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European Journal of Internal Medicine 15 (2004) 97–107

EUROPEAN JOURNAL OF INTERNAL MEDICINE

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Original article

Experimentally induced prolonged magnesium deficiency causes osteoporosis in the rat

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Received 11 March 2003; received in revised form 25 November 2003; accepted 27 November 2003

Abstract

Background: It has been shown that prolonged daily peroral magnesium (Mg) administration, as tabs of $Mg(OH)_2$, used as the only treatment in postmenopausal osteoporosis, causes a significant increase in BMD. *Objective*: In order to obtain definitive evidence of causality of magnesium deficiency in the etiology of osteoporosis, we spent 1 year examining rats given a daily Mg-deficient diet (200 ppm) and compared them with rats given a Mg-adequate diet (2000 ppm). *Methods*: Sixteen female Sprague–Dawley rats, mean weight 110 (S.D. 23) g, were divided into two groups and randomly assigned to a semisynthetic diet that differed only in Mg content. Urine samples were collected every 3 months and blood was collected at the end of the trial. After the animals were sacrificed, the L3–L5 vertebrae and the femoral regions were examined for bone density (BMD) using dual energy X-ray absorptiometry. The femoral bones were examined for bone fragility and the tibiae by histomorphometry, and the mineral content of the bones was estimated. *Results*: The mean BMD of L3–L5 vertebral bone was significantly higher in group A (adequately nourished) than in the Mg-deficient group B ($p = 0.035$, one-tail); in addition, the BMD of the femural region was significantly higher in group A ($p = 0.045$, one-tail). The bending stiffness of the femur was slightly higher in group A than in group B; however, after correction to diminish the influence of the difference in bone dimensions between the two groups, femur rigidity (i.e., the loss of elasticity) in group B became significantly higher than that in group A ($p = 0.024$). The force needed to break the bone was significantly higher in group A than in group B ($p = 0.024$) and it remained higher 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) clearly showed the presence of osteoporosis. On microscopy, focal osteoporosis of the metaphyseal spongious bone was observed in the bone rendered Mg-deficient. *Conclusions*: Because osteoporosis is characterized by lowering of BMD, increased bone fragility, and altered bone architecture, our study showed that maintaining rats for 1 year on a Mg-deficient diet gives rise to the appearance of osteoporosis.

Keywords: Magnesium deficiency; Osteoporosis; Rats

1. Introduction

In an earlier study, one of the authors (S.-L.G.), together with colleagues, established that a 2-year daily peroral treatment with magnesium (Mg) alone (250–750 mg as tabs of $Mg(OH)_2$), administered to 31 postmenopausal osteoporotics, prevented the yearly, statistically significant decrease in bone density (BMD) seen in untreated controls in 87% of the patients ($p = 0.001$). It also caused a significant increase in the mean BMD in the treated patients ($p = 0.020$). In 71% of the treated cases, the BMD increased by 1–8% [1]. Furthermore, in a recently conducted study, only the preliminary results of which have been published [2], oral administration of 250 mg Mg daily, as tabs of $Mg(OH)_2$, to two consenting osteoporotics for 10 and 14 years, respectively, after conclusion of the trial, resulted in a gradual rise in their BMD value (by 16% and 23%, respectively) until it reached a sustained level corresponding to that found in young, healthy women.

This indicates that achieving Mg saturation of the bone compartment and subsequently maintaining a steady state of Mg exerts a protective effect against osteoporosis.

Support for the above findings came from later studies. Tucker et al. [3] showed that elderly women with a low daily intake of Mg in their food had lower BMD values than

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doi:10.1016/j.ejim.2004.01.003

controls who received adequate daily amounts of 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 Olerich [4] studied patients with the latter diagnosis and found a significant increase in BMD among them as a result of Mg treatment. In order to investigate whether Mg depletion could actually give rise to osteoporosis, Rude et al. [5] induced Mg deficiency in the rat and, after a period of 3 months, found evidence of bone loss. In 1999, the same authors [6] found that experimentally induced Mg deficiency gave rise to osteoporosis in the rat. On the other hand, Riond et al. [7] reported that a suboptimal dietary supply of Mg benefited the rat bone.

Wishing to obtain definitive evidence with regard to the association of Mg deficiency with osteoporosis, we examined rats kept on a Mg-deficient diet for a period of 1 year and we compared them with rats fed a Mg-adequate diet. Because osteoporosis is characterized by a lowering of BMD, increased susceptibility to bone fractures (bone fragility) and altered bone architecture, we designed the study so as to highlight these three features.

2. Material and methods

2.1. The experimental animals

Permission to perform the experiment was obtained from the ''Ministerium fur Umwelt, Natur und Forsten des Landes Schleswig-Holstein'' (X 330a-72241.122-4). The experiment was performed on 16 female Sprague–Dawley rats (Charles River, Sulzfeld, Germany) initially weighing 110 g (S.D. 23 g). Four rats were housed per cage at 20 °C (S.D. 2 °C) with a controlled humidity of 55% and a constant light–dark cycle from 6 a.m. to 6 p.m.

2.2. Diets

Animals were randomly assigned to a semisynthetic diet (Ssniff, Soest, Germany) which differed only with respect to Mg content; it was enriched either with 200 or 2000 ppm MgO. The rats receiving 2000 ppm Mg daily constituted group A and those fed 200 ppm Mg daily, group B. The otherwise identical diet contained 9000 ppm calcium, 7000 ppm phosphate, 10 000 ppm potassium, and 2000 ppm sodium. It consisted of 24% casein, 50% starch, 11% glucose, 3% soybean oil, 5% cellulose powder, and 6% mineral mix. All animals had free access to tap water ad libitum. The animals were pair-fed. The average daily food intake per experimental animal in group A was 30 g and the daily water intake was 44 ml tap water. In group B, the average daily food intake per experimental animal was 29 g and the daily water intake was 43 ml tap water. The Mg content of the tap water was 8.9–11.8 mg/l.

2.3. Experimental procedure

The study was performed as an endpoint investigation. During 12 months of the study, the animals were weighed monthly and urine samples were taken every 3 months. For urine sampling, each animal was brushed to clean the animals' surface and put into a metabolic cage for a period of 6 h to collect urine separately from feces. During that time, no water was offered. Urine volume was measured, given as the nearest milliliter, and collected in Eppendorf vials. After the last urine sampling, the animals were anesthetized by i.p. injection of 25 mg/kg ketamine and 40 mg/kg diazepam. Blood was collected by heart puncture into heparinized syringes and centrifuged. After the animals were sacrificed, the BMD of the femoral and of the lumbar vertebral bones was estimated, the biomechanical properties of the bones were examined, and the histomorphometric studies carried out.

2.4. Bone density studies

To establish the possible presence of a lowered BMD as a result of experimentally induced Mg deficiency in the rats, we used a noninvasive method: dual energy X-ray absorptiometry (DEXA), which is the golden standard in the study and diagnosis of osteoporosis in human bone and which is routinely and widely used in clinical practice. After the animals were sacrificed, the BMD of the femoral bones and of the L3–L5 lumbar vertebral bones was estimated employing DEXA apparatus (Prodgy, LUNAR, Europe, Brussels, Belgium) used in patient surveys. For the study of rats we employed a special standardization technique. An area of exactly the same size and location was assessed in each animal [8]. The mean of three repeated measurements was used. Use of the standardization technique was facilitated by placing the animals between two plastic bags, each containing 2 l of water, in order to secure stable placement. The procedure used made it possible to achieve a precision of 12.5% CV. Results obtained in groups A and B were compared using independent Student's *t*-tests.

2.5. Biomechanical studies of the deformability and stability of the femoral bone

Seven femora from each group were carefully cleaned; that is, the adhesive muscle, tendon, and other soft tissue were removed. Before testing, each femur was microscopically inspected to ensure that every tested specimen was intact. In addition, the mean sagittal and the medial-lateral thickness of the middiaphysis (referred to as the ''breadth'' of the bones) were determined. From these values the area moment of inertia (J_x) and the moment of resistance (W_x) were calculated using the formulas for an ellipse.

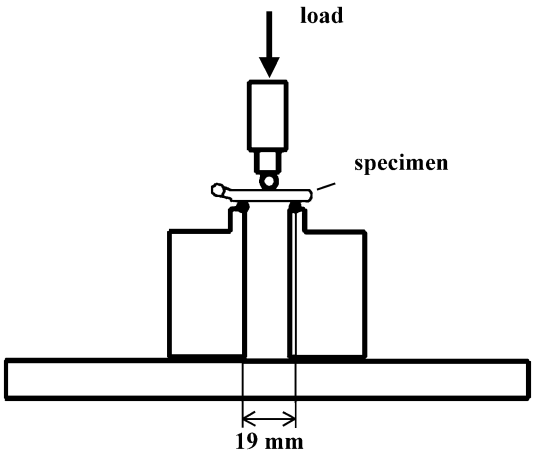

Fig. 1. Diagram of the experiment; three-point bending test.

The deformability and the firmness were determined in three-point bending tests (Fig. 1). The two supports were cylindrical and had a radius of 0.5 mm while the stamp had 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 19 mm; thus, the second support was 1–2 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 N, the load was increased to 50 N with a deformation rate of 0.2 mm/min. At this load level there was no damage to the bones. This was done five times to reach a steady state. Then, three load cycles followed to calculate the structural femoral 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 30 s. Between the load phases the specimen could recover over 30 s. Finally, the bones were loaded until the breakage point was reached to determine the failure load (F_{max}). All the time, the bones were kept moist by repeated spraying with Ringer solution.

As the rigidity of femur and F_{max} depend on the bone geometry, an attempt was made to reduce this influence. It was impossible to record and consider the geometry and the internal structure of the entire bone. Therefore, the material properties (elastic modulus and the strength) could not be determined. However, a rough elimination of the influence of geometry was reached by dividing the degree of the structural femoral stiffness by J_x and the F_{max} by the W_x values of the middiaphysis.

2.6. Histomorphometry studies

Six tibiae specimens from rats fed 2000 ppm Mg daily (group A) and six from rats fed 200 ppm Mg daily (group B) were examined at the Hamburg University Division of Osteopathology using their method of investigation [10]. Tibiae were embedded and decalcified. Sections 5-μm thick were stained using Goldner, von Kossa modification, and toluidine blue reaction. All morphologic measurements were done in the spongy bone of the tibiae. This was defined as an area at least 0.2 mm distant from the endostal site of cortical bone. The field of morphological evaluation was at least 1–2 mm, two in each case. Three single sections of each tibia were examined. The method makes use of two parameters that mirror the degree of osteoporosis present. The first is mean trabecular bone volume in relation to tissue volume (BV/TV), where the lower the value, the lower the mass of the trabecular bone. The second is the measure of trabecular interconnection (TBPf), which represents the measure of the amount of trabecular bone lattice. Here, the higher the value, the poorer the quality of the trabecular bone. The two parameters were measured directly. The BV/TV (%) 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), Trabecular Bone Pattern Factor, was measured as described in detail by Hahn et al. [11]. These estimates were made in blind and divided according to the group to which they belonged only at the end of the examination.

The limitation of the histomorphometry examination was that the tibia specimen had to undergo freezing prior to it because the DEXA procedure required intact frozen animals and, therefore, had to be carried out first. This did not allow for dissection of the animals and fixation with formalin before histomorphometry.

2.7. Biochemical estimations

2.7.1. Urinary, serum, and bone estimations of Mg, Ca, and P

Urinary and serum Mg (U-Mg and S-Mg) were estimated using atomic absorption spectrophotometry (Phillips, SP9)

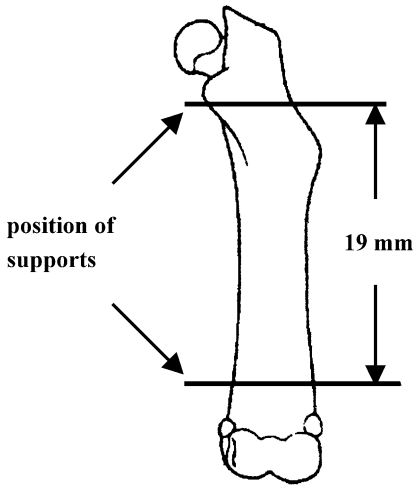

Fig.2. Sites where the femurs were supported.

and P and Cr were measured using a Hitachi 747 (Boehringer, Mannheim, Germany). For bone Mg estimations, rat femora were removed, dissected free of adhering soft tissue, 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 6 mol/l hydrochloric acid, and hydrolyzed by boiling for 20 h at 110 °C in calcium- and magnesium-free sealed boro-silicate glass reaction vials. Bone hydrolysates were tested for calcium 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) using the method routinely applied in laboratory diagnosis: formation of ammonium phosphomolybdate complex.

2.7.2. Estimation of parathyroid hormone (PTH)

Parathyroid hormone (PTH) was measured with a two-site homologous immunoradiometric assay (rat PTH, IRMA kit, Immunotopics Inc., San Clemente, USA; distributor IBL GmbH, Hamburg, Germany). This assay detects both intact PTH (1-84) and N-terminal PTH (1-34). The assay characteristics, as reported by the manufacturer, were: sensitivity 1.0 pg/ml; measurable range up to 2.500 pg/ml, recovery of added PTH 90–103%, intraassay and interassay CV 4.0% and 4.3% at 50 pg/ml, as well as 4.3% and 4.7 % at 485 pg/ml PTH, respectively. From our own results, an intraassay CV of 2.2% at a mean concentration of 12.4 pg/ml PTH, ($n = 16$), was derived.

2.7.3. Osteolytic bone markers

Pyridinium cross-links—pyridinoline (PYD) and deoxypyridinoline (DPD)—link the collagen molecules in the 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. [9]. Urine samples were acidified and hydrolyzed as described. Both urine and bone hydrolysates were extracted by column chromatography on cellulose and the extracts were analyzed by reversed-phase high performance liquid chromatography (HPLC) using fluorescence emission at 400 nm following excitation at 295 nm for analyte detection. The urinary pyridinoline (U-PYD) and the deoxypyridinoline (U-DPD) were expressed in nmol/mmol creatinine.

2.7.4. Statistical analysis

Comparisons between groups A and B were performed using independent Student's *t*-tests (one-tailed) and comparisons between consecutive values in the same group using dependent Student's *t*-tests and the Mann–Whitney *U* test. A Pearson correlation coefficient matrix was used to determine the associations between the parameters examined. Time effect on the parameters measured was analyzed using an analysis of variance (ANOVA) with repeated measures. Delta changes in values were computed and correlated with relevant variables. All data analyses were performed using SPSS, version 9. A p value below 0.05 was required to reject the null hypothesis.

3. Results

3.1. Vertebral and femoral bone density

The mean BMD in the L3–L5 region (BMDL) of the Mg-adequate group of rats (group A) was: × 0.291, S.D. 0.048, $n = 8$, range 0.230–0.271; that of the Mg-deficient rats (group B) was × 0.254, S.D. 0.015, $n = 8$, range 0.192–0.329; $p = 0.035$ (one-tailed).

The BMD in the femoral region (BMDF) in group A was: × 0.315, S.D. 0.047, $n = 8$, range 0.246–0.367 and in group B: × 0.275, S.D. 0.039, $n = 8$, range 0.238–0.329; $p = 0.045$ (one-tailed).

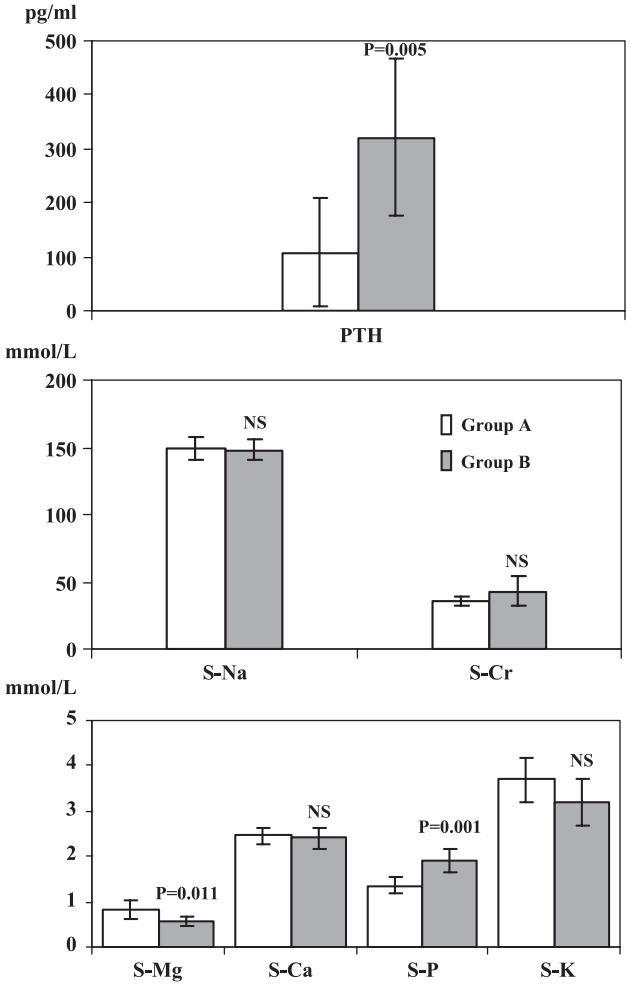

Fig. 3. Comparison of serum parameters of groups A and B at the end of the experiment.

3.2. Biomechanical studies

3.2.1. Difference in bone dimensions

The bones in rats rendered Mg-deficient were anticipated to be smaller than those of rats receiving Mg-adequate diets because Mg deficiency causes retarded growth in young organisms, including the growing bone. Indeed, the femora from rats rendered Mg-deficient were smaller. Although the anteroposterior thickness did not differ between the two groups, the mean breadth of the bones (i.e., the distance between the lateral and the medial aspects) was significantly smaller in group B than in group A ($p = 0.035$). Furthermore, the breadth of the femora from Mg-deficient rats was significantly correlated with S-Mg ($p = 0.042$) and with U-Mg at 12 months ($p = 0.042$).

In addition, although by the end of the experiment both groups showed a significant gain in body weight (p=0.05), the Mg-deficient rats began to gain less weight during the second part of the year. Body weight in group A was 367.0 g, S.D. 76, n=8 compared with 332.0 g, S.D. 52.3, n=7 in group B. The difference, 6%, was not statistically significant, but the bone weight in group B was 10% lower than that of group A (1.06 g, S.D. 0.08, n=7 and 0.95 g, S.D. 0.07, n=8, respectively) and that difference was statistically significant (p<0.017). The bone Mg content was also significantly decreased by the end of the experiment (p=0.001).

3.2.2. The bending stiffness (the degree of deformability)

The mean bending stiffness of group A (660.0 N/mm, S.D. 108, $n = 7$) was slightly, but not significantly, higher than that of group B (650.0 N/mm, S.D. 56.3, $n = 7$). On dividing the numbers by the area moment of inertia (J_x) to diminish the influence of difference in bone dimensions (see Materials and Methods), the mean normalized bending stiffness became much higher in group B (101.6 N/mm^5, S.D. 14.2, $n = 7$) than in group A (89.0 N/mm^5, S.D. 12.1, $n = 7$), and the difference was statistically significant ($p = 0.024$), showing a marked increase in rigidity in the rat bone rendered Mg-deficient.

3.2.3. The load to failure (the maximum force required to break the bone: F_{max})

The mean F_{max} required to break the bone was markedly higher in group A than in group B (163.0 N, S.D. 28.2, $n = 7$ and 137.0 N, S.D. 9.0, $n = 7$, respectively, $p = 0.024$), showing a significantly increased fragility of the bone rendered Mg-deficient. Furthermore, F_{max} 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 content ($p = 0.018$). On dividing the F_{max} by the moment of resistance (W_x) to diminish the influence of difference in bone dimensions, the normalized F_{max} still remained lower in group B, although no longer significantly so.

3.2.4. Histomorphometry

The proximal part of the tibiae showed superficial articular cartilage. There were no changes in the epiphysis or in the epiphysial cartilage. The cellular structures could not be distinguished because the specimens were frozen. The spongious bone showed a reduction in trabecular bone lattice in places. At the same time, one could see trabeculae reaching as far as the medullary canal of the diaphysis. The trabeculae of the diaphysis were broader than those of the metaphysis. The morphological findings showed a focal osteoporosis of the metaphyseal spongious bone in the rats rendered Mg-deficient.

There was a 24% diminution of the mean percentage of trabecular bone area (BV/TV), together with a 21% increase in connectivity measurement (TBPf), which are the two important indicators of the presence of osteoporosis.

The mean value of BV/TV in group A was 14.567, S.D. 4.824, $n = 6$; in group B, it was 11.047, S.D. 5.260, $n = 6$; the mean value of TBPf in group A was 6.607, S.D. 4.047, $n = 6$ and in group B was 8.398, S.D. 4.150, $n = 6$. The lack of statistical significance when comparing the results of the two groups is probably due to the small sample size.

3.2.5. Statistical analysis

The 24-h urinary Mg (U-Mg) values sampled at the beginning, after 6 months, and after 12 months in groups A and B are listed in Table 1. The values of the bone-related parameters in the two groups are given in Table 2. Serum parameter values in group A compared with those in group B are seen in Fig. 3. Other parameter values in group A compared with group B were as follows. Serum Mg (S-Mg) was significantly lowered in group B ($p = 0.011$). Bone weight and bone Mg content were also significantly decreased in group B ($p = 0.017$ and $p = 0.001$, respectively), as was BMDL, BMDF ($p = 0.035$, one-tailed and $p = 0.045$,

Table 1
24-h urinary parameter values

Variable	Months	Group A			Group B			
		x	S.D.	n	x	S.D.	n	p
U-Mg	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
U-Ca	6	7.78	2.84	7	1.47	0.52	8	<0.001
	12	7.78	1.79	8	3.67	1.77	7	<0.001
U-P	0	17.84	3.86	10	16.5	4.28	10	NS
	6	0.28	0.19	7	25.71	19.60	7	=0.014
	12	3.45	3.61	8	68.99	55.08	7	=0.020
U-K	0	162.1	27.7	10	158.7	23.9	10	NS
	6	147.5	27.3	7	163.1	65.6	7	NS
	12	132.3	28.6	8	167.7	86.6	7	=0.005
U-Na	0	132.6	24.7	10	137.8	20.0	10	NS
	6	108.3	38.7	7	137.6	22.5	7	=0.007
	12	86.3	26.8	8	91.2	38.5	7	NS
U-Cr	0	3.9	0.8	10	3.8	1.0	10	NS
	12	3.4	0.7	8	8.3	2.9	7	=0.004

Table 2
Bone-related parameters

	Group A			Group B			
	x	S.D.	*n*	*x*	S.D.	*n*	*p*
Bone Mg (mmol/g dry wt.)	0.181	0.007	8	0.126	0.01	7	=0.001
Bone Ca (mmol/g dry wt.)	7.14	0.30	8	7.35	0.24	7	NS
Bone P (mmol/g dry wt.)	4.20	0.19	8	4.31	0.14	7	NS
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
BMDL (g/cm^2)	0.291	0.048	8	0.254	0.015	8	=0.035 (one-tail)
BMDF (g/cm^2)	0.315	0.047	8	0.275	0.039	8	=0.045 (one-tail)
F_{max} (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 (nmol/mmol Cr)	265.7	22.4	8	285.0	25.7	7	NS
Bone DPD (nmol/mmol Cr)	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

Values in the two groups are compared using independent Student's *t*-tests. 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.

one-tailed, respectively), and F_{max} ($p=0.004$), whereas U-PYD I and II and U-PDP II showed a highly significant increase in group B ($p<0.001$).

Significant correlations of S-M and other serum parameters were as follows. S-Mg was positively correlated with U-Ca at 6 and 12 months ($r=+0.696$, $p=0.006$, $n=14$ and $r=+0.750$, $p=0.001$, $n=15$, respectively), and with U-Na at 6 months ($r=+0.591$, $p=0.026$, $n=14$). It was negatively correlated with U-Na at the beginning of the experiment ($r=-0.560$, $p=0.030$, $n=15$) and with U-K and U-Cr at 12 months ($r=-0.548$, $p=0.034$, $n=15$ and $r=-0.530$, $p=0.042$, $n=15$, respectively).

Significant correlations of S-M with U-Mg and bone-related parameters can be seen in Fig. 4. Significant correlations of U-Mg with PTH and bone-related parameters at 6 months are given in Fig. 5. Significant correlations of U-Mg with bone-related parameters at 12 months are given in Fig. 6.

3.2.6. Significant correlations of bone Mg content

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), F_{max} ($r=+0.619$, $p=0.018$, $n=14$), and negatively with PTH ($r=-0.594$, $p=0.020$), 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 ($r=-0.789$, $p=0.000$, $n=15$).

Significant correlations of PTH with bone-related parameters are given in Fig. 7.

3.2.7. Other significant correlations of PTH

U-Mg at 6 months (Fig. 6), S-P and U-P at 6 and 12 months ($r=+0.654$, $p=0.008$, $n=15$; $r=+0.708$, $p=0.005$,

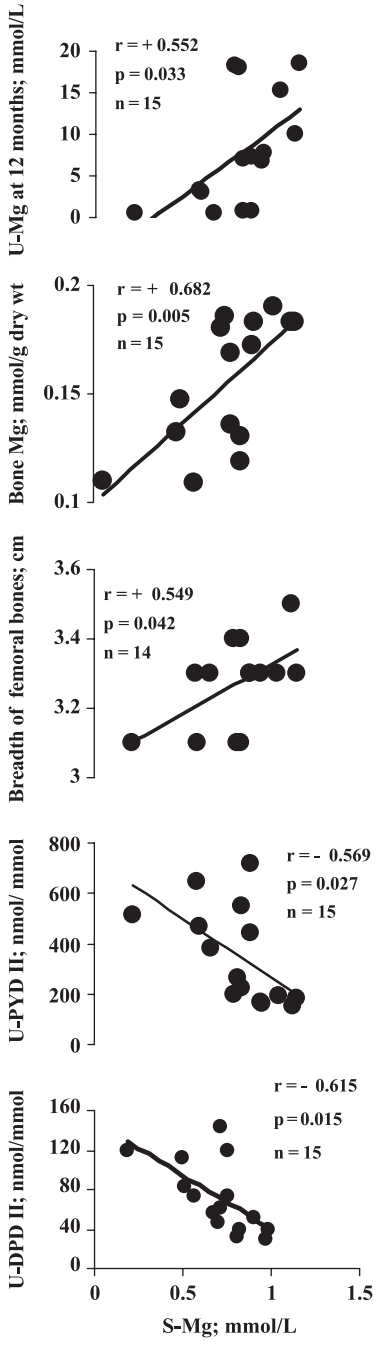

Fig. 4. Significant correlations of S-Mg with U-Mg and bone-related parameters.

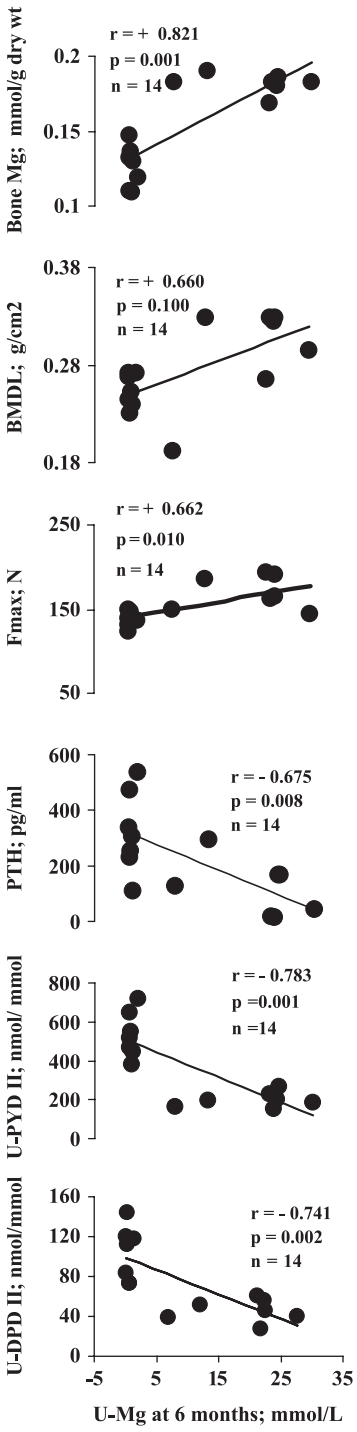

Fig. 5. Significant correlations of U-Mg with PTH and bone-related parameters at 6 months.

$n = 14$ and $r = +0.748$, $p = 0.001$, $n = 15$, respectively), U-Ca at 6 and 12 months ($r = -0.666$, $p = 0.009$, $n = 14$ and $r = -0.735$, $p = 0.002$, $n = 15$, respectively), U-Na at 6 months and U-K and U-Cr at 12 months ($r = -0.701$, $p = 0.005$, $n = 14$; $r = +0.760$, $p = 0.001$, $n = 15$ and $r = +0.720$, $p = 0.004$, $n = 15$, respectively).

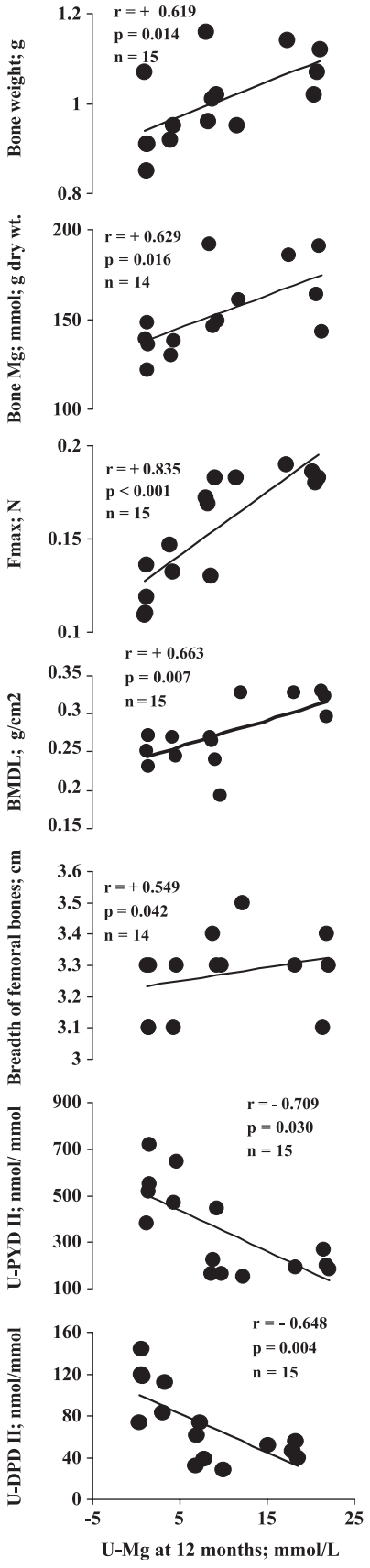

Fig. 6. Significant correlations of U-Mg with bone-related parameters at 12 months.

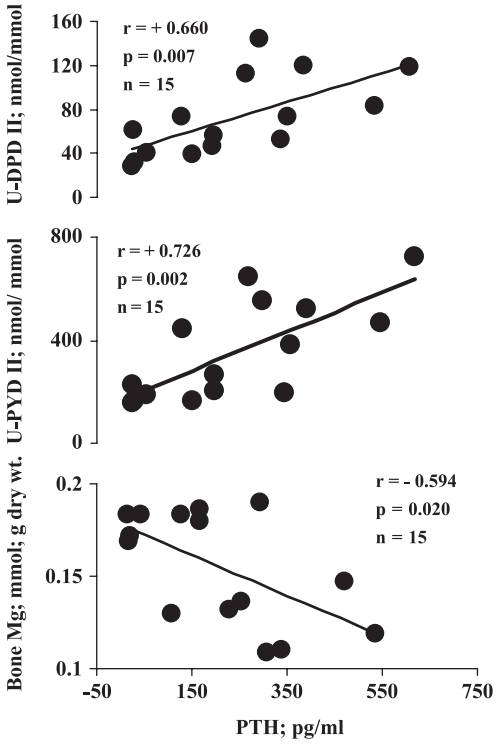

Fig. 7. Significant correlations of PTH with bone-related parameters.

3.2.8. Delta correlations

When considering the differences between the first and the second part of the year, Δ0–6 (delta I) and Δ6–12 (delta II), respectively, and that of the whole year, Δ0–12 (delta III) as regards U-Mg values, we found the following. Delta I of U-Mg was negatively significantly correlated with PTH, while delta II was positively correlated with PTH ($r=-0.595$, $p=0.025$, $n=14$ and $r=+0.578$, $p=0.030$, $n=14$, respectively). Deltas I and III of U-Mg were significantly correlated with BMDL ($r=+0.659$, $p=0.010$, $n=14$ and $r=+0.625$, $p=0.013$, $n=15$, respectively) and with F_{max} ($r=+0.604$, $p=0.022$, $n=14$ and $r=+0.520$, $p=0.057$: borderline 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. Deltas I and III of U-Mg were also negatively correlated with U-DPD ($r=-0.713$, $p=0.004$, $n=14$ and $r=-0.661$, $p=0.007$, $n=14$, respectively).

All three deltas of U-P were also positively significantly correlated with PTH ($r=+0.633$, $p=+0.015$, $n=14$; $r=+0.635$, $p=0.015$, $n=14$ and $r=+0.733$, $p=0.002$, $n=15$, respectively) and with U-PYD ($r=+0.851$, $p=0.001$, $n=15$; $r=+0.717$, $p=0.004$, $n=14$ and $r=+0.750$, $p=0.020$, $n=14$, respectively).

Deltas II and III of U-P were also significantly correlated with U-DPD ($r=+0.672$, $p=0.006$, $n=15$ and $r=+0.609$, $p=0.027$, $n=15$, respectively).

Deltas II and III of U-K were positively significantly correlated with PTH ($r=+0.718$, $p=0.003$, $n=15$ and $r=+0.537$, $p=0.046$, $n=14$, respectively) and delta I of U-Na was negatively significantly correlated with PTH ($r=-0.668$, $p=0.009$, $n=14$) whereas delta III of Na was positively correlated ($r=+0.788$, $p=0.001$, $n=14$).

There were no correlations between the deltas of U-Ca and PTH.

U-Mg is known to be associated with the Mg content of the mononuclear cells [12,13]; therefore, the delta III of U-Mg mirrors the change in intracellular Mg content throughout the span of the whole year of the trial. The correlates of delta U-Mg III therefore gain special significance and are presented in Fig. 8.

3.2.9. Analysis of variance (ANOVA) with repeated measures

On analysis of variance (ANOVA) with repeated measures, there was a significant interaction between time and group. That is, the effect of time differed between the two

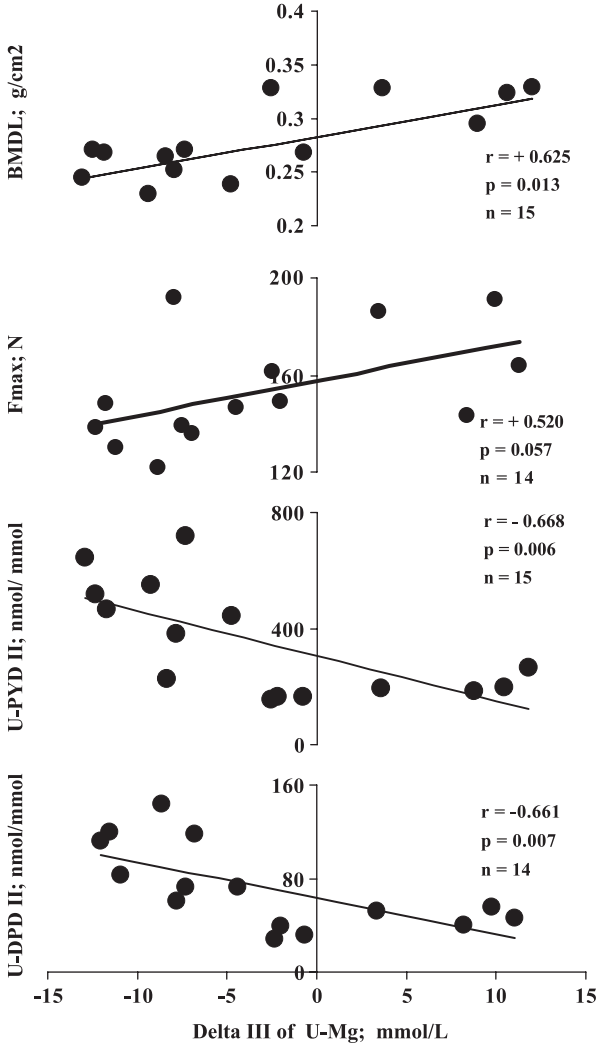

Fig. 8. Significant correlations of delta III of U-Mg.

groups fed 2000 ppm (group A) and 200 ppm (group B) Mg with respect to the following variables: U-Mg ($p < 0.001$), U-P ($p = 0.002$), U-K ($p = 0.004$), U-Na ($p = 0.020$), U-Cr ($p < 0.001$), and U-PYD ($p = 0.010$).

4. Discussion

Our findings are in agreement with those of Rude et al. [6] that experimentally induced Mg deficiency causes osteoporosis in the rat. We focused our study on identifying the three features characteristic of osteoporosis in the experimental animals kept on a Mg-deficient diet for a period of 1 year. To establish the possible presence of the first feature, a decrease in BMD, we used a noninvasive method, dual energy X-ray absorptiometry (DEXA). The lowered BMDL and BMDF that we found in the experimental animals rendered Mg-deficient, using exactly the same technique employed in diagnosing osteoporosis in humans, allows us to conclude that Mg deficiency induced a reduction in BMD in the experimental animals that is identical to that found in human osteoporosis.

To establish the possible presence of the second feature, the increased susceptibility of the bone to fractures (increased fragility), we used a biomechanical test defining the deformability of the bone and the maximal load needed to break it (F_{max}). We found that bone from rats fed an Mg-adequate diet (group A) were less deformable than those from group B and failed at higher loads (that is, they 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 with regard to the deformability (which became statistically significantly higher in group B) and diminished the difference with regard to the F_{max}, the latter nonetheless remained lower in the Mg-deficient bone, although no longer to a statistically significant degree.

Thus, the geometrical difference, accentuated by the smaller size of the Mg-deficient femora as a result of the thwarting effect exerted on the bone growth by the very same Mg deficiency that was being studied, was not the cause of the increased bone deformability. That is, the susceptibility to fractures in Mg deficiency and of the diminished F_{max} in the Mg-deficient bone. Other factors, such as changes in the material properties of the bone that occurred under low Mg intake and rendered the bone more rigid, must have been responsible. One of these presumably was the presence of the significant reduction in BMD demonstrated in the Mg-deficient bone. The small difference between groups A and B in the parameter "load to failure/moment to resistance", which was found after the correction, indicates that the lower load to failure of bones from rats rendered Mg-deficient may be due to their smaller cross-sectional areas and, therefore, to their lower resistance to bending. However, as the normalized mean load to failure still remained higher after the correction in group A than in group B (35.5, S.D. 2.4 and 34.4, S.D. 4.5 N/mm^3, respectively, $n = 7$), we have to conclude that other factors such as the BMD of the femur, which was significantly reduced in animals fed a Mg deficient diet, might be responsible, as well.

The change in the architecture in the bone of rats fed a Mg-deficient diet, the third feature characterizing the presence of osteoporosis, became apparent on morphological studies. A detailed histological examination was not carried out to allow us, e.g., to examine the number of osteoblasts and osteoclasts, the decrease in the former, and the increase in the latter reported as characterizing the effect of Mg depletion on bone [14] because the specimens were frozen. Nonetheless, the morphological findings, together with the diminution of BV/TV and the increase in the TBPf, clearly showed the presence of osteoporosis in the bone of the rats in which Mg deficiency was induced. This was especially due to the fact that a lowering of trabecular bone volume without a concomitant disturbance in mineralization characterizes the presence of osteoporosis. In addition, the urinary osteolytic marker, U-PYD, was significantly increased in the urine of the rats rendered Mg-deficient after 6 months. The increase was further augmented after the end of 1 year, when the experiment ended. The statistical work throughout also showed the close association of the increase in U-PYD and the indices of Mg deficiency, reinforcing the conclusion that the latter gave rise to the osteoporosis.

Thus, we found that whereas S-Mg and U-Mg were significantly decreased in group B, together with bone weight, bone Mg, BMDL, BMDF, and F_{max}, the osteolytic markers U-PYD II and U-DPD II were significantly increased.

S-Mg was significantly positively correlated with U-Mg, bone Mg content, and breadth of the femoral bone, and negatively correlated with U-PYD II and U-DPD II. After 6 months, U-Mg was significantly positively correlated with bone Mg content, BMDL, and F_{max}, and negatively with U-PYDII and U-DPD II; after 12 months, it was negatively correlated with bone weight and the breadth of the femoral bone as well. Delta, i.e., the change in values observed between consecutive measurements, such as the change between the initial and the end U-Mg value (a value that represents the total change that took place throughout the experiment and therefore may constitute a decisive factor responsible for the findings at the end of the experiment, which were not present at the beginning), was statistically significantly positively correlated with BMDL, and F_{max} and negatively correlated with the markers of the osteolytic activity (U-PYD II and U-DPD II), the parameters that constitute indices of osteoporosis. Furthermore, it is known that U-Mg is significantly associated with intracellular Mg content [12,13]. This lends weight to the importance of the

association of delta III of U-Mg with the indices of osteoporosis. The significance of this correlation supports the conclusion that the osteoporosis induced in the rat is not a random occurrence but rather a result of the experimentally induced Mg deprivation.

Of the total body Mg ($\pm$24 g), 50–60% is found in bone. Thirty percent of this is found in the readily available surface-limited pool, dissolved in the hydration shell, or absorbed on the surface of the hydroxyapatite crystals. This might serve as the primary source of Mg in states of Mg deficiency. The remaining 70% of the bone Mg forms an integral part of the bone crystals and is not readily available; its release probably involves the resorption of bone [15]. In our experiment, the U-Mg in group B was significantly decreased and reached its nadir at 6 months, when it approximates zero (Table 1). This shows an extreme Mg conservation and mirrors the presence of a severe intracellular deficit. At the same time, U-Ca was significantly lowered and U-P began to rise significantly in group B compared with group A. In Mg deficiency the cell retains Ca and loses phosphates [16], so the latter changes could be interpreted as secondary to the primary event, which is the loss of Mg. Mg deficiency causes rise of PTH [17]. We see in group B an approximate threefold rise in PTH. At the same time, there is a highly significant decrease in S-Mg and in bone Mg content (which is significantly negatively correlated to PTH) and a significant increase in S-P, whereas S-Ca, bone Ca, and bone P are virtually unchanged. While deltas I, II, and III of U-P are positively correlated to PTH, delta I of U-Mg is significantly negatively correlated to PTH, and delta II of U-Mg is significantly positively associated with PTH. There is no significant correlation between PTH and either of the three deltas of U-Ca. We assume that the rise in PTH occurred after U-Mg reached it nadir at 6 months. A rise in PTH increases proximal tubular reabsorption of Ca, diminishes that of P, and speeds up the reabsorption of Mg. We can observe these effects in the change in the respective values at 12 months (Table1). The significant positive correlation of PTH and of U-PYD II and U-DPD II, the osteolytic bone markers (estimated at 12 months) but not with U-PYD I and U-DPD I (estimated at 6 months) points to an association of the rise in PTH with osteolytic activity and confirms the presumed timing of PTH rise. We know that the effect of long-term excess of PTH is to activate osteoblastic and osteoclastic activity; with the passage of time, the bone absorption is more pronounced than the bone deposition. Rude et al. [14] described a preponderance of osteoclasts over osteoblasts in mice rendered Mg-deficient and a loss of trabecular bone in the bone rendered Mg-deficient. The bone absorption allows for release of Mg, as well as of Ca from the bone tissue. Consequently, we hypothesize that because Mg is essential for biological functions, the rise in PTH is instrumental in releasing Mg from the bone crystals in order to save the intact organism at the sacrifice of the integrity of the bone, which develops osteoporosis.

Acknowledgements

We are indebted to Dr. Leif Dibbelt, Department of Clinical Chemistry, University of Luebeck, who assisted with the biochemical analysis; to Prof. I. Leichter, Department of Radiology, Hadassah University Hospital, Jerusalem, for guidance in the application of DEXA to small animals; to Dr. Beate Stoeckelhuber, Department of Radiology, University of Luebeck, Germany, for supervising the osteodensitometry, and to Prof. Delling, Department of Pathology/Osteopathology, University of Hamburg Eppendorf, for assessment of the results of histomorphometry.

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