigating the occurrence of bone disorders in male rats to which LC histologically confirmed was induced through the validated procedure of CCl_4 inhalation. Length, anteroposterior and lateromedial diameters, densitometry, mechanical stress resistance, hydroxyproline (OHprol) and calcium and phosphate contents were measured in femurs from control (n = 10) and liver cirrhosis rats (n = 10). It has been found that femurs from liver cirrhosis rats showed a significant reduction ($p < 0.01$) in bone weight (0.254 ± 0.003 vs 0.230 ± 0.004 g/100 g b.w.), anteroposterior (4.08 ± 0.06 vs 3.69 ± 0.05 mm) and lateromedial (5.33 ± 0.05 vs 5.08 ± 0.04 mm, $p < 0.05$) diameters, resistance to mechanical stress (405.8 ± 9.5 vs 332.5 ± 9.1 N) and total densitometry (0.416 ± 0.005 vs 0.381 ± 0.004 g/cm^2). However, no significant differences were observed in bone length, calcium, OHprol and phosphate (all expressed as mg/100 mg fresh bone tissue) contents. Therefore, the proteins matrix to mineral contents ratio was not altered. These results indicate that in this model of experimental liver cirrhosis there is osteopenia characterized by bone frailty and reduced thickness, and it could offer an experimental model to study bone changes associated to liver cirrhosis.

Key words: Liver cirrhosis, CCl_4, Osteopenia, Bone densitometry, Rat.

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Regardless of its etiology, osteopenia has an elevated rate of prevalence among patients suffering from chronic liver disease. The pathogenesis of osteopenia in liver cirrhosis (LC) is not clearly under-

stood although it appears to be multifactorial (13, 21). Initially it was thought that the osteopenia was caused chiefly by an impairment of mineral deposition similar to that of osteomalacia (15, 29), which occurs in adults due to the vitamin D deficiency, and/or its activation. However, more recent human studies suggest that osteoporosis is the most common form of bone disease in patiens with liver disease (10, 12). Independently of its cause, severe osteopenia is a well-known risk factor for bone fractures, this being an additional complication which contributes to increase morbidity and mortality of patients already weakened by chronic liver disease (6, 24). An important step toward prevention of bone disorders in LC is to get an experimental model which allows to advance by improving the knowledge of the mechanisms leading to osteopenia in LC. So far, a valid model for studying osteopenia in liver disease is lacking. The aims of this study were: 1) to evaluate whether there is osteopenia in CCl_4-induced LC rats; and 2) to characterize changes in bone morphometry and composition in this animal model by assessing different bone parameters in order to evaluate whether rats with CCl_4 induced LC represent a useful tool to study bone disease associated to LC.

Materials and Methods

Animals and experimental design.– All the experimental procedures were performed in conformity with The Guiding Principles for Research Involving Animals. Liver cirrhosis was induced by carbon tetrachloride (Merck) inhalation in 30 Wistar male rats (5 week-old) weighing about 100-120 g. The organic solvent was administered twice a week, with a progressively increasing exposure time, ranging from 30 s to 5 min, during an 11 week period (18). From this time on, animals were maintained cirrhotic by a 3 min reexposure to CCl_4 weekly during 1 more month. To accelerate the development of liver cirrhosis, phenobarbital (Luminal, Bayer) was added to drinking water (400 mg/L) beginning one week before the first CCl_4 exposure and throughout the entire induction period (4, 18). Animals were housed in cages placed in a room provided with a 12 h light-darkness cycle and a constant room humidity and temperature (20 °C). Both water and food (standard semipurified diet for rodents, B.K. Universal, Sant Vicenç dels Horts, Spain) were given *ad libitum* until rats were sacrificed at the end of the 15th week. The mortality during this method of LC induction is about 30 %.

Immediately after decapitation, liver and femurs were carefully dissected out and weighed. A sample of the left major liver lobe was then excised and processed for histological examination. Tissue specimens were immediately frozen by immersion in liquid N_2 and stored at -80 °C until analysis.

Livers from CCl_4-treated rats were scored from 0 to 4, according to histopathological findings as follows (8): normal liver, 0; pericentral venous fibrosis, 1; fibrous septa not forming full-shaped regenerative nodules, 2; established cirrhosis with fibrous septa delimiting full-shaped regenerative nodules of variable size (mixed micro-macronodular pattern), 3; and established cirrhosis with fibrous septa delimiting full-shaped regenerative nodules with a difuse micronodular pattern, 4.

The preestablished criteria for retrospective inclusion of animals were the presence of histologically proven liver cirrhosis (score 3 or 4). Finally, only 10 cirrhotic rats (CI, n = 10) were chosen. Ten healthy rats of similar weight were used as control (CO, n = 10).

Morphological parameters, mechanical resistance and densitometry of bone.- Femur length was measured from the major trochanter to the end of distal epiphysis. Anteroposterior (AP) and lateromedial (LM) diameters were assessed at the center of medial diaphysis. All measurements were performed with a precision calliper, Mituyoto (± 0.05 mm) (30). Bone mechanical resistance was assessed with the aid of an Instron apparatus (Instron corporation, UK; model 4502), provided with three leaning points. Femurs were placed on the AP diameter leaned upon two points separated by 2.5 cm, the loading was executed on the anterior face of the diaphyseal middle point, the compression velocity being 2.5 mm/min. Resistance to mechanical stress was expressed as force (N) which was needed for breaking femurs.

Bone density was determined by dual-photon absorptionmetry (22) using a DP-3 instrument (DXA-HOLOGIC QDR 1000^{TM}, Hologic, INC, Waltham, MA, USA). Measurement stability was controlled by scanning a phantom every time. Femur densitometries were performed at environment temperature. Bones were placed on a polyester resin cell, opaqueless at X-ray. An "Ultra High Reach Program" (NEC APC IV, Power Mate 2, Boxboro, MA) was used to process the data. Bone density results were expressed as g/cm^2.

Analytical Methods. Liver hydroxyproline was quantified by HPLC using the Pico-Tag method for amino acids analysis (Waters. Division of Millipore, USA). To avoid the interference of fatty degeneration in cirrhotic livers, liver OHprol content was expressed as µmol/mg liver protein. Liver protein concentration was determined by the method of BRADFORD (3).

Bone OHprol was determined in whole tissue according to the method of WOESSNER (31) and it was expressed in mg/100 mg fresh bone tissue. Total bone calcium contents were assessed by atomic absorption espectrophotometry and total bone phosphate by the colorimetric reaction of acidic ammonium molybdate (26). In both determinations 200 mg of bone tissue was used. Femurs were calcinated and then dissolved in 0.1 % lanthane chloride for calcium assessment or in bidistilled water for phosphate determination. Results were expressed in mg/100 mg of fresh bone. All determinations were made in triplicate.

Statistical Analysis.– Data are expressed as mean±SEM. In order to assess intragroup comparisons, data were analyzed using a two-tailed Student's *t* test for unpaired data. Statistical significance was considered at $p < 0.05$ or less.

Results

The occurrence of LC (score 3 or 4) was confirmed in CI rats by histological examination. Animals which did not reach this LC score were excluded of the study. Besides, as compared to CO animals, all included LC rats showed significantly higher liver OHprol content (4.78 ± 0.70 vs 1.09 ± 0.19 µmol/mg liver protein).

Table I shows that LC rats exhibited a significant reduction in femur weight, as well as in lateromedial and anteroposterior bone diameters. However, no differences were observed in bone length. In addition, resistance to mechanical stress was significantly decreased in CI rats (405.8 ± 9.5 vs 332.5 ± 9.1 N).

Figure 1 displays total and regional femur densitometries. As compared to CO rats, total (0.416 ± 0.005 vs 0.381 ± 0.004), as well as diaphyseal (0.404 ± 0.007 vs

Table I. *Body weight, and femur weight, length, anteroposterior (AP) and lateromedial (LM) diameters of healthy control and CCl_4 liver cirrhotic rats.* Values are means ± SEM (n = 10).

	Control	Cirrhotic
Body weight (g)	634.70 ± 7.38	648.2 ± 4.71
Femur		
Weight – (g)	1.615 ± 0.029	1.493 ± 0.024**
– (g/100 g b.w.)	0.254 ± 0.003	0.230 ± 0.004**
Length (mm)	43.672 ± 0.301	43.804 ± 0.413
AP diameter (mm)	4.085 ± 0.059	3.695 ± 0.049**
LM diameter (mm)	5.335 ± 0.051	5.080 ± 0.042*

*p < 0.05 and **p < 0.01.

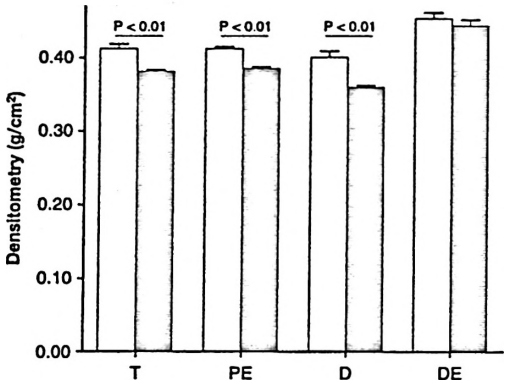

Fig. 1. *Densitometric data (g/cm^2) of total (T) as well as proximal (PE) and diaphysis (D) and distal epiphysis (DE) of femurs from normal healthy control (open bars) and CCl_4 liver cirrhotic (shaded bars) rats.* Values are means ± SEM (n = 10 in each group).

0.360 ± 0.005), and proximal epiphyseal (0.413 ± 0.005 vs 0.385 ± 0.004) bone mass was reduced in CI group. However, no differences were seen at the distal epiphyseal region (0.457 ± 0.007 vs 0.445 ± 0.010).

Regarding bone composition, no significant differences were found in calcium (20.83 ± 0.25 vs 20.96 ± 0.39) phosphate (10.672 ± 0.106 vs 10.366 ± 0.123) and OHprol contents (1.823 ± 0.020 vs 1.817 ± 0.024) between CO and CI rats (% of fresh bone tissue). Therefore, the ratio mineral/protein matrix were similar in both groups.

Discussion

This study shows that rats with carbon-tetrachloride-induced cirrhosis develop osteopenia and provides a potentially useful experimental model for the study of bone disorders associated to chronic parenchymal liver damage. The observed osteopenia is characterized by normal bone composition, reduced total bone mass and secondarily decreased bone resistance to mechanical stress.

Osteopenia is a common finding in patients with liver cirrhosis and accounts for the increased risk for bone fractures shown by these patients when compared with gender and age-matched controls (2, 28). Osteopenia has been shown to affect both trabecular and mixed (trabecular and cortical) bones (5, 23) and has clinical relevance since it leads to an increased morbidity in the advanced stages of liver cirrhosis and, in addition, constitutes a serious problem when these patients are subjected, as a rule, to high-dose glucocorticoid therapy early after liver transplantation (11, 23). However, a well character-

ized experimental model for the study of osteopenia in cirrhotics is not available.

The pathogenesis of bone disorders in cirrhotic patients is not yet completely understood (1, 11). Several factors such as deficient intake, malabsorption, undernutrition, decreased ability to synthesize bone protein matrix, abnormalities in vitamin D and calcium metabolism, alcohol toxicity and hypogonadism having been suggested to be involved (15, 24).

In the present study, bone density and bone thickness were significantly decreased whereas both the protein and mineral bone contents were normal. Indeed, the normality of the bone protein-to-mineral ratio allows a decreased mineralization of the osteoid to be ruled out as underlaying mechanism of osteopenia. Thus, osteoporosis rather than osteomalacia or osteitis fibrosa cystica appears to be the major disorder of bone in this model of cirrhosis.

The primary mechanism leading to osteopenia in these cirrhotic rats cannot be ascertained from data available in this study. A decreased osteoblastic activity would be the more likely possibility of a disease characterized by marked protein-caloric malnutrition in its late stages (20). Moreover, a low turn-over osteopenia seems to occur in chronic parenchymal liver disease (14, 27). Biochemical markers of bone resorption and formation will be assessed to clarify this issue and evaluation of the osteopenia in rats with far advanced stages of liver cirrhosis should be undertaken in additional studies.

The validity of the method requires discarding the direct toxic effects of carbontetrachloride on the bone (19).

The hepatotoxicity of CCl_4 is largely due to the generation of $CCl_3^{\bullet}$, a highly reactive metabolite arising from oxidation of the parent compound which is carried out by microsomal enzymes, mainly the cytochrome P450 system (7, 23). The severity of carbon-tetrachloride toxicity after acute challenge is proportional to the activity of these enzymes (7, 9). Moreover, induction of the microsomal system with alcohol or barbiturates greatly enhances the toxic effects of this compound (1, 4). Bone cells, are highly especialized for either bone formation or reabsorption but have a poor metabolizing ability, a condition that may be bone-protective against this toxic. The assessement in bone tissue of lipid peroxidation would be of interest to rule out definitively a significant contribution of the toxic to the bone disturbances observed in cirrhotic rats.

Carbontetrachloride-induced cirrhosis in the rat is the more extensively used experimental model of liver cirrhosis since it mimicks most of the systemic disturbances observed in cirrhotic patients (14, 16, 17), liver and kidneys (25) remaining as the major target organs for CCl_4 toxicity. Therefore, although bone toxicity by CCl_4 cannot be excluded at all until it is directly investigated, such a possibility seems to be remote.

In summary, rats with liver cirrhosis induced by administration of carbontetrachloride show normal bone composition but reduced bone mass resulting in a decreased resistance to mechanical stress. This experimental model could be a useful tool for the study of bone disorders in liver cirrhosis.

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tal en rata para el estudio de la osteopenia asociada a la cirrosis hepática. J. Physiol. Biochem. (Rev. esp. Fisiol.), 52 (2), 113-120, 1996.

La cirrosis hepática se asocia frecuentemente con osteopenia. Su fisiopatología, no bien conocida, parece ser multifactorial, y no se dispone de un modelo experimental válido para su estudio. El objetivo de este trabajo es investigar en rata si la cirrosis hepática inducida por CCl_4 cursa o no con osteopenia. Se estudian parámetros morfológicos, densitométricos y bioquímicos en fémur de ratas controles y cirróticas. Los resultados (media ± SEM; n = 10) muestran que los animales cirróticos presentan una reducción significativa ($p < 0,01$) del peso óseo (0,254 ± 0,003 vs 0,230 ± 0,004 g/100 g p.c.); densitometría de epífisis superior (0,413 ± 0,005 vs 0,385 ± 0,004 g/cm^2) y de diáfisis (0,404 ± 0,007 vs 0,360 ± 0,005 g/cm^2); de los diámetros lateromediano (5,33 ± 0,05 vs 5,08 ± 0,12 mm, $p < 0,05$) y anteroposterior (4,08 ± 0,06 vs 3,69 ± 0,05 mm); y de la resistencia mecánica (405,8 ± 9.5 vs 332,5 ± 9.1 N). No aparecen diferencias en la longitud ósea, en la densitometría de la epífisis inferior, ni en la proporción de calcio, fosfato e hidroxiprolina. En conclusión, las ratas con cirrosis hepática inducida por CCl_4 presentan una disminución de la masa ósea (osteopenia) caracterizada por fragilidad y reducción del espesor del hueso, sin que se altere la normal proporción mineral/proteína. Estos resultados permiten proponer este modelo experimental para el estudio de la osteopenia asociada a la cirrosis hepática.

Palabras clave: Cirrosis hepática, CCl_4, Osteopenia, Densitometría ósea, Rata.

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