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A Short-Term Low Magnesium Diet Reduces Autoimmune Arthritis Severity and Synovial Tissue Gene Expression.

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ABSTRACT

Magnesium has been suggested to have anti-inflammatory properties in short-term mostly in vitro studies. To examined the effect of dietary magnesium modifications in arthritis severity and joint damage DA rats were placed on one of three diet regimens before the induction of autoimmune pristane-induced arthritis (PIA): a four-week low-magnesium diet, normal diet and a magnesium-supplemented diet. The diets were switched to a normal diet fourteen days after the induction of PIA (typical time of disease onset). Arthritis severity was scored for 38 days, and joints examined by histology and qPCR for pro-inflammatory genes. Rats on the low-magnesium diet were significantly and reproducibly protected and had 70% lower median arthritis severity score, with preservation of normal joint histology without erosive changes. Rats on the normal or magnesium-supplemented diets were not protected and developed equally severe and erosive disease. While the dietary modifications were at disease onset (day fourteen post-induction), the protective effect of the short-term low-magnesium diet persisted, suggesting a lasting effect on a critical pathogenic pathway. Rats on the low-magnesium diet had significant reduction in synovial tissue expression of IL-6, RORA and RORC, which are genes required for the development of Th17 T cells. This study revealed a novel role for dietary magnesium in the regulation of autoimmune arthritis and opens new possibilities for the treatment of autoimmune diseases such as RA and psoriatic arthritis with short courses of dietary or drug-induced modulations of magnesium levels.

Keywords: *joint damage, erosions, diet, rheumatoid, models.*

INTRODUCTION

Magnesium is the second most abundant intracellular ion and plays an important role in enzymatic function and trans-membrane ion transport. Magnesium administration has been used to treat pre-eclampsia for decades and its use in short-term mostly *in vitro* experiments was recently shown to reduce levels of TNFα, IL-1β, IL-6 and other inflammatory mediators, as well as to prevent NFκB activation in experiments with endothelial cells, macrophages and placental explants, suggesting a suppressive effect predominantly on innate immune responses (1, 4, 8, 12, 13). TNFα, IL-1β, IL-6 and NFκB are central to rheumatoid arthritis (RA) pathogenesis and joint damage. Therefore, we hypothesized that magnesium supplementation would suppress these inflammatory mediators and reduce disease severity and joint damage in arthritis, while magnesium depletion would have the opposite effect.

In the present study we report the new discovery that a short-term low magnesium diet, and not magnesium supplementation, significantly reduced arthritis severity, joint inflammation and joint damage. This was associated with reduced synovial tissue expression of pro-inflammatory cytokines and transcription factors implicated in the development of Th17. These observations suggest that manipulation of magnesium levels could be a potentially novel strategy to treat RA, as well as other autoimmune and inflammatory diseases.

MATERIAL AND METHODS

Rats. Male DA/Hsd rats were purchased from Harlan (Indianapolis, IN) and housed under specific pathogen-free conditions. All experiments involving rats were conducted under a protocol approved by the Feinstein Institute's Institutional Animal Care and Use Committee (the Gulko lab has since moved to Mount Sinai).

Dietary magnesium modifications. Rats were assigned to one of three dietary treatments groups prior to the induction of pristane-induced arthritis (PIA): **a) *low-magnesium diet***: a diet containing 50ppm of magnesium, or 0.06 g/Kg (Harlan, IN) started two weeks before and continued for an additional two weeks following the administration of pristane, at which point the diet was switched to a regular diet for the rest of the 38 days of the arthritis experiments; **b) *a regular diet:*** a diet containing normal amounts of magnesium 500ppm or 0.8 g/Kg (Harlan, IN) administered before the induction of arthritis and throughout the 38 days of arthritis; **c) *magnesium-supplemented regular diet:*** the same regular diet described above with magnesium 500ppm or 0.8 g/Kg (Harlan, IN), with magnesium chloride 0.5% supplementation in the drinking water *ad lib* for one week before and two weeks after the administration of pristane to induce arthritis, followed by simple continuation of the regular diet for the remaining of the 38 days of the arthritis experiment. By the typical day of arthritis onset (day 14 post administration of pristane) all rats were on the regular chow diet.

Diet/chow specific details: The diets were purchased from Teklad/Harlan Laboratories (Madison, WI). Rats received identical diets, except for the amount of magnesium (low magnesium chow, normal magnesium chow, and normal magnesium chow plus magnesium choride 0.5% supplementation in the drinking water). Specifically the diets had the following contents (g/Kg) and were irradiated: protein (17.7), carbohydrates (64.4), fat (6.2), Casein (200), DL-Methionine (3.0), Sucrose (415), Corn Starch (250), Soybean Oil (60), Cellulose (30), Vitamin Mix (Teklad 40060) (10), Ethoxyquin (antioxidant) (0.01), Calcium Phosphate,

dibasic (13.7), Potassium Citrate (monohydrate) (7.7), Calcium Carbonate (4.8), Sodium Chloride (2.6), Potassium Sulfate (1.82), Ferric Citrate (0.25), Manganous Carbonate (0.12), Zinc Carbonate (0.056), Chromium Potassium Sulfate (dodecahydrate) (0.02), Cupric Carbonate (0.012), Potassium Iodate (0.0004), and Sodium Selenite, (pentahydrate) (0.0004). The only difference is in the amount of magnesium as the low magnesium diet has magnesium oxide 0.06g/Kg of chow (Mg 50ppm), the normal (regular) magnesium diet has magnesium oxide 0.822g/Kg of chow (Mg 500ppm).

Induction of PIA. Eight to twelve-week old rats received 150 µl of pristane (2,6,10,14-tetramethylpentadecane, SIGMA-Aldrich Chemical Co., Milwaukee, WI) by intradermal injection (day zero) (15) (2). The dose was divided in two injection sites at the base of the tail.

Arthritis scoring. We used a previously described arthritis scoring system (2) that evaluates individual joints and measures arthritis severity according to joint size as follows: a) interphalangeal, metacarpophalangeal and metatarsophalangeal joints in each one of the four lateral digits were scored 0=no arthritis; 1=arthritis present; b) wrist, mid-forepaw, ankle and midfoot joints were scored 0=normal; 1=minimal swelling; 2=moderate swelling; 3=severe swelling; 4=severe swelling and non-weight bearing. The scores from all involved joints were added (maximum score per rat=80). The same observer obtained all arthritis scores. The arthritis severity index (ASI), which is a measure of disease severity over time, was determined for each animal by adding the individual arthritis scores obtained over the course of the experiment. The ASI correlates with histological changes and damage (2).

Histology and histological scoring. At the end of the arthritis observation period the right hind paw was fixed in 10% formaldehyde, and then decalcified with a solution containing hydrochloric acid and 0.1M EDTA (Cal-Ex, Fisher Scientific, Fairlawn, NJ). Tissues were sectioned, embedded in paraffin, and slides prepared and stained with hematoxylin-eosin and safranin-O. Two slides were prepared per rat and the one with the best quality (section and staining) used for histology scoring without knowledge of treatment. A previously described comprehensive histological scoring system was used (2). Briefly, tibio-talar, talus-calcaneal and midfoot joints were histologically scored for the following parameters:

1) *Synovial inflammation.* Five high-power magnification fields (HMF) were scored for the percentage of infiltrating mononuclear cells as follows: 0=absent; 1=mild (1-10%); 2=moderate (11-50%); 3=severe (51-100%). The mean of the five HMF was used for analyses.
2) *Synovial hyperplasia.* 0=absent; 1=mild (5-10 layers); 2=moderate (11-20 layers); 3=severe (>20 layers).
3) *Extension of pannus formation.* Based on the reader's impression: 0=absent; 1=mild; 2=moderate; 3=severe.
4) *Synovial fibrosis.* 0=absent; 1=mild (1-10%); 2=moderate (11-50%); 3=severe (51-100%).
5) *Synovial vascularity (angiogenesis).* The number of vessels was counted in five HMF of synovial tissue, and the mean used for analyses.
6) *Cartilage erosion.* Percentage of the cartilage surface that was eroded: 0=absent; 1=mild (1-10%); 2=moderate (11-50%); 3=severe (51-100%).
   one erosion. 0=none; 1=minor erosion(s) observed only at HMF; 2=moderate erosion(s) observed at low magnification; 3=severe transcortical erosion(s).

Quantitative PCR (qPCR). Synovial tissues were collected from the rat ankles after the completion of the arthritis observation period and immediately frozen in liquid nitrogen. Tissues were subsequently homogenized and total RNA isolated with the RNeasy Kit (Qiagen) and digested with DNase (Qiagen). 200ng of total RNA from each sample were used for cDNA synthesis (ABI high capacity reverse transcription kit, Life Technologies). qPCR reactions were set using Thermo Scientific Absolute Blue qPCR mix (Waltham, MA), 200nM of forward and reverse primers, 100nM of gene-specific probes TaqMan (ABI) 5'exonuclease or Roche Universal Probe Library (Roche), and 150-200ng of cDNA. Reactions were run on a LightCycler 480 thermocycler (Roche) using Relative Quantitative Software (Roche). Primers and probes used for rat interleukin-1 beta (IL-1β), IL-6, IL-17A, C-X-C motif chemokine ligand 10 (CXCL10), RORA (RAR-related orphan receptor alpha), RORC (RAR-related orphan receptor C), and GAPDH genes have been previously reported (2, 5). GAPDH was used as endogenous control. *Ct* (threshold cycle) values were obtained and analyzed with the Sequence Detection System software version 1.9.1 (ABI). *Ct* (threshold cycle) values were adjusted for GAPDH in each sample (ΔCt). ΔCt values were compared using the t-test, and fold differences in gene expression between different concentrations of magnesium were determined using the $2^{-\Delta\Delta Ct}$ method (10).

Statistical analyses. Non-normally distributed data were compared with the Mann-Whitney test for two group comparisons. The t-test was used to compare means, and a paired t-test to compare serum levels of magnesium. One-way ANOVA with the Tukey's multiple comparison test was used to compare histology scores. A p-value of 0.05 or less was regarded as significant. All statistical analyses were done with SigmaStat 3.0 and GraphPad Prism.

RESULTS

A short-term low-magnesium diet reduces arthritis severity. Serum levels of magnesium were measured at the beginning of the diet and again on the last day of the diet (days -14 and 14 related to the pristane administration). Serum levels of magnesium remained stable in the normal magnesium diet, but were significantly decreased in the low magnesium diet (figure 1).

The low-magnesium diet group developed a significantly milder form of arthritis, with minimal disease (figure 2A) and a nearly 70% lower median ASI, compared with the regular diet and with the magnesium-supplemented group (low-magnesium group median ASI=93.5; normal-magnesium group median ASI=271.5; magnesium-supplemented group median ASI=251.75; low-magnesium versus each of the other groups P=0.004; Mann-Whitney test; figure 2A-C). These observations were confirmed in a second independent experiment with 12-13 rats per group (data not shown). The clinical benefit could be detected as early as day 18 and persisted throughout the 38-day scoring period despite switching the diet back to the regular normal magnesium diet on day 14.

The magnesium-supplemented group developed PIA as severe as the group receiving regular diet and had no evidence of protection (figure 2A).

Low-magnesium diet reduced synovial inflammation and joint damage. Histology analyses further confirmed the clinical data and revealed that the low-magnesium diet group had nearly no synovial abnormalities and preserved a normal joint architecture (figure 2E), while the regular diet and magnesium-supplemented groups had pronounced inflammatory cell

infiltration (P<0.05), synovial hyperplasia (P<0.05), and bone and cartilage erosions (P=0.04; figure 2D; Table 1). Three individual histology parameters (inflammatory infiltration, synovial hyperplasia and pannus extension) remained statistically significant after adjustments of P values for multiple comparisons (Bonferroni correction for seven variables). These observations demonstrated that short-term low magnesium intake and reduced serum levels of magnesium during the induction of PIA had a long-lasting and protective effect on arthritis severity and joint damage.

Low-magnesium diet rats had reduced synovial expression of key pro-inflammatory genes, including those required for Th17 development. qPCR analyses of inflammatory mediators in synovial tissues from low-magnesium diet rats with PIA revealed significantly reduced levels of IL-6, RORA and RORC, all which are required for T cell helper 17 (Th17) development (figure 3A and B). IL-17A was not expressed in the synovial tissues of rats in the low magnesium diet, but was still expressed (day 39) in three out of eight rats from the regular diet group. Additional genes involved in synovial inflammation and joint damage such as IL-1β and CXCL10 were also reduced in synovial tissues from the low-magnesium diet group.

DISCUSSION

Magnesium has been used for decades to prevent seizures in pre-eclampsia (14). Recently, pro-inflammatory mediators such as TNFα and IL-6 were implicated in the pathogenesis of pre-eclampsia (14) and studies conducted over short periods of time (hours) with macrophage, endothelial cells or placental explants showed that magnesium supplementation was able to reduce levels of these cytokines and suppress NFκB activation (4, 8, 12, 13). Given the central role that TNFα and IL-6 have in RA and joint damage, we hypothesized that magnesium supplementation would reduce levels of these cytokines, reducing disease severity and joint damage in PIA. However, we observed the opposite effect where magnesium supplementation had no effect in disease severity, while a short-term low-magnesium diet had a major, significant, lasting and protective effect rendering DA rats nearly resistant to PIA.

This new discovery raised the possibility that the low magnesium diet was targeting a cell type not examined in previous studies that mostly focused on short-term effects over hours on components of the innate immune response. We considered that magnesium could be affecting T cells, which were not examined in other studies and are required for the development of PIA (15).

Magnesium, including two magnesium channels TRPM7 and MAGT1, has been implicated in the regulation of thymic T cell development (6, 9). MAGT1 has also been shown to regulate TCR signaling (9). Therefore, we considered that the short-term low serum level of magnesium might affect the differentiation of T cell subsets such as pathogenic Th17. IL-17-producing Th17 T cells have been implicated in the pathogenesis of several autoimmune diseases including RA (3, 7), colitis, and psoriasis (11). Indeed, levels of IL-17A were undetectable in synovial tissues from rats fed the low magnesium diet, and the levels of Th17-driving transcription factors RORA and RORC were also significantly reduced. IL-6, which among several activities is involved in Th17 differentiation, also had significantly reduced expression in the low magnesium diet group. IL-1β and CXCL10 mRNA levels were also decreased in the low magnesium diet group. Therefore, the low magnesium diet could conceivably affect Th17 differentiation or migration into the synovial tissues.

IL-1β, IL-6 and CXCL10 are key pro-inflammatory mediators produced by fibroblast-like synoviocytes (FLS) and synovial macrophages, and implicated in arthritis pathogenesis, FLS invasiveness and joint damage. These observations suggested that the effect of reduced magnesium concentrations could include not only Th17, but also synoviocyte-mediated joint damage.

In conclusion, we describe a novel role for magnesium in the regulation of arthritis severity and joint damage. This new discovery and the effect of the low magnesium diet on Th17-related and other pro-inflammatory genes raise the possiblity of using a short-term diet or short-term use of magnesium lowering agents, or targeting magnesium channels or transporters as adjuvants in the treatment of inflammatory arthritis and autoimmune diseases such as RA.

Table 1. Histology scoring analyses. Paws from the experiment described on figure 2A scored using a previously described quantitative scoring system. The low magnesium diet group had significantly lower inflammatory cell infiltration (inflam infiltration) ($P<0.05$), lower synovial hyperplasia (synov hyperpl) ($P<0.05$) and lower bone erosions scores ($P=0.04$) compared with the magnesium supplemented group and with the normal diet group.

FIGURE LEGENDS

Figure 1. Serum Levels of magnesium at the beginning and end of the diet. Magnesium levels before starting the low magnesium or regular diet (day -14) and at the last day of the diet (day 14) relative to the day of induction of pristane induced arthritis (PIA, day zero) revealed a significant reduction in the low magnesium diet group (∗ $P<0.001$; paired t-test). At day 14 serum magnesium levels in the low magnesium diet group were also significantly lower than the regular diet group (# $P<0.0001$; t-test).

Figure 2. A magnesium-deficient diet had a significant and lasting protective effect on arthritis severity and joint damage. (A) Arthritis severity score curves over time with a cummulative arthritis severity score reduction of nearly 70% in the low magnesium diet group (Mg-) compared with normal magnesium chow (normal; $P=0.004$, Mann-Whitney test) and magnesium supplemented diet (normal chow plus magnesium chloride 0.5% in the water; Mg+) ($P\leq0.001$) (shown as mean$\pm$S.E.M). **(B)** Normal diet rat with PIA had severe arthritis and ankle/paw swelling while **(C)** the low magnesium diet rats had nearly no inflammation or arthritis. Histology of **(D)** normal diet rats with PIA revealed pronounced synovial hyperplasia, synovial inflammation and erosive changes (black arrow), while **(E)** the low magnesium rats developed nearly no disease with preservation of a normal joint histology. Histology (H&E; 100X magnification).

Figure 3. The low Magnesium diet group had reduced synovial expression of cytokines and genes implicated in the development of Th17 T cells, or on their homing into inflammatory sites. A. Cycle detection threshold (Ct) levels and standard deviation (S.D.) of qPCRs analyses of synovial tissues (∗ $P<0.01$, t-test). **B.** Fold-difference between low magnesium diet group and the regular diet group.

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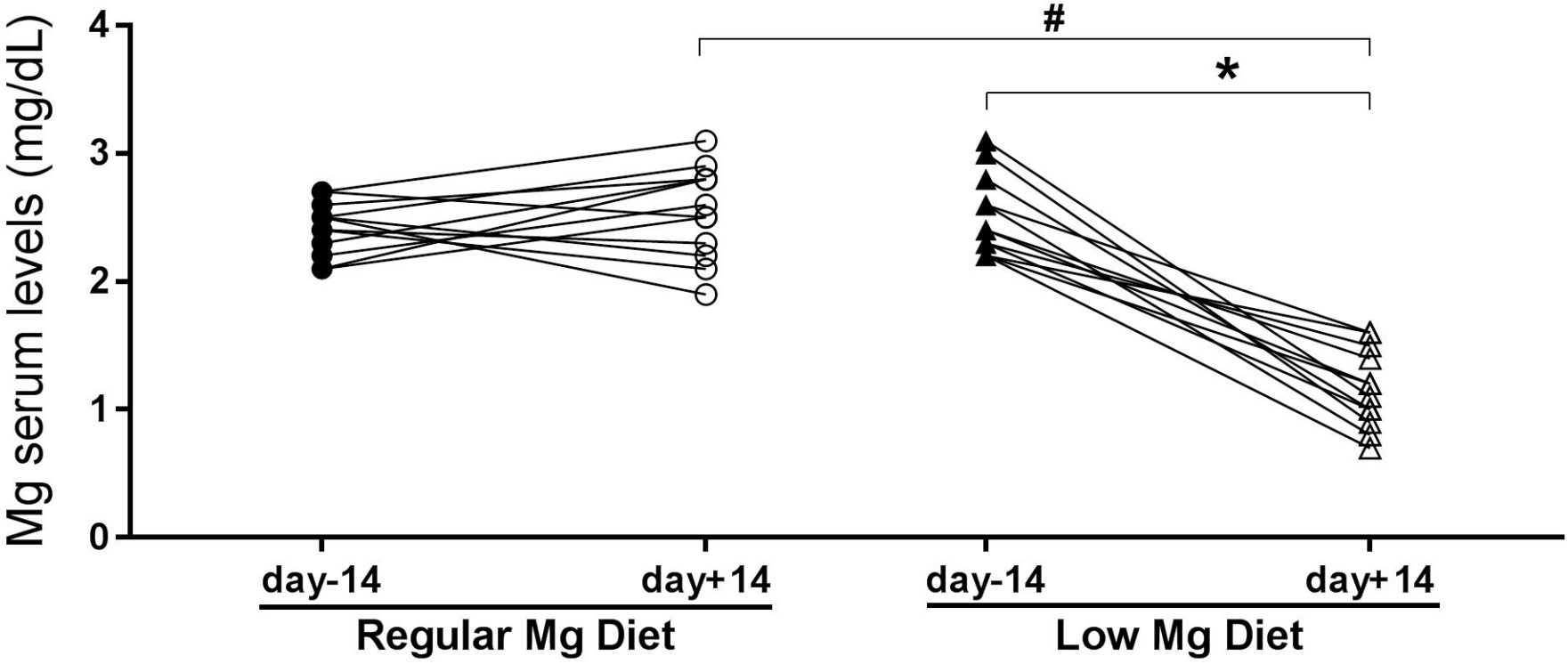

Mg serum levels (mg/dL)
4
3
2
1
0
#
*
day-14
day+14
Regular Mg Diet
day-14
day+14
Low Mg Diet

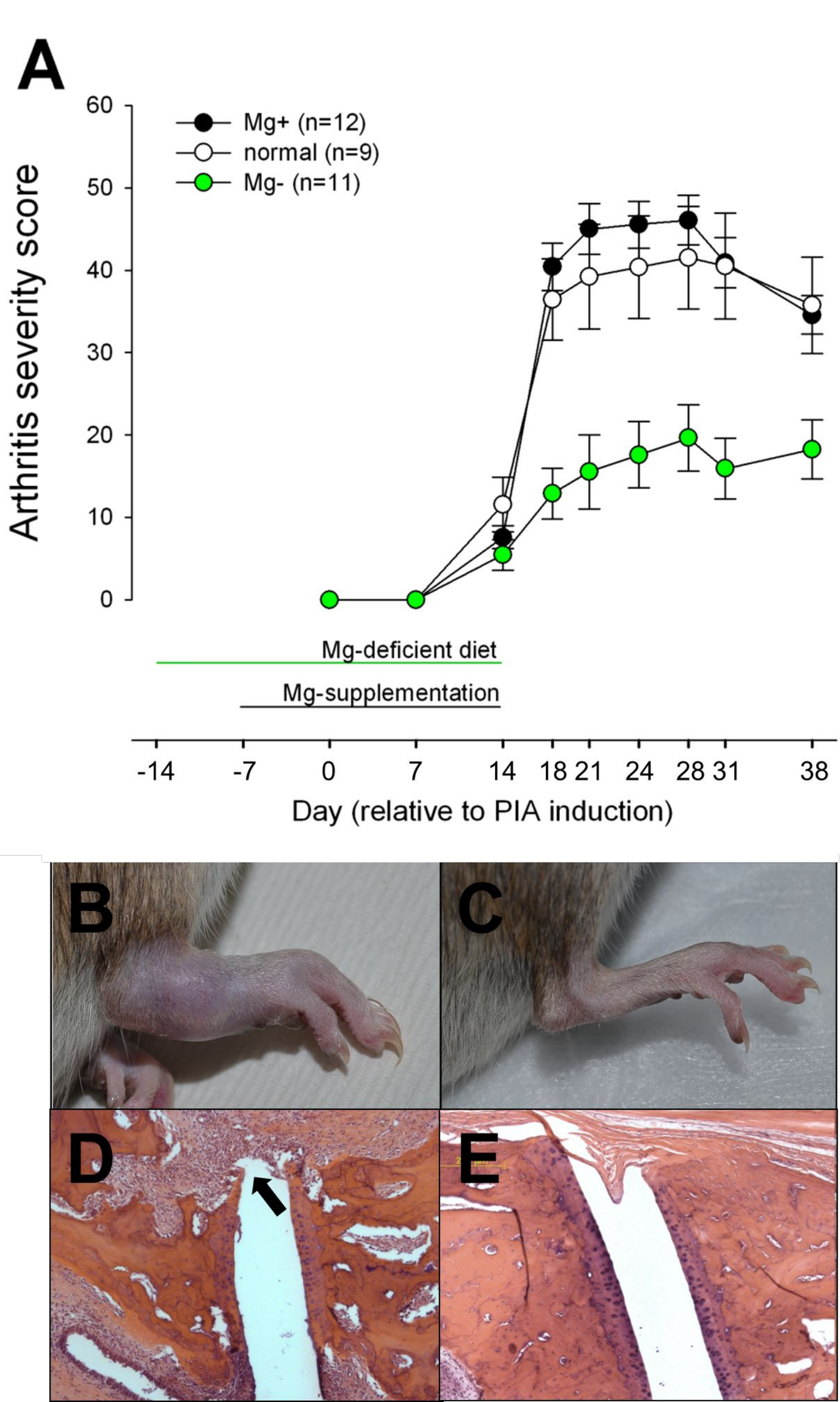
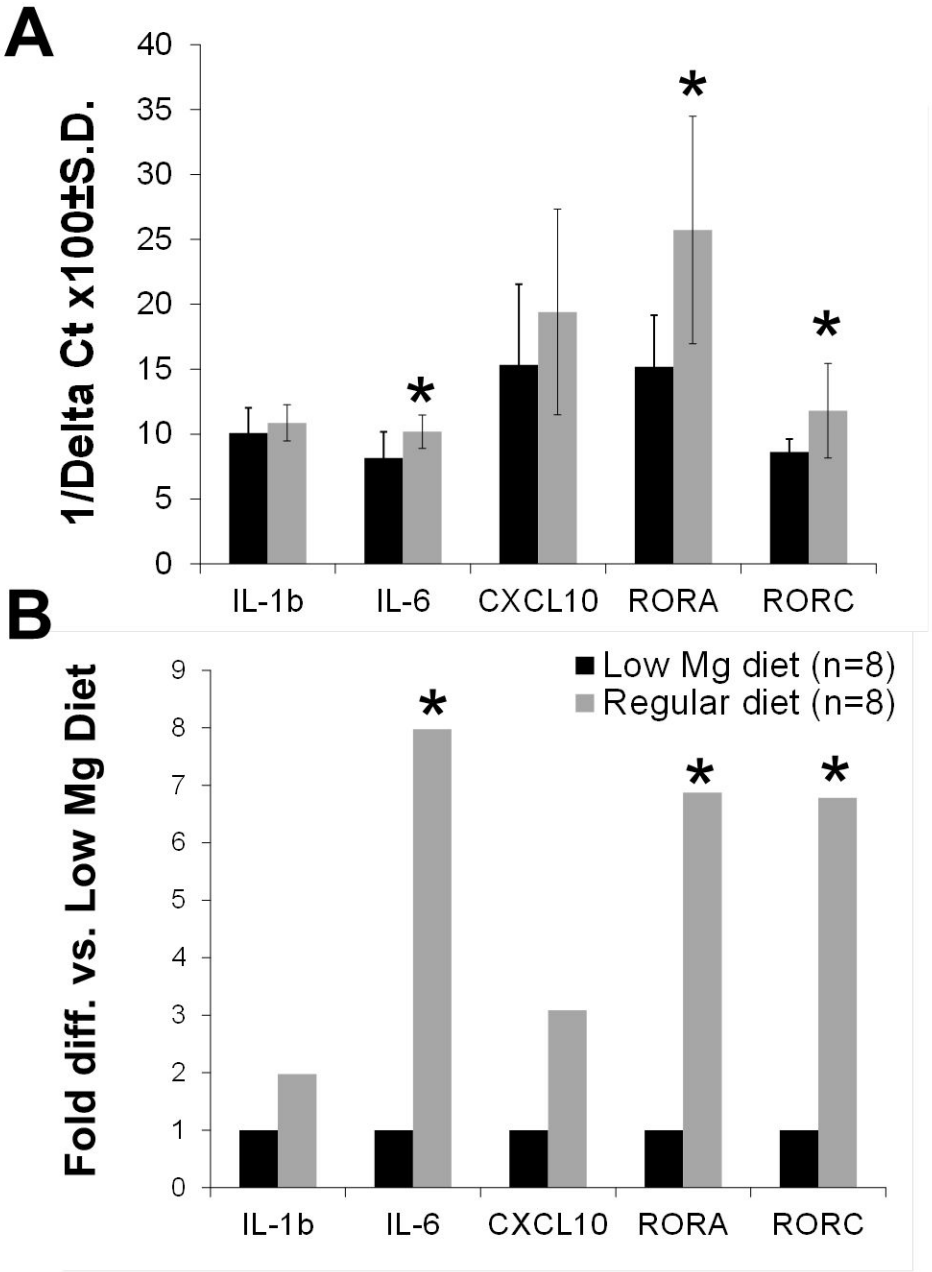

A
Arthritis severity score
60
50
40
30
20
10
0
Mg+ (n=12)
normal (n=9)
Mg- (n=11)
Mg-deficient diet
Mg-supplementation
-14
-7
0
7
14
18
21
24
28
31
38
Day (relative to PIA induction)
B
C
D
E

Table 1. Histology scoring and analyses of the ankles of rats with PIA*.

Scoring Parameter		Mg Suppl (n=5)			Regular (n=5)			Low Mg (n=5)			P-value [a]
		mean		S.D	mean		S.D	mean		S.D	
Inflammatory Infiltration	(0-3)	1.73	±	0.51	2.24	±	0.65	0.40	±	0.00	≤0.05; Low Mg vs Reg and Mg Supp
Synovial hyperplasia	(0-3)	2.00	±	1.41	2.60	±	0.55	0.00	±	0.00	≤0.05; Low Mg vs Reg and Mg Supp
Pannus extension	(0-3)	2.00		0.71	2.20		0.45	0.60	±	0.89	≤0.05; Low Mg vs Reg and Mg Supp
Fibrosis	(0-3)	0.20	±	0.45	0.20	±	0.45	0.20	±	0.45	>0.05
Vessels/HMF; counts.		4.92	±	2.16	3.36	±	2.09	3.40	±	1.46	>0.05
Bone erosions	(0-3)	2.40	±	1.34	2.40	±	1.34	0.40	±	0.89	≤0.05; Low Mg vs Reg and Mg Supp
Cartilage erosions	(0-3)	0.20	±	0.45	1.40		1.35	0.00	±	0.00	≤0.05; Low Mg vs Reg

* Mg Suppl=Mg Supplemented diet; Regular=regular diet; Low Mg = Low magnesium diet. HMF=high-magnification field.

[a] One-way ANOVA with Tukey's multiple comparisons test; Grey cell indicate signficant P≤0.05; S.D.=standard deviation.


A
1/Delta Ct x100±S.D.
40
35
30
25
20
15
10
5
0
IL-1b
IL-6
CXCL10
RORA
RORC
*
B
Fold diff. vs. Low Mg Diet
Low Mg diet (n=8)
Regular diet (n=8)
9
8
7
6
5
4
3
2
1
0
IL-1b
IL-6
CXCL10
RORA
RORC
*