Original Research

Prolonged Magnesium Deficiency Causes Osteoporosis in the Rat

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Key words: prolonged magnesium deficiency, osteoporosis, rats

Background: Peroral magnesium (Mg) administration, used as the only treatment in postmenopausal osteoporosis, has been shown to cause a significant increase of BD.

Objectives: To gauge the role of magnesium deficiency in the etiology of osteoporosis, we compared rats fed a Mg deficient diet daily with rats fed a Mg adequate diet over a period of one year.

Methods: Sprague-Dawley female rats (mean weight 110, SD 23g) were divided into two groups of 8 and randomly assigned to an identical semisynthetic diet, containing either 2000ppm (group A) or 200ppm Mg (group B). Urine samples were collected every 3 months and blood samples at end of trial. After sacrifice, L3–L5 vertebrae and the femoral regions were examined for bone density (BD) using dual energy X-ray absorptiometry. The femurs were examined for bone fragility, the tibias by histomorphometry and the mineral contents of the bones was estimated.

Results: The mean BD of L3–L5 vertebral bone (BDL) was significantly higher in group than in the Mg deficient group B ($p = 0.035$, 1 tail). The BD of the femoral region (BDF) was also significantly higher in group A ($p = 0.045$, 1 tail). The stiffness of the femur, as determined by resistance to bending, was slightly greater in group A than in group B, but after correction to diminish the influence of the difference in bone dimensions in the two groups, the stiffness (ie loss of elasticity) in group B became significantly greater than that in group A ($p = 0.024$). The force needed to break the bone (F-max) was significantly higher in group A, than in group B ($p = 0.024$) and remained so after correction, although no longer significantly. In Group B, the diminution of the trabecular bone volume, in relation to tissue volume (BV/TV) and the increase in the degree of trabecular interconnection (TBPf) indicated osteoporosis, and focal osteoporosis of the metaphyseal spongy bone was seen on microscopy.

Conclusion: Experimentally induced prolonged Mg deficiency causes osteoporosis in rats.

INTRODUCTION

An earlier study [1], established that two years of supplementation with daily peroral magnesium (Mg) alone (250–750mg Mg as tablets of $Mg(OH)_2$), administered to 31 postmenopausal osteoporotics, prevented—in 87% of the Mg-treated patients—the yearly, statistically significant decrease of bone density (BD) seen in untreated controls ($p = 0.001$). In 71% of the Mg-supplemented patients, the BD increased by 1–8% ($p = 0.020$) [1]. Two consenting postmenopausal osteoporotic patients, who were willing to continue to receive a daily maintenance dose of 250mg Mg for 10 and 14 years, respectively, exhibited gradual increase of BD by 16% and 23%, respectively, eventually reaching values that correspond to those found in young healthy women [2]. Tucker et al [3] subsequently showed that elderly women with low daily dietary Mg intake, had lower BD values than controls receiving adequate daily Mg in their food. Because several conditions associated with Mg deficiency, e.g. alcohol abuse, malabsorption, diabetes mellitus and gluten sensitive enteropathy are characterized by a high incidence of osteoporosis, Rude and Olrich [4] studied enteropathic patients and found that their BD increased as a result of Mg treatment. In a study to determine whether Mg depletion could give rise to osteoporosis, Rude et al 1998 [5] induced Mg deficiency in the rat and found that after a period of

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3 months there was evidence of bone loss. The following year they reported that rats with experimentally induced Mg deficiency developed osteoporosis, manifested by uncoupling of bone formation and bone resorption [6]. In contrast, Riond et al [7] reported that suboptimal daily Mg dietary supply benefits rat bone.

Wishing to obtain definitive evidence as regards the association of Mg deficit with osteoporosis, we examined rats kept over a period of one year on a Mg deficient diet for comparison with rats fed a Mg adequate diet with respect to a possible change in BD, bone fragility and bone architecture.

MATERIALS AND METHODS

Experimental Animals

Sixteen Sprague-Dawley female rats (mean wt 110, SD 23g) were randomly assigned to two groups receiving a semisynthetic diet that provided either 2000 ppm (group A) or 200ppm Mg (group B). The otherwise identical diet contained 9000 ppm calcium (Ca), 7000 ppm phosphate, 10000 ppm potassium (K) and 2000 ppm sodium (Na), 24% casein, 50% starch, 11% glucose, 3% soyabean oil, 5% cellulose powder and 6% mineral mix. All animals had free access to tap water *ad libitum*.

Experimental Procedure

The study was performed as an end-point investigation. During the 12 months of study the rats were weighed monthly and urine samples were taken every three months. Urine volume was measured to the nearest mL and collected into Eppendorf vials. After the last urine sampling the animals were anesthetized by i.p. injection of 25 mg/kg ketamine and 40mg/kg diazepam. Blood was collected by heart puncture into heparinized syringes and centrifuged.

Bone Density Studies

After sacrifice, the BD of the femoral bones and of the L3–L5 lumbar vertebral bones was estimated using a noninvasive method: dual energy X-ray absorptiometry, employing DEXA apparatus (Prodigy, LUNAR, Europe, Brussels) which is the gold standard in study and diagnosis of osteoporosis in human bone and is widely used in clinical practice. A special standardization technic was employed: an area of exactly the same size and location was assessed in each animal [8]. The mean of three repeated measurements was used. The procedure allowed for achieving a precision of 12.5% CV. Results, obtained in groups A and B, were compared using independent Student's t-test.

Biomechanical Studies of the Deformability and Stability of the Femoral Bone

Femurs of each group were carefully cleaned, with removal of the adhesive muscle, tendon and other soft tissue. Before testing, each femur was microscopically inspected to ensure that every tested specimen was intact. Additionally, the mean saggital and the medial-lateral thickness of the mid-diaphysis (referred to as the Breadth of the Bones) were determined. From these values the area moment of inertia (Jx) and the moment of resistance (Wx) were calculated using the formulas for an ellipse. The deformability and the firmness were determined in three-point bending tests (Fig. 1).

The two supports were cylindrical with a radius of 0.5mm and the stamp with a radius of 1 mm. Care was taken to guarantee a stable and reproducible position of the specimens on the supports. This was reached if the lesser trochanter was near one support as shown in Fig. 2. The distance between the supports was chosen to be 19mm, the second support was 1 to 2 mm near the condyles in all cases. The alignment of the femora was done under microscopic control.

All tests were performed using a universal material testing system (Zwick, Mod. 1456, Germany). After a preload of 2 Newton the load was increased to 50N with a deformation rate of 0.2 mm/min. At this load level no damage of the bones occurred. This was done five times to reach a steady state, then three load cycles followed to calculate the stiffness from the load deformation curve. After these tests six relaxation cycles were performed. The load was increased to 40 N and then the deformation kept constant over 30s. Between the load phases the specimen could recover over 30s. Finally, the bones were loaded until the breakage point was reached to determine the maximum load (Fmax). The bones were kept moist by repeated spraying with Ringer sol. The stiffness and Fmax depend on the bone geometry, therefore we attempted to reduce this influence. Since it was impossible to record and consider the geometry of the whole bone, only the Jx value and the Wx of the mid-diaphysis were calculated, as mentioned above. Dividing the degree of stiffness by Jx and the Fmax by the Wx value, eliminates partly the influence of the bone geometry.

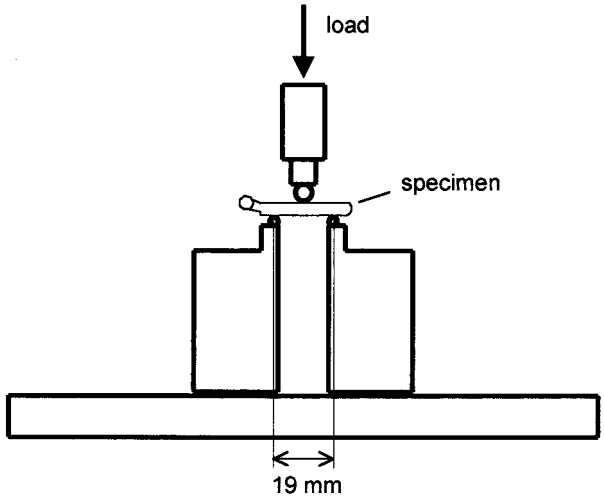

Fig. 1. Diagram of the experiment.

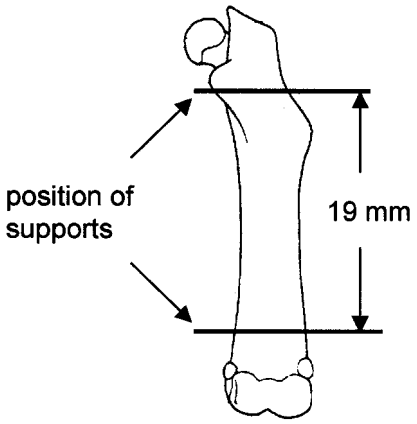

Fig. 2. Sites where the femurs were supported.

Histomorphometry Studies

Six specimen of tibias derived from rats fed 2000ppm Mg daily (group A) and six from rats fed 200ppm Mg daily (group B) were examined at the Hamburg University Division of Osteopathology, using the method of Delling [9]. Measurements were done in the spongy bone of the tibias, which was defined as an area at least 0.2mm distant from the endosteal site of cortical bone. The field of morphological evaluation was at least 1–2mm; two in each case. Three single sections of each tibia were examined. The method of Delling [9] makes use of two parameters which mirror the degree of osteoporosis present: the mean trabecular bone volume—in relation to tissue volume: BV/TV; the lower the value, the lower the mass of the trabecular bone, and the measure of trabecular interconnection: TBPf, which represents the measure of the amount of trabecular bone lattice; the higher the value, the poorer the quality of the trabecular bone. The two parameters were measured directly. The BV/TV (%), trabecular bone volume was measured by means of a point counting system (Merz-grid). All points covering mineralized bone and osteoid were divided by the number of points covering the tissue area. The TBPf (mm-1), the Trabecular Bone Pattern Factor was measured as described in detail by Hahn et al [10]. These estimates were made in blind and divided according to the group to which they belonged only at the end of the examination.

Biochemical Estimations of Urinary, Serum and Bone Mg, Ca and P

Urinary and serum Mg were estimated using ASS (Phillips, SP9). For bone Mg estimations, rat femurs were removed, dissected free and washed with water. Following fragmentation, pieces of cortical bone from the diaphysis area were collected, again washed with water, and stored in vacuo over phosphorus pentoxide. Dried bone fragments were weighed, suspended in an appropriate volume of 6mol/L hydrochloric acid and hydrolyzed by boiling for 20 hours at 110°C in Ca- and Mg-free sealed boro-silicate glass reaction vials. Bone hydrolysates were tested for Ca and Mg in a 4100 atomic absorption spectrometer (Perkin Elmer, Ueberlingen, Germany). Inorganic phosphate was measured photometrically on a Hitachi 717 analyzer (Roche, Mannheim, Germany).

Osteolytic Bone Markers

Pyridinium crosslinks: pyridinoline (PYD) and deoxypyridinoline (DPD) link the collagen molecules in bone if the bone is enzymatically degraded, they increase in the circulating blood and are eliminated in the urine, where they can be Easily measured. These markers of bone resorption were determined according to the method described by Acil et al [11]. The urinary pyridinoline (U-PYD) and the deoxypyridinoline (U-DPD) were expressed in nmol/mmol creatinine.

Statistical Analysis

Comparisons between groups A and B were performed using independent Student's t-test and comparisons between consecutive values in the same group by using dependent Student's t-test and the Mann-Whitney test. Pearson correlation coefficients matrix was used to determine the associations between the examined parameters. Time effects on the measured parameters were analysed by using analysis of variance with repeated measures (ANOVA). Delta change in values was computed and correlated with relevant variables. All data analyses were performed using SPSS, version 9. A p value of <0.05 was required to reject the null hypothesis.

RESULTS

Vertebral and Femoral Bone Density

The mean BD in L3 to L5 region (BDL) of group B was significantly lower than that of group A: $p = 0.035$ (1- tail) and the mean BD of the femoral region (BDF) was significantly lower that that of group A, as well: $p = 0.045$ (1- tail) (Table 1 and Fig 3).

Biomechanical Studies: Difference in Bone Dimensions

The bones in Mg deficient rats were, as anticipated, smaller than those of the controls, because Mg deficiency causes retarded growth in young organisms. Indeed, the femurs from Mg deficient rats were smaller. Although their antero-posterior thickness did not differ between the two groups, the mean breadth of the bones (= the distance between the lateral and the medial aspects) was significantly smaller in group B ($p = 0.035$), furthermore, the breadth of the femurs from Mg deficient rats correlated significantly with S-Mg ($p = 0.042$) and U-Mg at 12 months ($p = 0.042$). Although both groups

Table 1. Basic Data

Variable	Months	2000 ppm Mg			200 ppm Mg			*p*
		x	SD	n	x	SD	n	
U-Mg mmol/L	0	10.55	2.44	10	10.82	2.58	10	NS
	6	19.17	7.08	7	0.52	0.45	7	<0.001
	12	12.67	5.30	8	2.30	2.56	7	0.001
S-Mg mmol/L		0.824	0.121	8	0.573	0.203	7	0.011
Bone Mg mmol/g dry wt		0.181	0.007	8	0.126	0.01	7	0.001
Bone weight g		1.06	0.08	8	0.95	0.07	7	0.017
Breadth of femur cm		4.36	0.45	7	3.96	0.27	7	0.035
BDL g/cm2		0.29	0.048	8	0.254	0.015	8	0.035 (1tail)
BDF g/cm2		0.315	0.047	8	0.275	0.039	8	0.045 (1-tail)
Fmax N		163.0	26.1	7	137.0	9.0	7	0.004
U-PYD I nmol/mmol Cr		242.3	45.4	7	371.0	183.2	7	NS
U-PYD II nmol/mmol Cr		194.4	37.2	8	532.6	117.3	7	<0.001
U-DPDI nmol/mmol Cr		74.4	27.2	7	138.9	83.1	7	NS
U-DPD II nmol/mmol Cr		44.3	11.6	8	103.3	27.3	7	<0.001
Bone PYD		265.7	22.4	8	285.0	25.7	7	NS
Bone DPD		163.4	18.8	8	168.0	29.0	7	NS
BV/TV		14.6	4.8	6	11.0	5.36	6	NS
TBPf		6.6	4.0	6	8.4	4.2	6	NS

Rats fed 2000 ppm Mg diet daily belong to group A, rats fed 200 ppm, to group B.

Values in the two groups are compared using Independent Student's t-test.

U-PYD I and U-DPD I = values collected after 6 months, U-PYD II and U-DPD II = values collected after 12 months. NS = non-significant.

showed, by the end of the experiment, a significant gain in body weight ($p = 0.05$), group B gained less during the second year, than the controls. On comparing body weight in group A with that of group B, the difference amounted to 6%; not statistically significant, but the bone weight in group B was 10% lower than that of group A and that difference was statistically significant ($p < 0.017$). The Bone Mg content was also significantly decreased in group B by the end of the experiment ($p = 0.001$).

Bending Stiffness (Degree of Deformability)

The mean bending stiffness of group A (660.0 N*/mm, SD 108, n = 7) was slightly but not significantly higher than that of group B (650.0 N/mm, SD 56.3, n = 7). On dividing the numbers by the area moment of resistance (Wx) to diminish the influence of difference in bone dimensions (see under "Methods"), the mean became much higher in group B (101.6 N/mm, SD 14.2, n = 7) compared with group A (89.0 N/mm, SD 12.1,

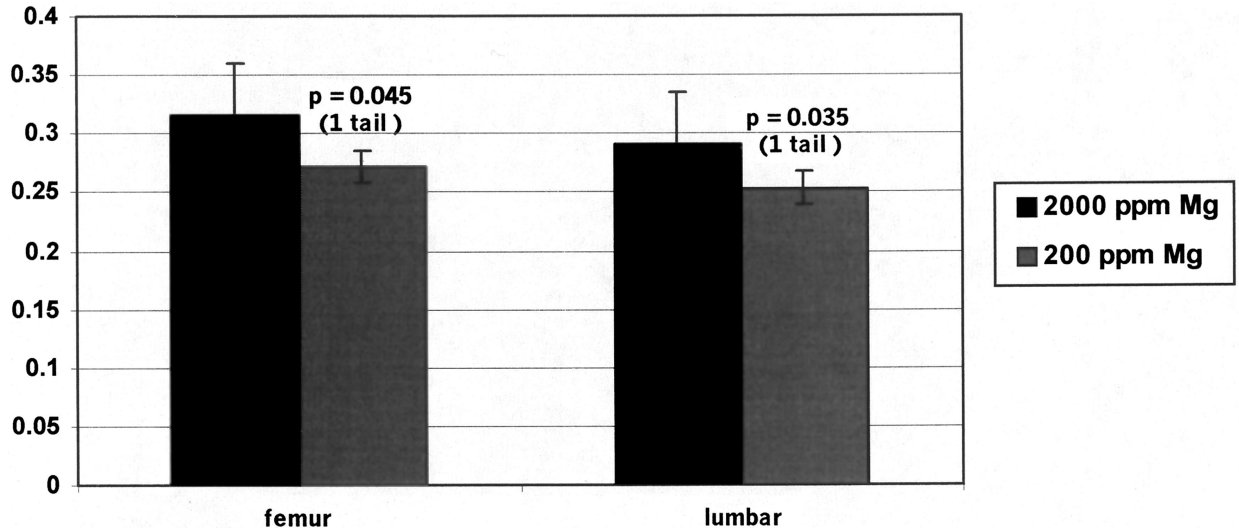

Fig. 3. Bone density (BD) of femur and of the lumbar vertebrae in rats fed 2000 ppm Mg compared with those fed 200 ppm Mg during one year.

n = 7) and the difference was statistically significant (p = 0.024) showing a marked increase in rigidity in the rat bone rendered Mg deficient.

Load to Failure (= Maximum Force Required to Break the Bone: Fmax)

The mean Fmax required to break the bone was markedly higher in group A than in group B (Table 1): p = 0.024, showing a significantly increased fragility of the bone rendered Mg deficient. Furthermore, Fmax was significantly correlated with U-Mg at 6 months (p = 0.010) and U-Mg at 12 months (p = 0.016) as well as with bone Mg contents (p = 0.018). On dividing the Fmax by the moment of resistance (Wx) to diminish the influence of difference in bone dimensions, the normalized Fmax still remained lower in group B, although no longer significantly.

Histomorphometry

The proximal part of the tibias showed superficial articular cartilage. There were no changes in the epiphysis and in the epiphysial cartilage. The cellular structures could not be distinguished because the specimen were frozen, due to the need to transport them a long distance. In group B, areas of the spongy bone showed reduction of the trabecular bone lattice, but some trabeculae reached as far as the medullary canal of the diaphysis. Diaphyseal trabeculae were broader than those of the metaphysis. The 24% diminution of the BV/TV together with a 21% increase in the TBPf (Table 1) indicated osteoporosis. This was reinforced by microscopy which showed focal osteoporosis of spongy bone in Mg deficient rats.

Statistical Analysis

The basic data obtained in group A and group B are presented in Table 1. The following parameters differed significantly on comparing group A with group B (Student's independent t-test): U-Mg after 6 months, was significantly decreased in group B (p = 0.001) and after 12 months it was further significantly decreased (p = 0.001). Serum-Mg (S-Mg) was also significantly decreased in group B (p = 0.011) as was bone weight, bone Mg content (p = 0.017 and p = 0.001, respectively) BDL, BDF (p = 0.035, 1- tail and p = 0.045, 1-tail, respectively) and Fmax (p = 0.004), whereas the osteolytic bone markers: U-PYD I and II and U-PDP II showed a highly significant increase (p < 0.001) in the bones of the Mg deficient rats (Figs 4 and 5).

Significant Correlations of S-Mg, U-Mg and Bone Mg (Pearson's Correlation Matrix)

S-Mg at the end of the experiment correlated significantly positively with the following variables: U-Mg at 12 months (r = + 0.552, p = 0.033, n = 15), bone Mg content (r = + 0.682, p = 0.005, n = 15), and Breadth of the Femoral Bones (r = + 0.549, p = 0.042, n = 14), and negatively with: U-PYD II (r = − 0.569, p = 0.027, n = 15) and U-DPD II (r = − 0.615, p = 0.015, n = 15). U-Mg at 6 months correlated positively with Bone Mg Content (r = + 0.821, p = 0.001, n = 14), BDL (r = + 0.660, p = 0.100, n = 14) Fmax (r = + 0.662, p = 0.010, n = 14) and negatively with U-PYD II (r = − 0.783 p = 0.001, n = 14) and U-DPD II (r = − 0.741, p = 0.002, n = 14). U-Mg at 12 month was positively correlated with bone weight (r = + 0.619, p 0.014, n = 15), bone Mg content (r = + 0.835, p < 0.001, n = 15), Fmax (r = + 0.629,

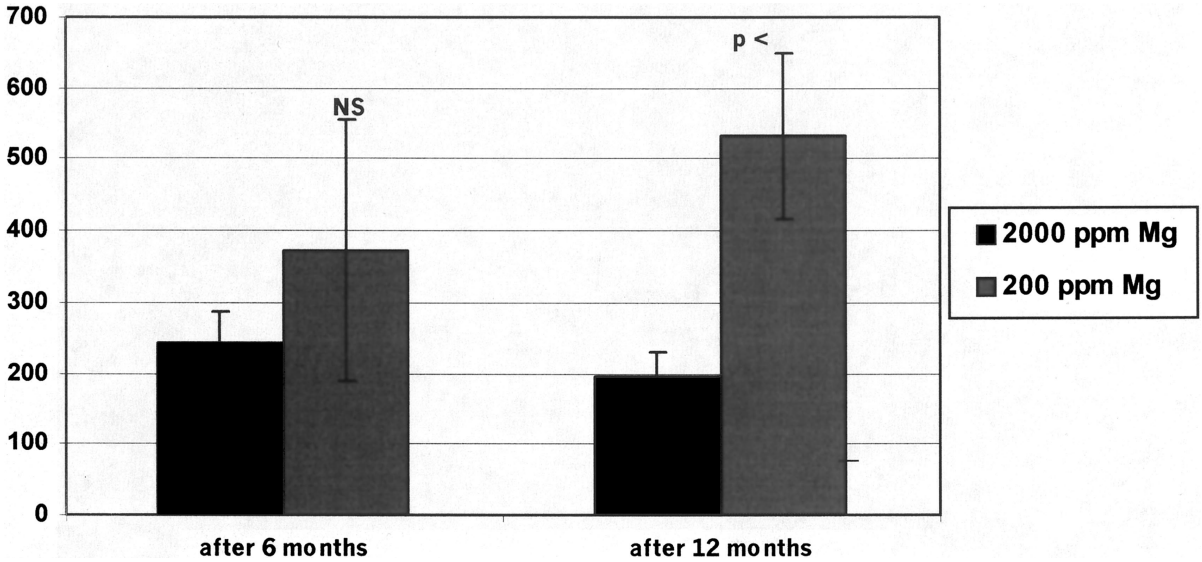

Fig. 4. U-PYD after 6, respectively, 12 months in rats fed 2000 (group A) and those fed 200 (group B) ppm Mg.

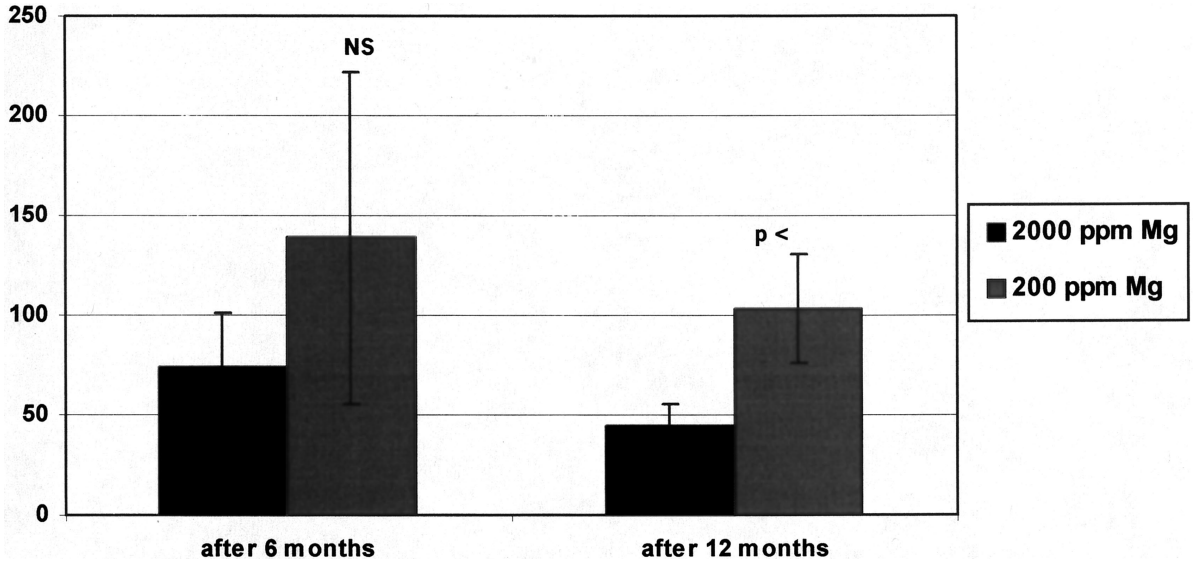

Fig. 5. U-DPD after 6, respectively, 12 months in rats fed 2000 (group A) and those fed 200 ppm (group B) Mg daily.

p = 0.016, n = 14) BDL (r = + 0.663, p = 0.007, n = 15), and Breadth of the Femoral Bones (r = + 0.549, p = 0.042, n = 14) and negatively correlated with U-PYD II (r = − 0.709, p = 0.030, n = 15) and U-DPD II (r = − 0.648, p = 0.004, n = 15). Bone Mg content was positively correlated with bone weight (r = + 0.519, p = 0.047, n = 15), S-Mg (r = 0.682, p = 0.005, n = 15; see above), Fmax (r = + 0.619, p = 0.018, n = 14), and negatively with U-PYD II (r = − 0.841, p = 0.000, n = 15) and U-DPD I (r − 0.568, p = 0.034, n = 14) and U-DPD II (r − 0.789, p = 0.000, n = 15).

Correlations (Regressions) of Delta Values of U-Mg

We gauged the change between the initial U-Mg value and that at 6 months and 12 months later respectively. The delta values represent the *change* which took place during the experiment. The differences during the first part of the year; $delta_{0-6}$ (delta I) of U-Mg and the second part of the year: delta 6–12 (delta II), respectively, and that of the whole year: o–12 (delta III), indicated that only deltas I and III of U-Mg were significantly correlated with BDL (r = + 0.659, p = 0.010, n = 14 and r = + 0.625, p = 0.013, n = 15, respectively) and Fmax (r = + 0.604, p = 0.022, n = 14 and r = + 0.520, p = 0.057: border line significance, n = 14, respectively). All three deltas of U-Mg were significantly correlated with U-PYD II (delta II positively and deltas I and III, negatively: r = +0.532, p = 0.050, n = 14, r = − 0.752, p = 0.002, n = 14 and r = − 0.668, p = 0.006, n = 15 respectively). Delta I and III of U-Mg were also negatively correlated to U-DPD II (r = − 0.713, p = 0.004, n = 14 and r = − 0.661, p = 0.007, n = 14, respectively). Thus, the significant correlations of delta III of U-Mg, e.g. the change in U-Mg value known to mirror intracellular Mg levels [13,14] throughout the year were: BDL, BDF (Fig 3), Fmax, U-PYD II (Fig 4) and U-DPD II (Fig 5) rats.

Analysis of Variance with Repeated Measures (ANOVA) of U-Mg, U-PYD and U-DPD

On analysis of variance with repeated measures (ANOVA), there was a significant interaction between time and group ie, the effect of time differed between Group A and group B with respect to U-Mg ($p < 0.001$), it increased in group A up to the 6th month, followed by a decrease and decreased in group B, reaching almost the baseline value at 6 months, followed by a slight insignificant increase between 6th and 12th month. U-PYD showed a significant interaction ($p < 0.010$), in group A showing a moderate gradual decrease throughout and in group B, a marked continuous increase. There was no significant interaction for U-DPD (p = NS); there was a parallel decrease in both groups throughout, the values being higher in group B.

DISCUSSION

Our findings agree with those of Rude et al [6] that experimentally induced Mg deficiency causes osteoporosis in the rat. The lowered BDL and BDF which we found in the Mg deficient bone, using exactly the same technic as employed in diagnosing osteoporosis in humans (dual energy X-ray absorptiometry (DEXA)), permits the conclusion that Mg deficiency in the rat reduced BD identical to that found in human osteoporosis. Bones from rats fed Mg adequate diet (group A) were less deformable than those from group B and required more

force to break. This was apparent even after dividing the results by the area moment of inertia and by the moment of resistance respectively in order to eliminate as far as possible the influence of bone geometry. Although this correction increased the difference between the groups, as regards deformability (which became statistically significantly higher in group B) and diminished the difference as regards the Fmax, the latter nonetheless remained still lower in Mg deficient bone—although no more statistically significantly. Thus, the geometrical difference, accentuated by the smaller size of Mg deficient femurs was not the cause of the increased bone deformability of Mg deficient bone. Other factors, such as changes of the material properties of the bone, which occurred under low Mg intake, and rendered the bone more rigid, must have been responsible; one of these presumably was the significant reduction of BD demonstrated in the Mg deficient bone. The small difference between group A and B, considering the parameter "load to failure/moment to resistance," found after the correction, indicates that the lower F-max of Mg deficient bones may be due to their smaller cross-sectional areas and, therefore, to their lower resistance to bending, however, as the F-max still remained higher after the correction in group A than in group B (35.5, SD 2.4, and 34.4, SD 4.5 N/mm^3 respectively, $n = 7$). Thus, we conclude that other factors, such as the significantly reduced BD in rats fed a Mg deficient diet, might be responsible. The change in architecture in the bone of rats fed Mg deficient diet became apparent on histomorphometry, even though a detailed histological examination was not carried out to allow us to examine the number of osteoblasts and osteoclasts [12]. The findings on microscopy in group B, the diminution of BV/TV and the increase in the TBPf clearly show existence of osteoporosis, which is also characterized by the lowering of trabecular bone volume without a concomitant disturbance in mineralization. S-Mg and U-Mg were significantly decreased in group B together with bone weight, bone Mg, BDL, BDF and Fmax, whereas the osteolytic markers U-PYD II and U-DPD II were significantly increased. The association of delta III of U-Mg (significantly associated with the intracellular Mg content [13,14]) augments the conclusion that the osteoporosis induced in the rat is not a random occurrence but a result of the experimentally induced Mg deprivation.

CONCLUSION

Prolonged experimentally induced Mg deficiency causes osteoporosis in the rat. Bones from rats fed Mg adequate diet (group A) showed a decrease of BDL and BDF, were less deformable than those from Mg deficient rats (group B) and required more force to break than bones with higher Mg content. In addition, the Mg deficient rats' bones exhibited architectural changes, reflected by diminution of BV/TV, increase in the TBPf, and by signs of focal osteoporosis on direct microscopy.

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