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Suppression of Atherogenesis in Female Low-Density Lipoprotein Receptor Knockout Mice following Magnesium Fortification of Drinking Water: The Importance of Diet

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Key Words
Atherosclerosis · Cholesterol · Female · Drinking water · Magnesium

Abstract
Objective: Magnesium (Mg) has previously been found to modulate blood lipid levels, atherogenesis and atherosclerosis in rabbits when used as a dietary supplement. In addition, we have reported that Mg fortification of drinking water can attenuate atherogenesis in male low-density lipoprotein (LDL)-receptor-deficient mice, but had a mild and nonsignificant effect on female mice fed a high-cholesterol diet supplemented with cholic acid. The aim of this study was to examine whether Mg has an antiatherogenic effect in female mice fed a high-cholesterol diet without cholic acid. ***Methods:*** Two groups of female LDL-receptor-deficient mice were included. The mice received either distilled water or water with 50 g of Mg sulfate per liter. In the first (12 weeks) and second (6 weeks) stages of the experiment, the mice received low- and high-cholesterol diets, respectively, both without cholic acid. At the end of each stage of the experiment, blood was drawn for the determination of plasma Mg, calcium and lipid levels. In addition, the extent of atherosclerosis was determined at the aortic sinus level. ***Results:*** Mg fortification was associated with higher levels of plasma Mg while the mice were on a high-cholesterol diet, and the extent of atherosclerosis at the aortic sinus was significantly decreased in the female mice that received high levels of Mg compared with the female mice that received distilled water. The female mice that received water fortified with Mg had lower levels of triglycerides after stage 2, whereas no differences regarding cholesterol levels were found. ***Conclusion:*** These results confirm that Mg fortification of drinking water is capable of inhibiting atherogenesis also in female LDL-receptor-deficient mice fed a high-cholesterol diet, and demonstrate the importance of the nutritional composition of diet in this experimental model.

Introduction

Magnesium (Mg), the fourth most abundant cation in the human body, plays an important role as a cofactor of more than 300 enzymatic reactions [1]. Mg deficiency is very common in Western society, since the average Western diet does not provide the recommended daily intake of Mg [2, 3]. The importance of a sufficient amount of dietary Mg stems from its antiatherogenic effect, as re-

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ported previously in rabbits [4–6]. These appealing studies [4, 5] emphasized that both Mg aspartate hydrochloride and Mg sulfate were equipotent in atherogenesis inhibition. Recently, we investigated whether Mg fortification of drinking water (without change in Mg diet content) can also inhibit atherogenesis. We found that the fortification of drinking water with Mg sulfate resulted in atherogenesis inhibition to a level of about one third of that in controls in male low-density lipoprotein (LDL)-receptor-deficient mice, whereas no significant difference in atherosclerosis extent was found in female mice, even though a tendency towards reduction of atherosclerosis was observed [7]. These LDL-receptor-deficient mice develop atherosclerotic lesions throughout the arterial tree that have many similarities with human atherosclerosis with respect to the distribution pattern and morphological features, and thus they provide a good model for human atherosclerosis research [8]. Upon being fed a low-cholesterol diet (chow diet), the mice do not develop significant atherosclerotic lesions, whereas with a high-cholesterol diet the mice develop marked hypercholesterolemia and lesions throughout the aorta within 3–4 months [9].

The aim of this study was to examine the potential antiatherogenic effect of Mg fortification of drinking water in female LDL-receptor-deficient mice, using the same protocol as was used before [7], with a change only in diet content from a diet that included cholic acid and very high cholesterol levels to a diet without cholic acid, with a smaller amount of cholesterol and with a lower Mg concentration.

Materials and Methods

Mice and Diets

LDL-receptor-deficient mice were bred in the local animal house of the Institute of Lipid and Atherosclerosis Research (Sheba Medical Center, Tel-Hashomer, Israel). The mice were created by homologous recombination as described by Ishibashi et al. [8]. Two groups of 10 female mice were studied. The mice were 3 weeks old when entered into the study. They were on 12-hour dark/light cycles and were allowed access to food and water ad libitum.

The study involved two stages. Stage 1 lasted 12 weeks, in which the mice were fed a normal chow diet containing 4.5% fat by weight and 0.02% cholesterol. One group of female mice was allowed access to distilled water (Mg concentration < 10 mg/l), while the other group was given only water containing 50 g of Mg sulfate per liter. Stage 2 lasted 6 weeks, and in this period the mice were fed an atherogenic Western-type diet containing 0.15% cholesterol, 21% anhydrous milk fat and 19.5% casein, and there was no change with respect to the Mg content of their drinking water. At the end of stage 2, the mice were sacrificed. The mineral content of both diets was measured in an ICP machine (Spectro, Germany); the chow diet contained 2,470 mg/kg diet of Mg and 15,400 mg/kg diet of calcium (Ca), while the Western-type diet contained 510 mg/kg diet of Mg and 6,400 mg/kg diet of Ca.

Measurement of Plasma Mg and Ca

Blood was collected from the retroorbital plexus of the mice at the end of stage 1, and 1,000 U of heparin per milliliter of blood was added to each sample for plasma Mg and Ca determination. At the end of stage 2, blood was obtained by cardiac puncture, and plasma Mg and Ca were determined using the same method. Briefly, 10 µl of plasma was deposited on either Mg or Ca slides (Vitros Chemistry Products) which have a correlation coefficient of 0.999. Mg, both free and protein-bound, from the sample reacts with the formazan dye derivative in the reagent layer and the high Mg affinity of the dye dissociates Mg from its binding proteins. The Mg-dye complex formed was read at a wavelength of 630 nm. Ca forms a complex with arsenazo III dye and this complex was read at a wavelength of 680 nm.

Determination of Lipid Profile

At the end of both stages 1 and 2, blood was collected from the retroorbital plexus and by cardiac puncture, respectively, after the mice had undergone 12 h of fasting. One milligram of ethylenediaminetetraacetic acid per milliliter of blood was added to each sample. Total plasma cholesterol and triglyceride levels were determined using an automated enzymatic technique (Bochringer Mannheim, Germany) [10].

Assessment of the Extent of Atherosclerosis at the Aortic Sinus

Quantification of atherosclerotic fatty streak lesions was carried out by calculating the lesion size in the aortic sinus, as previously described [11], with a few modifications. Briefly, the heart and upper section of the aorta were removed from the animals and the peripheral fat was cleaned carefully. The upper section was embedded in OCT medium and frozen. Every other section (5 µm thick) throughout the aortic sinus (400 µm) was taken for analysis. The distal portion of the aortic sinus can be recognized by the three valve cusps which form the junctions of the aorta with the heart. The sections were evaluated for fatty streak lesions after staining with oil red O. The number of lesion areas per section was counted using a grid by an observer unfamiliar with the tested specimen.

The extent of atherosclerosis was evaluated at the level of the aortic sinus. Processing and staining of the tissue was carried out according to the method of Paigen et al. [11]. Lesion area was quantified by the modified method of Rubin et al. [12].

Statistical Analysis

Statistical analysis was carried out using Student's t test. $p < 0.05$ was considered statistically significant.

Results

Mg is used in clinical practice as a laxative, and therefore the body weights of both mouse groups were compared at the end of the study. No significant difference was found; body weights were 28.5 ± 6.4 g vs. 25.0 ± 4 g ($p = 0.24$) in the control and Mg groups, respectively.

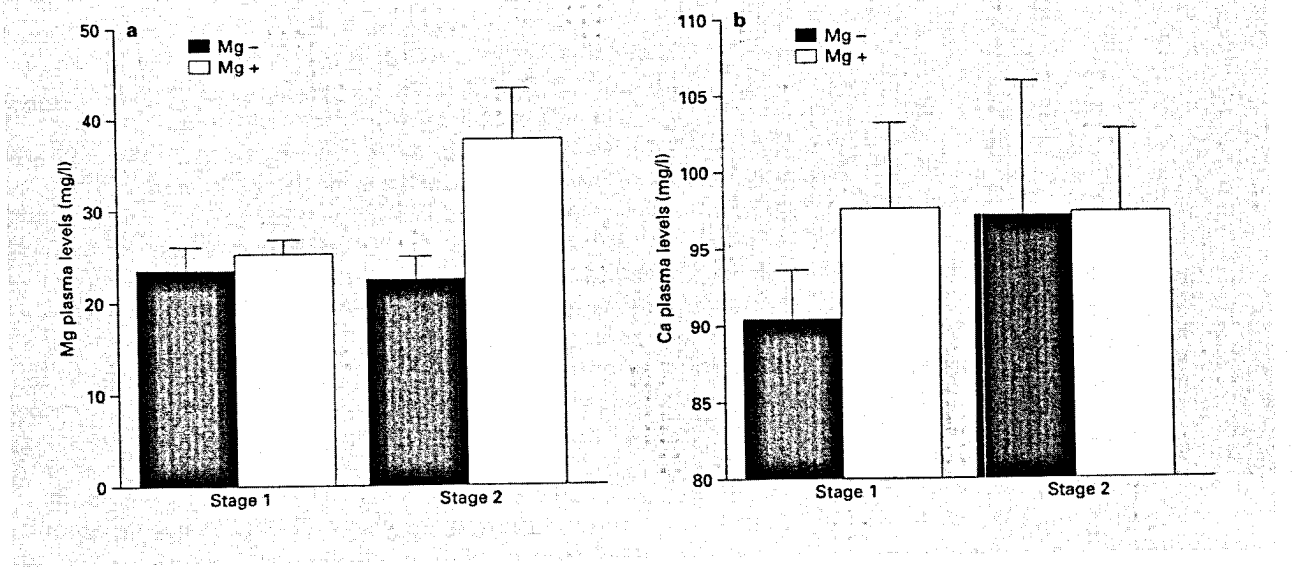

Fig. 1. a Average Mg levels (mg/l) after stages 1 and 2 for the two groups of mice. $p < 0.001$ in a comparison of Mg levels between the groups after stage 2. **b** Average Ca levels (mg/l) after stages 1 and 2 for the two groups of mice. $p = 0.01$ in a comparison of Ca levels between the groups after stage 1.

Table 1. Plasma lipid levels in female mice with (+Mg) and without Mg (–Mg) (mg/dl) in their drinking water

Study stage	Lipids	–Mg	+Mg	p value
Chow diet: after stage 1	triglycerides	121 ± 25	101 ± 21	0.13
	cholesterol	248 ± 43	231 ± 41	0.46
Western-type diet: after stage 2	triglycerides	214 ± 81	134 ± 37.1	0.03
	cholesterol	927 ± 206	926 ± 245	0.99

Plasma Mg and Ca Levels

At the end of stage 1, plasma Mg levels were 23.2 ± 2.7 mg/l in the mice that received distilled water and 25.1 ± 1.6 mg/l in the mice that received water fortified with Mg ($p = 0.13$). However, as emphasized in figure 1a, after stage 2 the mice that received water with Mg developed significant hypermagnesemia (37.3 ± 5.6 mg/l) compared with the control mice (22.0 ± 2.6 mg/l) ($p < 0.001$). As opposed to the differences in Mg concentration at the end of stage 2, there was no difference in Ca concentration (97.1 ± 8.8 vs. 97.3 ± 5.5 mg/l; $p = 0.97$). Surprisingly, there was a slight but significant difference in Ca levels at the end of stage 1 (fig. 1b), i.e. 90.2 ± 3.3 mg/l in the control group versus 97.4 ± 5.7 mg/l in the Mg group ($p = 0.01$). However, this difference in Ca concentrations did not affect the mean Mg to Ca ratio of both groups after stage 1 (25.7 ± 2.4% vs. 25.8 ± 1.1%; $p = 0.92$).

Lipid Profile

Lipid levels were determined at the end of stages 1 and 2. Significant differences in cholesterol levels were not found between the groups after either stage 1 or 2. Nevertheless, both groups of mice developed severe hypercholesterolemia after the second stage of feeding with a high-cholesterol diet (table 1). Even though the high-cholesterol diet did not cause significant hypertriglyceridemia, the mice that received Mg had significantly lower levels of triglycerides compared with the control group (table 1).

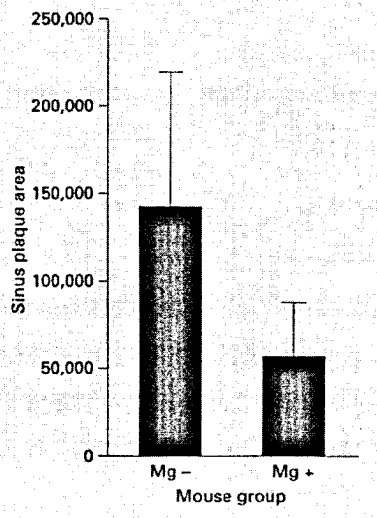

Fig. 2. Average atherosclerosis area at the aortic sinus (μm^2) in the two groups of mice.

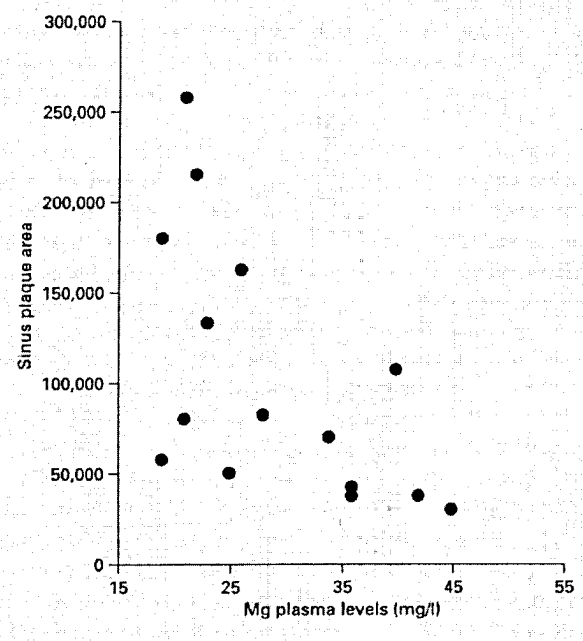

Fig. 3. A comparison of atherosclerosis area at the aortic sinus (μm^2) and Mg plasma levels of individual mice from both groups.

Extent of Atherosclerosis

The extent of the atherosclerosis at the aortic sinus differed significantly between the groups (fig. 2), i.e. 142,000 ± 76,000 μm^2 in the control group vs. 58,000 ± 29,000 μm^2 in the Mg group ($p = 0.017$). Within the Mg group, lesions with an area greater than 50,000 μm^2 were found only in 3 mice. These mice had lower plasma Mg levels than the other mice in the Mg group, i.e. 34 ± 6 vs. 40 ± 5 mg/l ($p = 0.1$). None of the mice in the control group had atherosclerotic lesions with an area less than 50,000 μm^2. A comparison of aortic sinus lesion area and Mg plasma levels in individual mice from both groups is demonstrated in figure 3.

Discussion

The results presented here demonstrate that Mg fortification can attenuate early atherogenesis in female LDL-receptor-deficient mice. Upon being fed a high-cholesterol diet, these mice develop marked hypercholesterolemia and lesions throughout the aorta within 3–4 months [8]. In this study, the mice were sacrificed after 6 weeks of being fed a Western-type diet in order to find differences in early atherogenesis, and hence the aortic sinus lesions, from which the lesions progress, were chosen as the most appropriate site for comparison of the extent of atherosclerosis. The results presented here support our previous findings that Mg fortification of drinking water is capable of attenuating atherogenesis without any change in the dietary content of Mg [7], but they also provide new evidence that Mg can significantly inhibit atherogenesis in female LDL-receptor-deficient mice.

Previously, we have proposed that the difference in atherosclerosis extent (albeit small and not statistically significant) found in female mice fed a Paigen diet suggests that Mg might also have an antiatherogenic effect in these mice, and that in another experimental design which includes another diet, this difference might reach statistical significance [7]. Herein, we have demonstrated that this assumption was correct; a change in the high-cholesterol diet (the diet of stage 2) from a Paigen diet containing cholic acid, 1.25% cholesterol and an Mg concentration of 1,430 mg/kg diet [7] to a Western-type diet that contained no cholic acid, only 0.15% cholesterol and a lower level of Mg of 510 mg/kg diet, changed the trend of atherogenesis inhibition into a highly significant one in female mice as well. These results are in agreement with a recent report that compared atherosclerosis development in LDL-receptor-deficient mice fed high-cholesterol diets

with and without cholate (cholic acid) [13]. It was found that cholate did not result in an altered atherosclerosis pattern or a significantly greater level of atherosclerosis, and thus the authors concluded that dietary cholic acid was unnecessary in atherogenesis studies in these mice [13]. It should be noted, however, that dietary supplementation with cholic acid results in aggravated hypercholesterolemia, presumably due to interference with the hepatobiliary excretion of cholesterol; this is evident from a comparison of the cholesterol levels of female mice in this study (around 1,000 mg/dl) and those in the female mice that received a diet with cholic acid in a prior study (around 2,000 mg/dl) [7]. However, this can also be explained by the fact that the Western-type diet contained much less cholesterol than the Paigen diet. It is not known why the antiatherogenic effect of Mg was clearly evident in female mice only on a diet without cholic acid; however, this difference can also be explained by the lower content of Mg and cholesterol in the Western-type compared to the Paigen diet. Hence, the Mg in drinking water turned out to be a more important source of Mg in the mice fed a Western-type diet, and possibly was more effective in these lower cholesterol concentrations.

These findings are further strengthened by studies regarding the ability of Mg to modulate blood lipid levels and atherosclerosis in rabbits [4, 5]. It has been found that Mg deficiency could augment, while Mg supplementation can suppress, atherogenesis in these animals. Altura et al. [5] reported both a reduction in atherosclerosis extent in the aorta and a reduction in serum cholesterol and triglycerides in rabbits fed a diet with higher Mg content, compared with rabbits fed a diet with lower Mg content. The Mg salt used in that report was Mg aspartate hydrochloride, while the Mg salt used by Ouchi et al. [4] was Mg sulfate, which failed to modulate plasma lipid levels but succeeded in decreasing atherosclerosis. We have also previously used Mg sulfate, and found that it did not result in a reduction of either plasma triglycerides or cholesterol level, but surprisingly led to an increased cholesterol level in females [7]. In the present study, no change in cholesterol levels was found, while the Mg group had significantly lower triglycerides than the control group. These inconsistencies regarding the effect of Mg on plasma lipid levels are also evident from other human and animal studies. Mg administration to hypomagnesemic renal transplant patients has been shown to result in a significant decrease in total cholesterol and LDL concentration [14]. Mg induced a decrease in triglycerides in patients with ischemic heart disease [15], whereas in mice, Mg led to decreased total cholesterol but did not change triglyceride levels [16].

The mechanisms of atherogenesis inhibition by Mg are not clear, but suggested mechanisms include increased excretion of lipids, decreased Ca influx into vascular smooth muscle and decreased oxidation of LDL. Decreased levels of lipids cannot properly explain the beneficial effect of Mg, since findings in respect of this are not consistent, as detailed above. An alternative hypothesis would be the prevention of Ca influx into vascular smooth muscle cells by Mg, as demonstrated in an in vitro study of endothelial cells incubated with different Mg concentrations [17]. Finally, as a decrease in lipid oxidation has been reported in the presence of elevated Mg [18], decreased oxidation of LDL may also be involved in the prevention of atherogenesis. Bussiere et al. [19] found that triglyceride-rich lipoproteins from Mg-deficient rats were more susceptible to oxidation by Cu^{2+}, and that a culture of vascular smooth muscle cells with these lipoproteins resulted in greater cell growth than with lipoproteins isolated from control animals. They suggest that Mg deficiency affects atherogenesis by both increased lipoprotein peroxidation and vascular smooth muscle cell proliferation [19].

In conclusion, the presented data emphasize that Mg fortification of drinking water can attenuate atherosclerosis in female LDL-receptor-deficient mice fed a high-cholesterol diet without cholic acid. The mechanisms of the antiatherogenic effect of Mg should be further elucidated.

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