Magnesium Supplementation in Rats with Hydronephrocalcinosis

Electrolyte and Histologic Studies

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Zusammenfassung

Weibliche, 53 g schwere S.D.-Ratten (n = 130) wurden zufällig auf 13 Gruppen verteilt und erhielten 66 Tage lang Futter mit unterschiedlichen Mengen an Magnesium. Bei 6 Gruppen wurde nach einer 10tägigen Mg-Mangeldiät (75 ppm Mg) das Futter auf 9500 ppm Mg supplementiert. Proben wurde an den Tagen 0, 1, 3, 7, 15, 28 und 56 entnommen. An den Tagen 0 und 56 standen außerdem Kontrollgruppen mit durchgehender Ernährung mit 9500 ppm Mg haltigem Futter zur Verfügung. Als weitere Vergleichsgruppen dienten ausschließlich mit Mg-Mangeldiät (75 ppm Mg) ernährte und an den Tagen 0, 7, 15, 28 und 56 untersuchte Ratten.

Plasma- und Femur-Mg spiegelten deutlich den Magnesiummangel und die anschließende Supplementation wieder. Histologische und histochemische Untersuchungen ergaben in den Magnesiummangelgruppen eine Nephrohydrose, die durch eine Verlegung distaler Hauptstücke mit Konglomeraten aus Kalzium, Phosphat und einer organischen Matrix bedingt war. Die organische Substanz besitzt vermutlich ursächliche Bedeutung für die Entstehung der Nierenschädigung. Hochdosiertes Magnesium bewirkte bei den Mangeltieren eine beträchtliche Verminderung des Nierenkalziums mit großen Unterschieden von Tier zu Tier. Eine vollständige Ausscheidung oder Auflösung der Präcipitate war jedoch nicht die Regel.

Summary

Female S. D.-rats (b. w. 53 g, n = 130) were divided into 13 groups and fed with different Mg schedules for 66 days. The diet of 6 groups was supplemented to 9500 ppm Mg after a 10 day period of Mg deficiency (75 ppm Mg). The animals were examined on day 0, 1, 3, 7, 15, 28 and 56. Control groups were given 9500 ppm Mg continously and examined on day 0 and 56, and other with an exclusively Mg deficient diet (75 ppm Mg) on day 0, 7, 15, 28 and 56.

Plasma- and femur-Mg clearly reflected the Mg deficiency and the subsequent supplementation. Histological and histochemical examinations showed a nephrohydrosis in the Mg deficient groups caused by an obstruction of the distal part of proximale tubules, with conglomerates of calcium, phosphate and an organic matrix; the latter may be especially significant for the pathogenesis of the lesions. High Mg doses led to a remarkable decrease in renal calcium with conspicuous differences between the animals. A complete excretion or dissolution of the precipitates has not been the rule.

Résumé

Des rats femelles S. D. pesant 53 g (n = 130) ont été répartis au hasard en 13 groupes et nourris pendant 66 jours avec des mélanges contenant différentes quantités de magnésium. Six groupes ont reçu une supplémentation en magnésium de 9500 ppm après une période de 10 jours de carence en magnésium (75 ppm). Les animaux ont été examinés aux jours 0, 1, 3, 7, 15, 28, et 56. Des groupes témoins recevant de façon ininterrompne 9500 ppm de Mg dans l'alimentation ont été examinés aux jours 0 et 56, tandis que d'autres groupes témoins recevant une alimentation carencée en Mg (75 ppm) ont été examinés aux jours 0, 7, 15, 28 et 56.

Les concentrations de Mg dans le plasma et le fémur ont clairement mis en évidence la carence en magnésium ainsi que la supplémentation ultérieure. Les examens histologiques et histochimiques ont montré la présence, dans le groupe carencé en magnésium, d'une hydronéphrose provoquée par l'occlusion de la partie distale des tubules proximaux, avec des conglomérats de calcium, de phosphate et d'une matrice organique, cette dernière intervenant probablement de façon significative dans la pathogénie des lésions rénales. Des doses élevées de magnésium ont permis d'obtenir chez les animaux carencés une diminution considérable de la concentration rénale de calcium, avec des différences importantes entre les animaux. Cependant, le traitement n'a généralement pas induit l'élimination ou la dissolution complète des précipités.

Introduction

The development of nephrocalcinosis in rats as a consequence of imbalanced diets has often been described in recent reviews. The most frequently used treatment for producing calculous deposits in kidneys is to feed a low magnesium (Mg) diet (*Watchorn* and *Mc Cane,* 1937; *Leder* et al., 1981), but it is also possible to use a calcium:phosphorus ratio less than 1.0 (*Al-Modhefer* et al., 1986), to vary both calcium and magnesium (*Woodard* and *Jee*, 1984) or to decreasc the chloride content of a high phosphorus diet (*Levine* et al., 1974). In cases of Mg deficiency most of the calcium phosphate deposits occur as intraluminal calculi concentrated at the corticomedullary junction (*Oliver* et al., 1966; *Bunce* and *King,* 1980).

In spite of many investigations little is known about the nature and origin of the intratubulary calcium deposits and about its correction. In this study we wanted to see whether high doses of dietary Mg are able to reverse nephrocalcinosis.

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Methods

130 female S.D.-rats (Interfauna, Tuttlingen, FRG) averaging 53 g (49 g–68 g) at the beginning of the experiment were randomly allotted to 13 groups of equal size. After a two day period of acclimatisation they were treated with two different diets under standard conditions. A Mg deficient semipurified powdered diet (Altromin C 1035, Lage, FRG; composition determined by analysis: 75 ± 8 ppm Mg, 8734 ± 165 ppm Ca, 7757 ± 783 ppm P) was used as basal nutrition. This substance was enriched with magnesium-aspartatehydrochloride (MAH)[1] to 9500 ppm Mg to produce the supplemented diet. To include time-dependent alterations in plasma and tissues, we devided the animals up as follows:

— Repletion groups: 6 groups received the basal diet during 10 days (the end of this period was called day 0); thereafter 5 groups received the supplemented diet for 1, 3, 7, 15, 28 or 56 days.
— Mg deficient groups: 5 groups were fed the basal Mg deficient diet for 10 (day 0), 17 (day 7), 25 (day 15), 38 (day 28) or 66 (day 56) days.
— Controls: 2 groups received the supplemented diet, 1 for 10 days (up to day 0), the other for 66 days (up to day 56). The diets and deionised water were given ad libitum. Since, contrary to former experience, convulsions followed by death occurred after 6 days of Mg deficiency, drinking water was constantly enriched with 2 mmol/l of Mg (as MAH) in all groups. Body-weight and water consumption were recorded daily. Animals were anesthetized with 6 mg/100 g b. w. Nembutal® (Ceva, Bad Segeberg, FRG).

Analysis

Organs were lyophilisised for three days and ashed twice for 24 hours (550 °C), and electrolyte content of the tissues and plasma were determined with an atomic-absorption-spectrograph (AAS, Perkin Elmer 1100).

Creatinine estimation was carried out on the principle of decomposing sarcosin and determination of the H_2O_2 produced (Creatinine PAP, Boehringer, Mannheim, FRG).

Blood-gas parameters were analysed using an automatic blood-gas-analyser (MT33R, Eschweiler, Kiel, FRG).

Statistics

After analysis of variance (ANOVA), the Scheffe-test (0.01 level) was used if homogeneity of variance was given (*Schubö* and *Uehlinger*, 1984).

Histological and Histochemical Methods

The right kidneys of the rats were subsampled for studies on unfixed and formol fixed material. Thickness of the slices was 10 µm or for the fixed kidneys 7 µm. Unfixed tissue was used for the Kossa method (*Leder*, 1981), for staining with alizarin red S, for fluorescence microscopy of calcein or murexid treated slices, for polarization, the Normarski interference contrast and Jamin-Lebedeff interference microscopy, for REM and energydispersive X-ray analysis (cf. *Leder, Frey, Fleckenstein*, 1987, *Leder* et al., 1989, 1990). The formol fixed tissue was embedded in paraplast; the slices were stained with HE, PAS and Alcianblue at pH 1.0 or 2.5.

Results

All groups showed nearly equal increase in body weight although, as a consequence of the Mg deficient diet, typical erythema occurred during day –6 (4 days of Mg deficiency) and day 18. Drinking water consumption raised, as a consequence of the high salt concentration, from 25 ml to about 35 ml per day in the rats receiving the supplemented diet. No change in drinking water occurred in the Mg deficient groups.

Magnesium

The experimental outcome is reflected by the Mg concentrations, measured in plasma and thigh bones (Figs. 1 and 2). Repletion led to normal values in plasma within the first 7 days (0.88 mmol/l); the corresponding Mg deprived groups showed significantly lower Mg levels (0.25 – 0.39 mmol/l), representing a definite state of Mg deficiency.

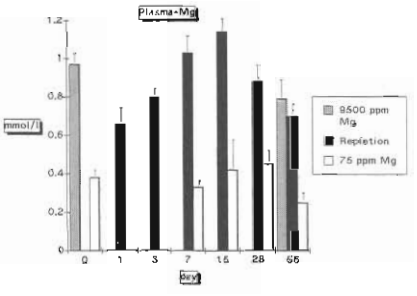

Fig. 1: Plasma-Mg (mean and SD, n = 10 per group) in rats receiving a Mg deficient diet (75 ppm Mg, white columns) from days –10 up to day 56, or excess Mg (= controls, 9500 ppm Mg, striped columns). Black columns designate Mg depleted rats (–10 days) with a subsequent repletion period of 1 up to 56 days.
There were highly significant differences ($p < 0.001$) between the 75 ppm Mg groups and the other treatments.

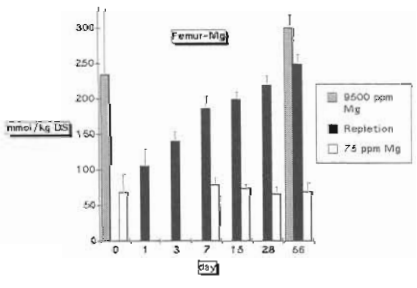

Fig. 2: Femur-Mg: There were highly significant differences ($p < 0.001$) between the 75 ppm Mg groups, the controls and repletion groups except day 1.
For further details see Fig. 1.

[1] Verla Pharm, Tutzing, FRG, Batch Nr. 87/87/238

Data from the thigh bones showed nearly the same effect, although at day 56 concentrations were still lower in the repleted group than in the group supplemented from the beginning. As demonstrated in earlier studies (*Classen* et al., 1988) the Mg content of femora decreased within 10 days of Mg deficiency to a level of about 80 mmol/kg d. s., which remained constant despite further feeding with the Mg insufficient regimen.

Both plasma and bone Mg showed that after a period of 10 days a steady state of Mg concentration was reached, indicating that no more Mg pools were available.

Calcium

Femur calcium (Ca) remained uneffected; plasma Ca increased insignificantly (max. +10.4 %) during Mg deficiency and fell to normal values within the first 7 days of the repletion period.

Kidney parameters

pCO_2, pO_2, base excess (BE) and pH were measured in whole blood. Surprisingly, not any alterations occurred to suggest a decrease of renal functions. This was confirmed by the results of plasma creatinine determination of day 56. There was no difference of creatinine level either in the control, the repletion or Mg deficient groups (data not shown in detail).

Fig. 3 presents renal Ca. As expected, there was a remarkable calcium enrichment in the kidneys after only

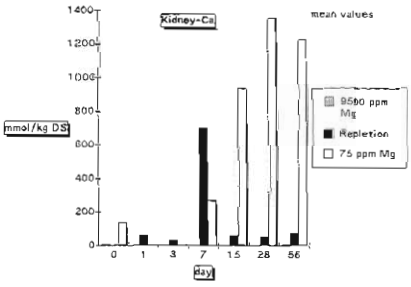

Fig. 3: Renal-Ca: There were at day 15, 28 and 56 highly significant differences ($p < 0.001$) between 75 ppm Mg groups and repletion groups. For further details see Fig. 1.

10 days of Mg deficiency (day 0). But in fact, values differed in most groups with Mg supplementation after 10 days of Mg deficiency by between 5 and 3000 mmol Ca/kg d. s.. Likewise, the animals without supplementation over the whole time of the experiment exhibited Ca concentrations in a range between 100 – 2400 mmol/kg d. s.. Only the rats with a sufficient diet showed coefficients of variance below 10 %. Only in these groups mean and median were essentially equal.

In spite of the broad unsymmetrical distributions, it is quite clear that renal Ca decreased during the first 3 days or repletion (from 135 to 32 mmol/kg d. s.). Both control groups which were fed the supplemented diet, showed very low Ca values (5.5 ± 0.5 mmol/kg d. s.). There were kidneys with and without any calcurous deposits in the Mg deficient group up to day 7, (i. e. 17 days of Mg shortage), and also in the repletion groups. All the kidneys of rats developed an extreme calculosis when subsequently fed the Mg deficient diet rising up to day 28. The calcium deposits reached at this time seem to reflect a superior limit with no further increase up to day 56 (66 days of Mg lack). No concrements were found in the urinary bladders of repleted and inadequately Mg supplied groups by macroscopic inspection on days 28 und 56.

Histology, Histochemistry

Calcification of the kidneys could already be observed by macroscopic inspection in the rats inadequately supplied with Mg (Fig. 4), and even in the replenished groups. There were enlargements of the thick descendent tubules of Henle's loop in the outer strip of the outer zone of the medulla with atrophic or destroyed epithelial cells and a further expansion of the tubules proximally to the damaged parts (cf. Fig. 5; for further figures see *Leder* et al., 1989, 1990; cf. also *Leder* and *Classen*, 1989). The alizarin redstain for calcium revealed remarkable calcium deposits with occlusions of the lumen, especially in the thick descending segment of Henle's loop (Fig. 6). The deposits are produced essentially by spheroid structures which are often agglomerated. This may explain the enlargement of the tubuli and the atrophy of tubule cells. Similar, but smaller calculi could be observed in the thin part of Henle's loop; they had been transported to these segments with the tubular fluid. Further fluorescence histochemical work with calcein and murexid, as

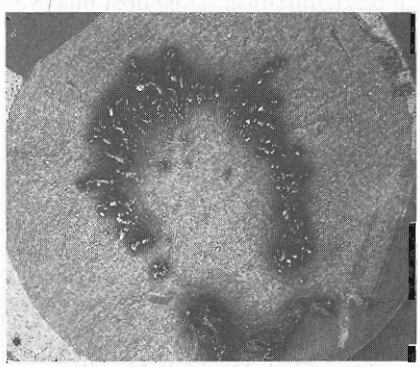

Fig. 4: Unfixed, frozen dried section of animal XIII/2 (2411 mmol Ca/kg d. s.), 66 days Mg deficiency, 10 µm thick. Scale 10:1. REM. The most calcification are seen in the outer strip of the outer zone of the medulla. Others are observed in the inner strip. Whilst the regions without calcification give a clear image, around the calcium deposits are black zones without any contrast.

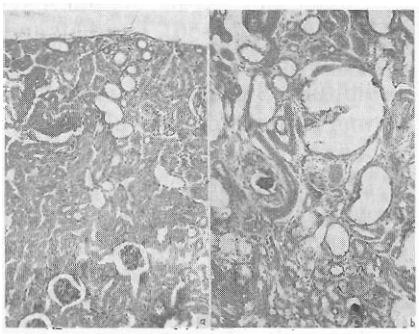

Fig. 5: Formol fixed kidney, animal XII/7 (1570 mmol Ca/kg d. s.), 38 days Mg deficiency; section thickness 7 µm, scale 160:1. a) Outer cortex with enlarged lumen of a proximal tubule. b) Outer strip of the outer zone of the medulla. There are remarkable enlargements of the tubules with atrophy of their epithelium. Some cell infiltrations in the interstitium and deposits in two tubules are likewise seen. The comparison of the cortex with the outer zone within single kidneys and between them demonstrates that the damage is restricted to a part of the nephron only.

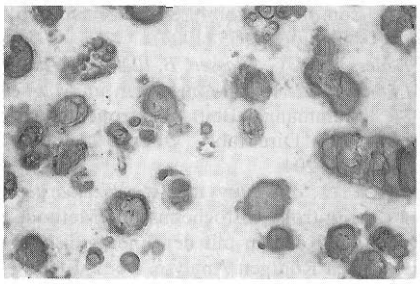

Fig. 6: Unfixed, frozen dried section, 10 µm, stained with alizarin red S. Scale 160:1. Outer strip of the outer zone of the medulla. Tubules are obstructed by conglomerates, in which globular structures can be detected. Similar images gives the Normarski interference contrast.
The concentric layers of the globules had been especially demonstrated with Jamin-Lebedeff-Interference (Leder et al., 1989).

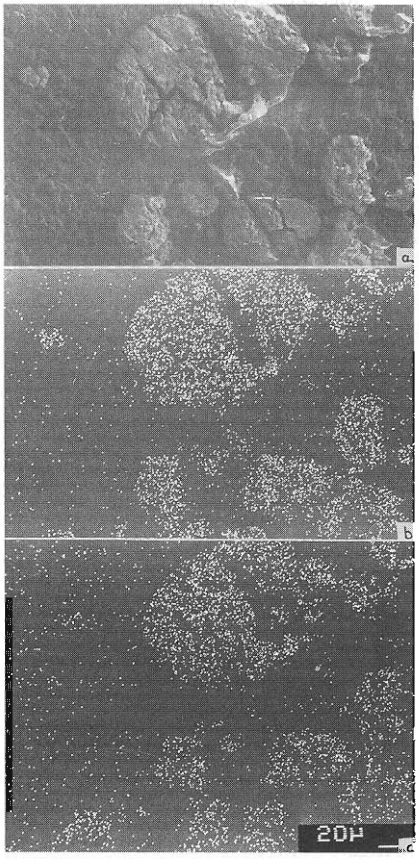

Fig. 7: Animal XIII/9 (609 mmol Ca/kg d. s.), Mg deficiency 66 days. Unfixed, frozen dried sections, 10 µm thick. a) REM image, b) Ca mapping, c) P mapping. Both elements show essentially the same distribution.

well as energy dispersive X-ray analysis (Fig. 7) confirms these findings. Moreover, the latter method shows P in the calcification, which corresponds to the histochemical results of the von Kossa reaction. The spheroids exhibit a radially symmetrical structure as seen by polarisation microscopy (Fig. 8). There are differences in the extension of the birefringence and uptake of dye or silver. Staining of decalcified sections with PAS and alcianblue produced concentric layers of a glucosaminoglycan and glykoproteids, and this arrangement confirmed the interference microscopic observations on the uncalcified sections.
Obviously the calcurous deposits possessed an organic matrix, on which layers of calcium phosphate had been precipitated. Our results correspond especially to the finding of Ca, P and a glycoproteid in intratubular calculi by *Oliver* et al., (1966). The histochemical findings agree likewise to the chemical analysis of Ca. Furthermore, the decrease of Ca could be observed in the Mg replenished groups by histochemical techniques. It is surprising that there are extremely small alterations to observe in those kidneys which had lost most Ca after the replenishment of Mg. Cell infiltrations or scarces were found only in some animals and nothing of enlarged and destroyed tubules could be detected if the spheroid deposits had disappeared. No pathological alterations were detected on histological and histochemical examination in renal tissues of rats receiving the highly Mg-supplemented diet during 66 days.

Discussion

It was the intention of the present study to produce a definite degree of Mg deficiency at a fixed point of time (day 0), characterized by low plasma-Mg and depleted Mg-pools in bone. This condition was obtained with 0.4 mmol Mg/l plasma and an inferior threshold of 80 mmol Mg/kg bone (d. s.). On the other hand, after supplying Mg in the drinking water daily, the Mg intake was sufficient to support live.
As reported by other authors (for example *Watchorn* and *Mc Cane*, 1937; *Greenberg* et al., 1938; *Fischer* et al., 1984) even a few days of Mg

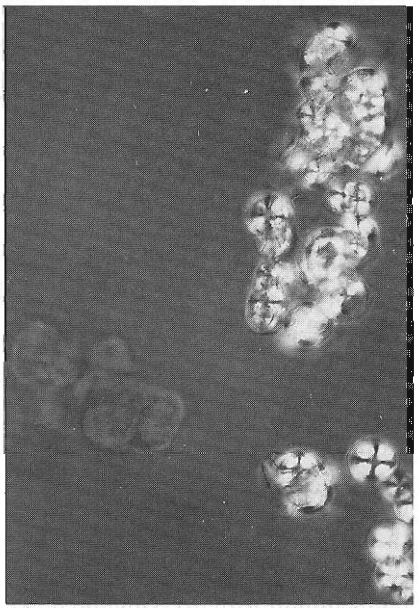

Fig. 8: Animal XI/5 (740 mmol Ca/kg d. s.), Mg deficiency 25 days. Unfixed, frozen dried section, 10 µm thick. Scale 580:1. Globules with or without strong birefringence.

deficiency induced nephrocalcinosis in rats. Although these kidneys were enlarged, white and speckled, and on a microscopical level exhibited frequent obstructions of proximal tubules, there was no evidence for changes in renal function, checked by plasma-creatinine and blood-gas-parameters. This is in agreement with the findings of *Al Modhefer* et al. (1986). The development of comparable calcified deposits in the kidneys can also be evoked by diets with a deleterious Ca:P ratio (*Woodard* and *Jee*, 1984). In our experiments we therefore used a Ca:P ratio of 1:1 to exclude causes other than Mg deficiency. More than 20 years ago *Oliver* et al. reported a similar experiment: They fed Mg deficient rats with a "„normal diet" during a repletion period, but did not give any further description of its composition. After 5 months, they still found calcium complexes of the same kind just as in this study, and noted alterations of their crystalline structure accompanied by an actual loss of total renal calcium. *Classen* and *Schumacher* (1987) showed a decrease in renal Ca within only 5 days during repletion with a diet contain-

ing only 500 ppm Mg. Following their results it seemed to be of interest to study the characteristics of the relationship of calcium deposits to a high oral Mg intake. The demonstrated decrease of renal Ca after repletion agrees with *Schumacher's* results. Whereas the variance in the groups with enriched Mg or with deficient Mg supply do not deserve special attention, the variability in the others is surprising.

Some indication may be derived from the fact that the Ca deposits in the kidneys are not pure Ca salts, but rather an organic matrix that has been calcified layer by layer, and that this process may be dissimilar in the different animals. After the replenishment with Mg, calcium phosphate globules, localised in tubuli may be rinsed out and excreted. Such a transport by flow of the primary urine can be supposed, because calculi are found in tubules distal from the thick descending part of Henle's loop. Moreover, a dissolution of the precipitates should be considered. Further calcification, on the other hand, could occur if additional organic substance accumulate in the tubules, expecially on spheroids acting as crystallisation centres. Although the differences in Ca concentration are surprising, one may wonder more about the fact that little damage is seen after decalcification of the replenished groups. The calcium deposits certainly represent intratubular changes and no primary calciumoverload of the epithelium. The spheroids produce more atrophy than cell death and therefore the kidney structure may return to normal after supplementation of the diet with Mg.

It should also be noted that excess Mg, offered as Mg-Asp.-HCl, did not induce adverse reactions; this observation confirms the data of *Fischer* et al., 1984.

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