HEPATOBILIARY

Hepatic osteodystrophy in rats results mainly from portasystemic shunting

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Background and aims: In chronic liver disease, bone disease frequently develops. The contributions of different features of liver disease such as parenchymal inflammation, portal hypertension, and portasystemic shunting on bone metabolism have not been systematically studied. The aim of this study was to identify the features of liver disease contributing to bone disease using rat models.

Methods: Parenchymal liver disease was induced by carbon tetrachloride administration, portal hypertension by partial portal vein ligation, and portasystemic shunting by end to side anastomosis of the portal vein to the inferior vena cava. Normal and sham operated surgical animals served as controls. Serum calcium, 25-hydroxy vitamin D (25-OH vit D), and osteocalcin levels, and urinary deoxypyridinoline excretion were analysed. Testosterone and oestradiol levels were determined in male and female rats, respectively. Interleukin 1, interleukin 6, and tumour necrosis factor α (TNF-α) were determined in serum. Bone density was measured in all groups and in addition, in the surgical groups, histomorphometry was performed on undecalciﬁed specimens of the proximal tibia. The calcium content of the femurs, removed at termination and ashed, was determined.

Results: Early parenchymal disease and portal hypertension did not affect bone metabolism or body mass. Portasystemic shunting increased bone resorption, decreased bone formation, bone density, and trabecular bone volume which were commensurate with a reduction in body mass. TNF-α levels were elevated and testosterone levels were low in male portasystemic shunted rats.

Conclusions: Portasystemic shunting in the rat adversely affects bone metabolism as part of a generalised catabolic state where high TNF-α and low testosterone and 25-OH vit D levels may play a role.

Patients with chronic parenchymal and cholestatic liver disease often have metabolic bone disorders. The pathogenesis of hepatic osteodystrophy is complex and incompletely understood. Alcohol is toxic to osteoblasts but may also affect bone indirectly through its effects on the liver. In autoimmune liver disease, treatment with corticosteroids promotes bone loss. Bone disease has been reported in patients with chronic parenchymal and cholestatic liver disease. Osteopenia is particularly common in cholestatic liver disease. Severe cholestasis accelerates bone loss in primary biliary cirrhosis. In general, the severity of bone disease correlates with the extent and duration of liver disease. Abnormal vitamin D metabolism has been reported in liver disease but other studies failed to confirm this. The impact of bone disease may even extend into the post liver transplantation setting where osteopenic fractures commonly occur.

The aim of the present study was to determine the effects of early hepatic parenchymal disease, isolated portal hypertension, and portasystemic shunting on the development of bone disease in rat models.

Materials and methods: Ten week old male and female Sprague-Dawley rats were used in all experiments. Rats were housed in individual stainless steel cages in a controlled environment at a constant room temperature (22°C), humidity, and 12 hour light-darkness cycles. Rats were fed standard chow (Epol, Johannesburg, South Africa) and water was given ad libitum. The approval of the ethics committee of the University of Pretoria was obtained and animals were treated according to ethical guidelines established by the University of Pretoria.

Animal models and experimental design: The experimental design consisted of two non-concurring protocols. In the first protocol, parenchymal liver disease was established and compared with a non-surgical control. In the second protocol, portal hypertension and portasystemic shunting was established and compared with a surgical control group.

Early parenchymal liver disease model: n=10 (males=5; females=5) Liver cirrhosis in rats can be induced by weekly administration of carbon tetrachloride (CCL4) for 16 weeks. To induce early parenchymal disease, animals were dosed for eight weeks with CCL4 (Merck, Darmstadt, Germany). No phenobarbital was added to the drinking water because of its recognised effects on bone metabolism. Animals received 80 µl of CCL4 at the outset with subsequent dosing based on weight loss. The intended weekly weight loss was 6–9%. An additional five CCL4 exposed rats were sacrificed between five and 10 weeks and the liver removed for histology.

Control rats (n=8) received 0.9% sodium chloride weekly by gavage.

Abbreviations: PSS, portasystemic shunting; PPVL, partial portal vein ligation; SC, sham operated surgical controls; 25-OH vit D, 25-hydroxy vitamin D; CCl4, carbon tetrachloride; u-DPD, urinary deoxypyridinoline; BMD, bone mineral density; BMC, bone mineral content; CRP, C reactive protein; TNF-α, tumour necrosis factor α; ALT, alanine aminotransferase; ALP, alkaline phosphatase; IL, interleukin.
Presinusoidal portal hypertension model (PPVL): n=9 (males=5; females=4)

Portal hypertension was induced by reduction of the diameter of the portal vein. A midline laparotomy was performed and a blunt 21 gauge needle was placed alongside the portal vein. A 3-0 silk ligature was placed around both needle and portal vein and the needle was carefully removed. Portal hypertension was confirmed at termination by portal vein pressure measurements in all PPVL animals.

Portasystemic shunt model (PSS): n=10 (males=5; females=4)

A laparotomy was performed, the portal vein, inferior vena cava identified, and the pyloric vein ligated. The adventitia of the inferior vena cava was carefully removed. The portal vein was ligated at the hilum of the liver, transected, and the distal limb Anastomosed end to side to the inferior vena cava. The patency of the shunt was confirmed histologically in all animals at termination.

Sham operated surgical controls (SC): n=10 (males=5; females=5)

A laparotomy was performed at baseline. Portal pressure measurements were conducted at termination.

The surgical groups were terminated after 16 weeks. At termination, the liver, femur, and tibia of all animals were carefully removed. The bones were carefully stripped of all soft tissue.

Analytical methods in sera and urine

Blood samples were obtained at baseline and at week 8 by puncture of the right jugular vein with a 22 gauge needle, as well as at termination in the surgical groups. Urine was collected from rats individually housed in metabolic cages following an adaptation period of 24 hours. Blood samples were obtained at baseline and at week 8. Bone densitometry was performed in the surgical groups on whole body and high resolution scans of the femur at baseline, and on individual tibiae and fibulae removed at termination. The tibiae and fibulae were placed on a non-opaque polyester resin block and high resolution bone densitometry was performed using appropriate software for small animals (Hologic, Inc). Tibial measurements were used for analysis.

Portal vein pressure measurements

Portal pressure measurements were performed at termination in the SC and PPVL animals under isoflurane 2% anaesthesia. In control rats, portal pressures were measured by insertion of a fluid filled 20 gauge needle directly into the portal vein and in PPVL animals by inserting the needle into the distal superior mesenteric vein. The needle was connected to a transducer (Medex Medical Instrumentation, UK) calibrated for venous pressure. The external zero reference point was placed at the level of the atria. The pressure was recorded as soon as a stable pressure. The external zero reference point was placed at the level of the atria. The pressure was recorded as soon as a stable

Bone densitometry

Rats were anaesthetised and bone densitometry performed by dual energy x ray absorptiometry using a DXA Hologic QDR 4500 (Hologic, Inc, Waltham, Massachusetts, USA). The stability of the measurement was controlled daily by scanning a phantom. Whole body and high resolution scans of the femur were carried out in CCl4 exposed animals and their controls at baseline and at week 8. Bone densitometry was performed in the surgical groups on whole body and high resolution scans of the femur at baseline, and on individual tibiae and fibulae removed at termination. The tibiae and fibulae were placed on a non-opaque polyester resin block and high resolution bone densitometry was performed using appropriate software for small animals (Hologic, Inc). Tibial measurements were used for analysis.

Histomorphometry

Individual tibiae removed at termination in the surgical groups were processed undecalcified, embedded in methyl-methacrylate, and cut at 7 μm thickness on a Jung K heavy duty microtome. Sections were stained with Masson-Goldner stain and reading with respiratory variations was observed. There were no significant differences between control and CCl4 treated animals at baseline.

Liver histology

Liver sections were stained with haematoxylin and eosin and Masson’s trichrome.
were then dissolved in 2 ml of 6 N HCl and diluted 400 × with demineralised/deionised water. Calcium content was determined using a Perkin-Elmer 3030 atomic absorption spectrophotometer.

Statistical analysis
Data were analysed using SigmaStat for Windows version 2.0 and SigmaPlot for Windows version 4.0. Data were checked for normality and equal variance; if passed, one way analysis of variance (ANOVA) was performed and where it failed, analysis of variance on ranks (Kruskal-Wallis) was conducted. Results are presented as means (SD), medians, or range where appropriate. Results were considered significant at p<0.05.

RESULTS
Early parenchymal liver disease model
CCl₄ exposure led to a significant increase in aspartate aminotransaminase and alanine aminotransferase (ALT) levels (table 1). At histology, characteristic changes associated with CCl₄ exposure, consisting of inflammatory cell infiltrates, necrosis, and fatty changes, were observed, as has been reported previously by others. Except for 25-OH vit D levels, no statistically significant differences between control and CCl₄ exposed rats were observed at eight weeks in any of the variables studied relating to bone metabolism, including bone density. 25-OH vit D levels at eight weeks were significantly lower (table 1) in the treated animals compared with controls.
Liver histology showed mild fatty change in PSS animals compared with controls, but calcium per mg of bone did not differ between groups. This absence of an excessive osteoid seam is interpreted as an expression of an overall failure to thrive, and of stunted growth. Indeed, the reduction in bone size is interpreted as an expression of an overall failure to thrive, and of stunted growth. The present study demonstrated that experimental portasystemic shunting had the most deleterious effect on bone.

Complete portasystemic shunting led to significantly lower bone formation, greater bone resorption, lower trabecular bone volume, and smaller bone size compared with controls. The lower trabecular bone volume was reflected in lower bone density, and both lower trabecular bone volume and smaller bone size in lower BMC in PSS rats. The smaller bone size is interpreted as an expression of an overall failure to thrive, and of stunted growth. Indeed, the reduction in bone mass together with body mass suggests that bone loss occurs as part of a generalised catabolic state. The presumed partial shunting induced by collaterals in the portal vein ligation model did not have the same adverse effect on body mass, growth, or bone, nor did early parenchymal disease.

Suppression of bone formation in the PSS group was expressed by lower values for osteocalcin, osteoid surface, and osteoid volume, and greater bone resorption by higher values for u-DPD and increased resorption surfaces observed in PSS animals but not in controls. This picture suggests uncoupling of bone formation from resorption in favour of resorption. Contributions to these bone abnormalities by failure to thrive, stunted growth, and malnutrition cannot be ruled out.
The discrepancy between the rise in femur mass per 100 g of body weight and the decline in trabecular bone volume in the PSS group may be due to the fact that the femur consists predominantly of cortical bone whereas histomorphometry is routinely carried out on trabecular bone. Trabecular bone loss is known to occur earlier and more rapidly than cortical bone loss because of the larger surface area available for resorption. The duration of the experiment may have been insufficient for cortical bone loss to become apparent. The rise in femur mass per 100 g of body mass in PSS rats at 16 weeks may be explained on similar grounds: body fat and lean body mass are presumably lost earlier and more rapidly than cortical bone mass in the femur so that the lag in cortical bone loss gives rise to an apparent increase in bone mass relative to body mass.

Whether the findings in the rat can be applied to adult humans remains unclear: rat bone continues to grow throughout life and does not exhibit remodelling. A similar study in a larger animal that has both closed physes in adulthood and bone remodelling may be more applicable to human bone pathology.

The cytokines IL-1, IL-6, and TNF-α have been shown to influence different aspects of bone metabolism.\textsuperscript{24–28} IL-1, IL-6, and TNF-α are known to be elevated in liver disease.\textsuperscript{24} In our study, TNF-α levels were increased in PSS animals at termination and may have contributed to the development of osteopenia.

Chronic liver disease, especially in males, can result in gonadal dysfunction with low testicular mass and decreased...

| Table 3 | Comparison of histomorphometric parameters: trabecular bone volume, osteoid volume, and osteoid surface, and resorption surface in the proximal tibia of rats at 16 weeks |
|-----------------|-------------|-------------|-------------|-----------|-----------|
|                | SC          | PPVL        | PSS         | p Value  | p Value  |
| Trabecular bone volume (%) | 13.2 (1.35) | 14.17 (2.23) | 7.92 (0.99) | 0.36      | <0.001    |
| Osteoid volume (%) | 7.16 (1.23) | 5.17 (1.47)  | 4.74 (0.74) | 0.031     | 0.001     |
| Osteoid surface (%) | 9.71 (1.04) | 11.33 (2.34) | 6.06 (1.33) | 0.234     | <0.001    |
| Resorption surface (%) | 0.94 (0.07) | 1.73 (0.59)  | 3.38 (0.96) | 0.061     | <0.001    |

SC, sham operated surgical controls; PPVL, partial portal vein ligation; PSS, portasystemic shunting.

| Table 4 | Comparison of femur dimensions, composition, and tibial bone mineral density in rats at termination at 16 weeks |
|-----------------|-------------|-------------|-------------|-----------|-----------|
|                | SC (n=10)   | PPVL (n=9)  | PSS (n=10)  | p Value  | p Value  |
| Femur length (mm) | 30.47 (2.36) | 30.78 (1.94) | 27.83 (1.25) | 0.86      | 0.008     |
| Mid shaft thickness (mm) | 4.13 (0.46) | 3.98 (0.31) | 3.63 (0.30) | 0.44      | 0.017     |
| Femur mass (g) | 0.416 (0.09) | 0.385 (0.06) | 0.295 (0.03) | 0.37      | <0.001    |
| mg Ca\textsuperscript{2+}/femur | 70.92 (14.52) | 66.29 (14.62) | 50.69 (5.40) | 0.50      | 0.003     |
| mg Ca\textsuperscript{2+}/100 g body mass | 0.108 (0.009) | 0.100 (0.02) | 0.137 (0.03) | 0.08      | 0.01      |
| mg Ca\textsuperscript{2+}/mm bone | 2.31 (0.30) | 2.18 (0.36) | 1.82 (0.13) | 0.40      | 0.002     |
| mg Ca\textsuperscript{2+}/mg bone | 0.164 (0.03) | 0.172 (0.02) | 0.172 (0.03) | 0.21      | 0.34      |
| Tibial BMD (g/cm\textsuperscript{2}) | 0.223 (0.005) | 0.218 (0.009) | 0.199 (0.01) | 0.12      | <0.001    |
| Tibial BMC (g) | 0.244 (0.05) | 0.253 (0.05) | 0.184 (0.02) | 1.00      | 0.004     |

SC, sham operated surgical controls; PPVL, partial portal vein ligation; PSS, portasystemic shunting; BMD, bone mineral density; BMC, bone mineral content.
estrogen levels. Interestingly, postsystemic shunting, but not portal hypertension, has been reported to result in gonadal injury in a rat model. Low testosterone levels were confirmed in the male PSS rats in our study and may have contributed to the development of osteopenia. Despite similar changes in bone parameters, oestradiol levels did not seem to have the same influence in female rats.

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