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Effects of IGF-I treatment on osteopenia in rats with advanced liver cirrhosis

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IGF-I is an anabolic hormone which has been reported to increase bone formation in several conditions of undernutrition. Advanced liver cirrhosis is associated with osteopenia and also with low serum levels of IGF-I. Previous results showed that low doses of IGF-I increase osteoblastic activity and decrease bone reabsoption in early liver cirrhosis. The aim of this study was to evaluate whether IGF-I-treatment also induces beneficial effect on osteopenia associated with advanced cirrhosis. Rats with ascitic cirrhosis were divided into two groups: group CI (n=10) which received saline and group CI+IGF (n=10) which were treated with IGF-I (2 μg/100 g bw. day, sc, during 21 days). Healthy controls which received saline were studied in parallel (CO n=10). On the 22nd day, the animals were sacrificed, and bone parameters were analyzed in femur. Posterior-anterior diameter was similar in all groups. No significant differences were observed in bone content of calcium, total proteins, collagen and hydroxyapatite in cirrhotic rats as compared with controls. However, CI rats showed significant reductions in total bone density (–13.5%, $p<0.001$) assessed by densitometry and radiological study. In CI+IGF rat bone density (assesed by densitometry) improved significantly as compared with CI animals. In summary, osteopenia characterized by loss of bone mass and preserved bone composition was found in rats with advanced cirrhosis induced by CCl_4 and phenobarbital in drinking water. This bone disorder is partially restored by treatment with low doses of IGF-I during only three weeks. Thus, IGF-I could be considered as a possible therapy for osteopenia associated with advanced liver cirrhosis.

Key words: Liver cirrhosis, Osteopenia, Insulin-like Growth Factor-I (IGF-I), Undernutrition.

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Bone is one of the target organs for the Insulin-like growth factor I (IGF-I), an anabolic hormone produced in the liver upon growth hormone stimulation (20). Different tissues other than the liver, including bone, synthesize IGF-I which acts locally in an autocrine and/or paracrine manner (23). In fact, IGF-I stimulates osteoblastic proliferation and differentiation (4, 23), acting through specific receptors, which are present in bone cells of osteoblastic lineage (2) and in bone-derived endothelial cells (13). Furthermore, clinical studies have shown beneficial effects of IGF-I treatment on bone in several clinical conditions (12, 14, 19).

The prevalence of osteopenia is significantly higher in cirrhotic patients than in an age-matched normal population (3, 11, 14-17). Consequently, these patients are exposed to an increased risk of bone fractures, which are a source of morbidity in advanced stages of the disease and after liver transplantation (3, 18, 28). The pathogenesis of osteopenia in liver cirrhosis is not well understood, although malabsorption, undernutrition, vitamin D deficiency, reduced levels of sexual hormones and alcohol toxicity appear to be some of the factors involved in disturbed bone metabolism (10, 17, 31). In addition, in advanced liver cirrhosis, IGF-I levels decrease (16, 17, 30) as a result of diminished hepatocellular biosynthetic function (5, 16, 27, 30). Although low IGF-I levels characterize advanced cirrhosis, the role of IGF-I in the genesis of osteopenia has not yet been established. Previous data from our laboratory showed that IGF-I has a beneficial effect on osteopenia in early liver cirrhosis (induced by CCl_4 ip), by increasing osteoblastic activity and decreasing bone resorption (9). In this study we evaluate whether low doses of IGF-I increases bone mass in rats with advanced liver cirrhosis induced by CCl_4 and phenobarbital in drinking water.

Materials and Methods

Animals and experimental design.– Thirty, 4-week-old, male Wistar rats, weighing about 110-120 g, were included in this protocol. Animals were housed in cages placed in a room provided with a 12-h light-darkness cycle and at constant humidity and temperature (20 °C). Both food (standard semi purified diet for rodents, purchased from B.K. Universal, Sant Vicenc dels Horts, Spain) and water were given *ad libitum* until rats were sacrificed at the end of the study. All the experiments were performed in conformity with The Guiding Principles for Research Involving Animals.

Liver cirrhosis was induced in 20 rats by carbon tetrachloride (CCl_4, Merck, Darmstadt, Germany) inhalation and phenobarbital in drinking water during 30 weeks as previously described (5). CCl_4-treated animals were randomly divided into two groups: CI+IGF group (n =10) which received IGF-I (2 µg/100 g bw x day, subcutaneous, in two divided doses) for 21 days; and group CI (n =10) which received saline following the same schedule. Ten age-matched healthy rats maintained under the same conditions during the period of the study, excluding CCl_4 inhalation, were used as the control group (CO). These rats were given saline, as in group CI. The day before starting the administration of IGF-I or placebo, body weight was recorded and blood samples were obtained from retroocular venous plexus with capillary tubes (70 mm, Marienfeld, Germany). Serum was stored at –20°C for analytical determinations.

On the 22nd day, animals were sacrificed by decapitation. Immediately thereafter, liver and femurs were carefully dis-

sected out. Samples of the left major liver lobe were immediately frozen by immersion in liquid N_2 and stored at –80 °C until analysis. Histological liver sections (4 mm), stained with Masson's tricrome, were used for histopathological confirmation of liver cirrhosis. Ascites was approximately calculated by direct sucking syringe.

Morphological parameters, densitometry and radiograph of bone.– Bone morphometry, densitometry and X-ray studies, as well as analysis of biochemical composition of bone were performed in the femur. Femur length was measured from the major trochanter to the end of the distal epiphysis. Anteroposterior (AP) and lateromedial (LM) diameters were assessed at the mid point of medial diaphysis. All measurements were performed with a precision calliper, Mituyoto" (±0,05 mm). Bone density was determined by dual-photon absorptiometry using a DP-3 instrument (DXA-HOLOGIC QDR 1000^{TM}, Hologic, INC, Waltham, MA, USA). Scanning a phantom every time controlled measurement stability. Femur densitometries were performed at environment temperature. Bones were placed on a polyester resin cell, nonopaque at X-ray. An "Ultra High Reach Program" (NEC APD IV, Power Mater 2 Boxboro, MA, USA) was used for processing the data. Bone density results were expressed as g/cm^2 (24). Radiographs were performed on dissected bones which were carefully aligned (anteroposterior and lateral positions successively) on Min-R, E, MRE-1 chassis (10 x 24 cm) (Kodak Diagnostic Film, Windsor, Colorado, USA), using a tube of X-rays (Siemens, Münchem, Germany) coupled to Polumat-PC computer, 100/60 Farb-Monitor MCM 1404 (Siemens, Nixford, China).

Analytical methods in bone tissue.– Bone hydroxyproline was determined in whole bone tissue according to the method of Woessner (34). Total bone calcium content was assessed by atomic absorption spectrophotometer (33) and total bone phosphate by the colorimetric reaction of acidic ammonium molybdate, respectively (33). Femurs (200 mg) were calcinated and then dissolved in either 0.1% lanthanum chloride for calcium assessment or bidistilled water for phosphate determination. Results were expressed in mg/100 mg of dry bone. All determinations were made in triplicate. Total collagen and hydroxyapatite can be indirectly assessed from hydroxyproline and phosphate values, respectively (29). Total nitrogen content was assessed by Kjendal's method in triplicate and <5% intraassay variation was considered acceptable. Protein content was calculated from these data, using x6.25 as constant (35). Malondialdehyde (MDA, measured after heating samples at 45 °C for 60 min in acid medium, was used as an index of membrane lipid peroxidation (24). This product was quantitated by colorimetric assay in liver and bone homogenates, using a chromogenic reagent (LPO-586, Bioxytech, OXIS International, France), which, after reacting with MDA, generates a stable chromophore that can be measured spectrophotometrically at 586 nm.

Analytical methods in serum.– Basic biochemical parameters were determined in serum obtained the day before beginning treatment (day 0) from all animals. Serum levels of albumin, total proteins, glucose, cholesterol, bilirubin, alkaline phosphatase and aminotransferases (AST and ALT) were determined by routine laboratory methods using a Cobas Mira autoanalyzer (ABX, Spain).

Statistical analysis.– Data are expressed as mean ± SEM. Non-parametric tests were used for sample size: Kruskall-Wallis analysis was used for simultaneous comparisons of the three groups, and the group-to group significance was evaluated by *a posteriori* Mann-Whitney test. Statistical significance was accepted at $p < 0.05$. Calculations were performed with SPSS-Win v.6.0. Program PC.

Results

Liver cirrhosis was histopathologically proven in rats from CI and CI+IGF groups at the end of the study. Therefore, all animals were included for evaluation of results. The day before starting the experimental period, the two groups of cirrhotic rats showed, as compared with control animals, significantly lower body weight and significantly abnormal serum levels of amino transferases, albumin, total proteins, cholesterol, alkaline phosphatase and bilirubin (table 1), showing an altered liver function. The extent of these alterations was the same in the two cirrhotic groups, thus indicating the absence of differences in the severity of liver disease and desnutrition at baseline. Ascites was observed in all cirrhotic rats (CI=8.0±1.8 ml; CI+IGF=5.2±1.4 ml, p<0.001 CO vs CI and p<0.01 CO vs CI+IGF).

At the end of the study (day 22), body weight was diminished in CI group (g, 417±7 vs CO= 510±4, p<0.001), but it was significantly increased in CI+IGF group (445±13; p<0.05). Femur length was significantly reduced in both cirrhotic groups (mm, CI=39.65±0.24; CI+IGF = 39.91±0.30) as compared with controls (41.5±0.23; p<0.001), while no significant differences were observed in antero-posterior diameter between groups. However, external latero-medial diameter was significantly reduced in cirrhotic groups as compared with controls (4.68±0.04) but less in CI+IGF group (CI=4.35±0.06, p<0.001; CI+IGF=4.49±0.08, p<0.05).

Densitometry studies (fig.1) demonstrated a significant reduction in bone mass of the whole femur in both CI (g/cm^2, 0.32±0.004) and CI+IGF (0.34± 0.005) groups as compared with control animals (0.37±0.006; p<0.001). However, bone mass was significantly lower in untreated the cirrhotic group than in IGF-

Table I. *Baseline biochemical data and body weight of control and cirrhotic animals before treatment (on the day 0).*

	Healthy control group (n=10)	Cirrhotic animals (n=20)
Body Weight (g)	510 ± 4	402 ± 10.5***
Aspartate aminotransferase (UI/L)	35 ± 2	98 ± 20**
Alanine aminotransferase (UI/L)	21 ± 1	52 ± 5**
Glycemia (mg/dL)	193.37 ± 13.84	172.86 ± 6.6
Albumin (g/dL)	3.4 ± 0.00	2.4 ± 0.2**
Total Proteins (g/dL)	6.7 ± 0.1	5.8 ± 0.2*
Cholesterol (mg/dL)	97 ± 4	162 ± 26*
Alkaline phosphatase (IU/L)	240 ± 17	570 ± 90**
Bilirubin (mg/dL)	0.3 ± 0.1	1.0 ± 0.3*

Values are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

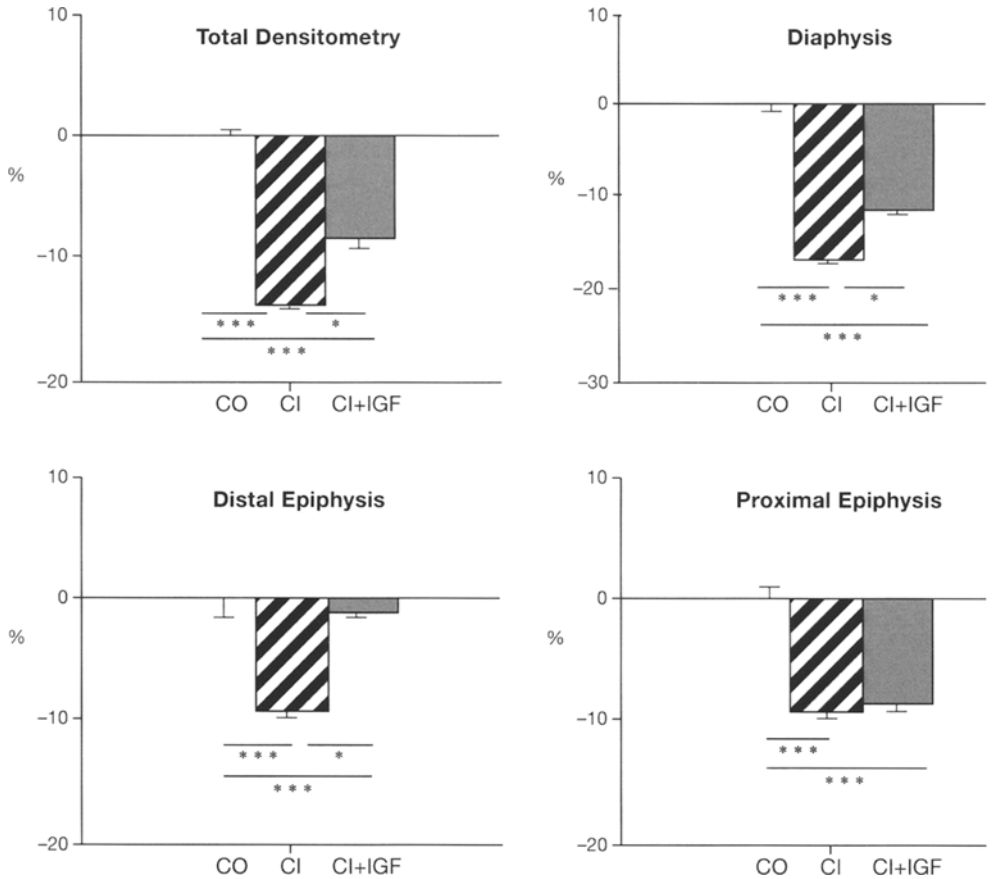

Fig. 1. *Decrement on densitometries in cirrhotic animals.*
The reduction in density (expressed as percent deficiency with respect to mean values of the control group) was significantly lower in CI+IGF group than in untreated cirrhotic rats.

I treated cirrhotic rats ($p<0.05$). A similar effect of IGF-I was observed in femur diaphysis and distal epiphysis, but not in proximal epiphysis (fig. 1).

Demonstrative X-ray films (fig. 2) show that cortical femoral density was lower in CI animals than in control rats, whereas in the CI+IGF group bone density was intermediate between the other two groups. Although X-ray images cannot replace quantitative measurements, they are useful to illustrate bone architecture in three experimental groups.

There were no significant differences among groups regarding bone content of hydroxyapatite (CO=33.62±2.85; CI=35.53±2.71; CI+IGF=35.97±2.50), calcium (CO=24.12±2.80; CI=24.99±1.27; CI+IGF=24.48±1.61), total proteins (CO=43.75±6.16; CI=46.02±6.0; CI+IGF=47.05±5.43), and collagen (CO=28.39±1.70; CI=31.48±0.96; CI+IGF=28.34±

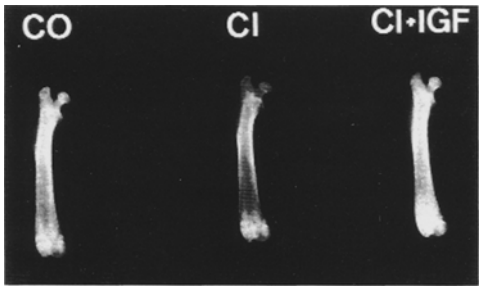

Fig. 2. *Radiograph of femur from the three experimental groups.*

0.87), expressed as percent of dry bone weight. As a result, the protein to mineral ratio of bone was similar in the three groups.

In order to rule out the direct action of CCl_4-induced tissue damage, malondialdehyde (MDA) was determined in liver and bone homogenates from control and CCl_4-treated rats to assess whether lipid peroxidation could be involved in bone changes observed in cirrhotic animals. Tissue MDA was significantly increased in liver homogenates from CCl_4-treated rats (133±27 nmol/g tissue, p <0.05) as compared with controls (61±2), but products of lipid peroxidation were undetectable in bone homogenates from both groups (< 0.25 nmol/g tissue, which is the minimal limit of detection the assay).

Discussion

The present study has demonstrated that osteopenia in advanced liver cirrhosis may be partially corrected by a short-term course of IGF-I at low doses, as it was demonstrated in early cirrhosis (9). In this protocol, osteopenia in cirrhotic rats was also characterized by reduced bone weight and total bone mass with preserved bone composition. This kind of osteopenia is similar to osteoporosis in patients with chronic liver disease (3, 9, 17). Thus, this animal model might be useful to study the osteopenia which develops in patients with cirrhosis as was previously suggested (9, 24).

In the present study, cirrhosis was induced by CCl_4 inhalation and phenobarbital added to the drinking water to potentate CCl_4 toxicity (24). It was used despite producing changes in bone metabolism and osteopenia induced by barbiturates (25, 32). However, a question arises as to whether direct toxicity of CCl_4 to bone could contribute to osteopenia. This possibility has been reasonably ruled out by showing that MDA, a marker of lipid peroxidation, was undetectable in bone homogenates from both controls and CCl_4-treated rats, suggesting that lipid peroxidation does not occur in bone under these experimental conditions.

The mechanisms responsible for osteopenia in our experimental model of liver cirrhosis cannot be fully ascertained from present data. It seems possible that osteopenia might be related, at least in part, to malnutrition (14, 27). However, in a previous work (9) a marked increase was observed in deoxypyridoline cross-links in untreated cirrhotic rats, indicates enhanced bone resorption and osteoclast activity. Under these conditions the maintenance of bone mass would require a compensatory increase in osteoblastic function. But, in the same study (9), histopathologycal data suggested that new bone formation is not increased in untreated cirrhotic group while in IGF-I-treated group, an increased newly formed tissue was observed (9). Although malnutrition occurs in cirrhotic rats, bone is not the most affected tissue. In fact, reductions of fat stores and muscle mass have been reported to be the main contributors to weight loss in clinical malnutrition

(22, 25), and we have recently shown that significant muscle wasting occurs early in the course of experimental liver cirrhosis (28). It seems possible that the positive effects of low doses of IGF-I on nutritional state, intestinal absorption and liver function, which have been previously demonstrated in rats with liver cirrhosis (5, 6, 27) might have contributed to improvement of bone metabolism. In this series, in IGF-I treated cirrhotic animals body weight improved significantly as compared to untreated cirrhotic group.

In the present study, rhIGF-I was able to counteract the reduction of bone mass observed in untreated cirrhotic rats, improving densitometry values. As was previously reported, histological findings suggested an increased bone forming activity (9) as demonstrated by other teams (1, 2, 3). In addition, previous data (9) showed that IGF-I reduces bone resorption in cirrhotic animals as was reported by other groups (31, 32) demonstrated the inhibitory effect of IGF-I on osteoclastic activity.

Since IGF-I is an important anabolic hormone for bone formation, decreased bioavailability of this hormone in liver cirrhosis may play a role in osteopenia accompanying cirrhosis. Of interest, in the present work IGF-I was able to improve bone mass despite possible changes in bone metabolism induced by phenobarbital in drinking water. Reduced levels of sexual hormones and malabsorption have been reported as factors involved in the pathogenesis of osteopenia. In fact, cirrhotic animals included in these series had reduced levels of testosterone (7) and sugar and amino acid malabsorption (8, 26). These alterations also improved with low doses of IGF-I (7, 8, 26).

In conclusion, rats with advanced cirrhosis (induced by CCl_4+Ph) show osteopenia of osteoporosis-type. A short treatment course (three weeks) with low doses of IGF-I increases bone density, probably by inhibiting bone resorption and increased bone formation, as it has been shown by our team in rats with early cirrhosis. IGF-I should be considered as a possible therapy for this common complication in advanced stage of hepatic disease.

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IGF-I es una hormona anabólica con efecto sobre el metabolismo óseo. La cirrosis hepática avanzada está asociada con osteopenia y también con bajas concentraciones de IGF-I en suero. Resultados anteriores de nuestro grupo muestran que dosis bajas de IGF-I aumentan la actividad osteoblástica y reducen la resorción ósea en estadíos compensados de cirrosis hepática. El objetivo de este estudio consiste en evaluar si el tratamiento con IGF-I induce también un efecto beneficioso en la osteopenia asociada a cirrosis avanzada. Ratas con cirrosis ascítica se dividen en dos grupos: grupo CI (n=10), que reciben salino, y grupo CI+IGF (n=10) tratadas con IGF-I (2mg/100 g peso

corporal /día , sc, durante 21 días). Controles sanos, que reciben salino, se estudian al mismo tiempo (CO n=10). El día 22, los animales se sacrifican y se analizan parámetros óseos en fémur. El diámetro antero-posterior es similar en todos los grupos. No se observan tampoco diferencias significativas en el contenido óseo (calcio, proteínas totales, colágeno, hidroxiapatita) en las ratas cirróticas al compararlas con los controles. Sin embargo, en los animales cirróticos se observa una reducción significativa de la densidad total ósea (-13.5%, $p<0.001$) medida por densitometría, reducción que se aprecia también en el estudio radiológico. En las ratas CI+IGF la densidad ósea mejora significativamente en comparación con los animales cirróticos sin tratamiento. En resumen, se observa osteopenia caracterizada por pérdida de masa ósea, en la que se preserva la normal composición del hueso, en ratas con cirrosis avanzada inducida por CCl_4 y fenobarbital en el agua de bebida. Esta disminución de la masa ósea se recupera parcialmente por el tratamiento con dosis bajas de IGF-I durante sólo tres semanas. Por lo tanto, IGF-I podre ser considerado como una posible terapia para la osteopenia asociada a la cirrosis hepática en estadíos avanzados.

Palabras clave: Cirrosis hepática, Osteopenia, IGF-I, Desnutrición.

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