Oral magnesium supplementation improves endothelial function and attenuates subclinical atherosclerosis in thiazide-treated hypertensive women

ABBREVIATIONS DEFINITION LIST

NO	Nitric oxide
BP	Blood pressure
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
HOMA	Homeostatic Model Assessment
IR	Insulin Resistance
ABPM	Ambulatory Blood Pressure Monitoring
FMD	Flow-mediated dilation
PAT	Peripheral arterial tonometry
PWV	Pulse wave velocity
DT	Distance travelled
TT	Transit time
MAP	Mean arterial pressure
AP	Augmentation pressure
Aix	Augmentation index
IMT	Intima-media thickness

Condensed Abstract

This study evaluated the effects of magnesium supplementation on blood pressure (BP) and vascular function in thiazide-treated hypertensive women, mean 24-h BP≥130/80 mmHg, in a randomized, double-blind, clinical trial comparing placebo and magnesium chelate 600 mg/day supplementation. Brachial flow-mediated dilatation (FMD) and central hemodynamic parameters were measured at inclusion and after 6 months. The magnesium group demonstrated a significant reduction in systolic and diastolic BP, and a significant increase in variation of FMD versus the placebo group (+3.7±2.1 vs 2.4±1.2 %, p=0.015). In conclusion, magnesium supplementation was associated with better BP control and improved endothelial function in thiazide-treated hypertensive.

Oral magnesium supplementation improves endothelial function and attenuates subclinical atherosclerosis in thiazide-treated hypertensive women

Short title: Magnesium in endothelium and atherosclerosis

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Abstract

Epidemiological studies demonstrate an inverse association between serum magnesium and incidence of cardiovascular disease. Diuretics commonly cause hypomagneseamia. We evaluated effects of magnesium supplementation on blood pressure (BP) and vascular function in thiazide-treated hypertensive women in a randomized, double-blind, clinical trial. Hypertensive women (40- 65 years)- on hydrochlorothiazide and mean 24-h BP≥130/80 mmHg were divided into placebo and supplementation (magnesium chelate 600 mg/day) groups. Patients were evaluated for nutritional and biochemical parameters, office and ambulatory blood pressure monitoring (ABPM), brachial flow-mediated dilatation (FMD), peripheral arterial tonometry, assessment of carotid intima-media thickness (IMT), central hemodynamic parameters and pulse wave velocity at inclusion and after 6-month follow-up. The magnesium group had a significant reduction in systolic BP (144±17 vs 134±14mmHg, p=0.036) and diastolic BP (88±9 vs 81±8mmHg, p=0.005) at 6 months, without effect on plasma glucose, lipids or arterial stiffness parameters. The placebo group showed a significant increase in carotid IMT (0.78±0.13 vs 0.89±0.14mm, p=0.033) without change in the magnesium group (0.79±0.16 vs 0.79±0.19mm, p=0.716) after 6 months. The magnesium group demonstrated a significant increase in variation of FMD versus the placebo group (+3.7±2.1 vs 2.4±1.2 %, p=0.015). There was a significant correlation between the intracellular magnesium variation and FMD (r=0.44, p=0.011). In conclusion, magnesium supplementation was associated with better BP control, improved endothelial function and amelioration of subclinical atherosclerosis in these thiazide-treated hypertensive women.

Keywords: hypertension; endothelial dysfunction; flow-mediated dilation; magnesium; arterial stiffness; pulse wave velocity

Introduction

Many factors have been implicated in the pathogenesis of hypertension, such as activation of the renin-angiotensin-aldosterone system and intracellular changes in ions like calcium, sodium, potassium and magnesium [1], [2], [3]. Magnesium has been the subject of experimental and clinical studies in hypertension [4-8] on the background that epidemiological studies showed significant inverse correlation between serum magnesium levels and incidence of cardiovascular disease [9] [10, 11].

Magnesium is the second most abundant intracellular cation and is involved in several important biochemical reactions [12]. Magnesium regulates vascular tone, by influencing formation and release of nitric oxide (NO), and competing against effects of calcium resulting in changes in the vascular smooth muscle tone and contractility [13, 14]. In addition, magnesium deficiency has previously been related to oxidative stress, inflammation, endothelial dysfunction, platelet aggregation, insulin resistance and hyperglycemia [15-19]

Hypomagnesemia has been shown to negatively influence functional and structural vascular alterations in hypertension [20] but the influence on arterial stiffness has not yet been demonstrated been. Hypomagnesemia has been implicated in the pathogenesis of high blood pressure (BP), endothelial dysfunction, dyslipidemia and inflammation [21-23], with these factors being associated with arterial stiffness [24, 25]. Furthermore, epidemiological studies indicate a direct relationship between atherosclerosis and low serum magnesium [26], probably associated with insufficient dietary intake of this mineral [27]. In addition to reduced dietary intake of magnesium, drugs, such as thiazide diuretics, also lead to hypomagneseamia. Whether patients treated with such drugs have associated vascular damage remains unclear. Thus, the aim of this study was to evaluate the effects of 6-month magnesium supplementation on functional

and structural vascular changes as well as blood pressure control in hypertensive women treated with diuretics.

Methods

Study Population

A double-blind randomized clinical trial was carried out to evaluate women with uncontrolled hypertension, aged 40–65 years, on stable monotherapy with hydrochlorothiazide 25 mg at least in the last 4 weeks. Exclusion criteria were BP ≥ 160/100 mmHg, body mass index≥ 35 kg/m^2, diabetes mellitus, kidney disease, clinical evidence of heart failure, coronary artery disease and previous stroke. Participants were randomized to receive placebo or 600 mg of magnesium chelate orally twice a day for 6 months. Clinical evaluation, laboratory tests and vascular studies were performed before and after the 6-month intervention period. The local ethics committee approved the protocol, and all subjects gave their written informed consent. This study was registered at ClinicalTrials.gov (NCT01151683).

Biochemical evaluation

Venous blood samples were collected after 12 hours of fasting. Serum lipids (total cholesterol, high-density lipoprotein [HDL]-cholesterol, triglycerides), and blood glucose were measured using an auto analyzer technique (Technicon DAX 96; Miles Inc). Low-density lipoprotein (LDL)-cholesterol was calculated with the Friedewald formula when triglyceride values were <400 mg/dl. Serum magnesium was measured by colorimetry and intracellular (intra erythrocyte) magnesium was evaluated by atomic absorption spectrometry. Insulin was measured by radioimmunoassay, and the Homeostatic Model Assessment – Insulin Resistance (HOMA-IR) index [fasting

glucose (mmol/l) x fasting insulin (mUI/ml)/22.5] and HOMA-beta [fasting insulin x 20/(fasting glucose – 3.5)] were calculated to estimate insulin sensitivity and secretion [28].

Ambulatory Blood Pressure Monitoring

The patients underwent 24-hour ambulatory blood pressure monitoring (ABPM) in non-dominant arm with SpaceLabs 90207 monitor (Spacelabs Inc., Redmond, WA, USA), validated by the British Hypertension Society and the Association for the Advancement of Medical Instrumentation protocol [29]. Readings were taken every 20 minutes during the day and every 30 minutes at night. The patients recorded their sleep and wake times during the monitoring. ABPM was considered adequate if >70% of measurements were successfully obtained. The percentage decline in nocturnal blood pressure was calculated as follows for systolic and diastolic BP: % BP fall = [(daytime BP−night-time BP) /daytime BP]* 100.

Assessment of endothelial function

Flow-mediated dilation (FMD) was assessed as a measure of endothelial function. The participant was positioned supine with the arm in a comfortable position, and the brachial artery was imaged above the antecubital fossa. After 10 minutes of rest, the right brachial artery was scanned in longitudinal section, 5 cm above the antecubital fossa, using a linear array transducer to acquire the baseline diameter of the brachial artery. A cuff was then inflated to at least 50 mmHg above systolic BP and deflated after 5 minutes to induce reactive hyperemia. A pulse wave Doppler recording in the artery lumen documented the flow increase, and the maximal diameter after cuff release was registered. FMD was calculated as the maximal percentage change of brachial artery diameter from baseline.

Peripheral arterial tonometry (PAT)

Endothelial function was also evaluated in microcirculation with the Endo-PAT system (Itamar Medical Ltd, Caesarea, Israel). During the measurement, the subjects remained in a chair with their hands at the level of their heart and fingers hanging freely. Fingertip probes were placed on both index fingers, and pulse wave amplitudes were detected and recorded during the study. After a 5-minute baseline measurement, arterial flow was occluded with a cuff on the non-dominant arm. After 5 minutes of occlusion, the cuff was rapidly deflated to allow reactive hyperemia. Pulse wave amplitudes were recorded again for at least 5 minutes. The software calculated a reactive hyperemia index, which was a ratio of the average pulse wave amplitude measured over 60 seconds, starting 1 minute after cuff deflation, to the average pulse wave amplitude measured at the baseline. The other arm served as a control, and the ratio was corrected for changes in the systemic vascular tone.

Pulse wave velocity

The same investigator using a Complior device (Alam Medical, France) after the patients had rested for 10 minutes in supine position in a quiet room with a stable temperature measured carotid-femoral pulse wave velocity (PWV). All measurements were performed between 8 a.m. and 11a.m. During the measurements, speaking or sleeping was not allowed and no meal, caffeine or smoking was allowed within 3h before measurement. Pulse wave forms were obtained transcutaneously from the right common carotid artery and femoral artery. Aortic PWV was calculated by dividing the distance travelled by the time travelled. The time travelled was obtained by measuring the time difference between the arrival of the pulse wave at the femoral and carotid

arteries. The distance travelled (DT) was estimated as 80% of the direct tape measure distance between carotid and femoral artery. Carotid-femoral PWV was calculated as distance (DT) in meters divided by transit time (TT) in seconds (PWV = DT/TT). The mean of two measurements was calculated and when the difference between them was more than 0.5 m/s, a third measurement was obtained. All PWV values were adjusted by mean arterial pressure (MAP) to obtain normalized PWV (PWV norm) as 100 x (PWV/MAP).

Central Hemodynamic Parameters

Applanation tonometry was performed with the SphygmoCor system (Atcor Medical, Sydney, Australia) with the patient in the sitting position, resting the arm on a rigid surface, and a sensor in the radial artery. Central aortic pressure was calculated from the radial pulse wave analysis with the use of a validated transfer function. Wave reflection parameters, such as augmentation pressure (AP) and augmentation index (AIx), were also obtained by this method.

Carotid Ultrasound Scan

The patient was supine with slight hyperextension and rotation of the neck in the direction opposite the probe. A linear array transducer with a multiple-frequency (7–12 MHz) attached to a high-resolution B-mode ultrasound system was used to acquire images by a single sonographer blind to clinical data of subjects. Simultaneous electrocardiogram was recorded to assure the timing of end-diastolic images. Manual measurement of intima-media thickness (IMT) was performed in the common carotid artery, at both sides, in a region free of plaque located approximately 20 mm from bulb. At least three values were obtained in different sites of this segment, and the mean value

of six measurements (three from each side) was used for analysis.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation. Baseline differences between the two study groups were evaluated with Student *t*-tests for continuous variables and Fisher's exact test for categorical variables. For intra-group comparisons between the baseline and study-end, paired *t*-test was used. Between-group differences were compared using unpaired *t*-test or Mann–Whitney *U*-test where appropriate. To identify possible relationships between the changes in the study parameters, bivariate correlation Pearson coefficients (*r*) were calculated. A *P* value <0.05 was considered statistically significant. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 18.0 for Windows (SPSS, Chicago, IL, USA).

Results

Fifty-three hypertensive women treated with thiazides were screened for this study. After initial evaluation, 16 patients were not included due to BP < 130/80 mmHg and two other patients had fasting plasma glucose higher than 126 mg/dl and were also excluded.

Baseline clinical characteristics in the placebo and magnesium groups are presented in Table 1. The mean age and anthropometric data were not significantly different, and both groups presented intermediate risk prediction for general cardiovascular disease with normal liver and renal function. The magnesium group demonstrated a significant reduction in office systolic and diastolic BP between baseline and 6-month evaluation, and a slight decrease in mean 24-h systolic and diastolic BP but

without statistical significance. The same was observed in both daytime and nigh-time periods. The changes in systolic and diastolic BP nocturnal fall were not significantly different between the groups (Table 2).

Fasting plasma glucose, insulin and HOMA indexes were similar at baseline and final evaluation in both groups (Table 3). Total cholesterol was significantly decreased at the end of the study in the magnesium group. In this group, HDL-cholesterol was increased and LDL-cholesterol was reduced but did not reach statistical significance. There were no significant changes in the lipid profile in the placebo group. With regards to plasma electrolytes, there was a small but significant reduction in serum sodium in the placebo group but not in the magnesium group. Serum potassium and calcium were not significantly different in both groups. Serum magnesium was significantly reduced in the placebo group and did not change in the supplementation group. On the other hand, intracellular and urinary magnesium were significantly increased only in the group receiving supplementation (Figures 1a-1c). Although there was no significant difference in intracellular sodium between the groups, there was a negative correlation between intracellular magnesium and intracellular sodium ($r=-0.48$, $p=0.023$; Figure 1d).

Vascular results are shown in Table 4. Carotid-femoral PWV was similar between the groups in baseline and after 6-month supplementation even after adjusting for MAP. Likewise, the central hemodynamic parameters were not significantly different between the groups before and after intervention. The brachial FMD was reduced in the placebo group and increased in the magnesium supplemented subjects although not statistically significant. However, the magnesium group presented significantly higher values and changes of brachial FMD after supplementation when compared to the control group (Figure 2a). The variation of intracellular magnesium was positively correlated to the variation ($r=0.40$, $p=0.015$) and to the end-study values

(r=0.44, p=0.011) of brachial FMD (Figures 2c-2d). RHI obtained by peripheral arterial tonometry was also reduced in the placebo group and increased in the magnesium group but without statistical significance (Figure 2b). After 6 months of study, the placebo group demonstrated a small but significant increase in carotid IMT and media-to-lumen ratio which were not changed in the magnesium supplemented patients (Figures 3a-3b). Serum magnesium was inversely correlated to mean (r=-0.37, p=0.046) and maximum (r=-0.52, p=0.004) carotid IMT (Figures 3c-3d).

Discussion

The therapeutic value of magnesium supplementation for human hypertension is still unclear , with some studies showing benefit and others failing to demonstrate therapeutic advantage on BP control [30, 31]. In the present study, oral magnesium supplementation for six months significantly decreased office systolic and diastolic BP in hypertensive women. On the other hand, there was a small and non-statistically significant lowering of 24-h BP levels after magnesium supplementation. Nevertheless, the reduction of 5 mmHg in systolic and diastolic BP might be clinically relevant concerning the prevention of cardiovascular events such as myocardial infarction and stroke. This finding is in agreement with a recent meta-analysis that demonstrated a small but clinically significant decrease, around 3 mmHg, in systolic and diastolic BP determined by magnesium supplementation [32]. In fact, a previous meta-analysis had already detected dose-dependent BP reductions from magnesium supplementation [33]. Recently, a slight but significant lowering effect of oral magnesium supplementation on 24-h BP levels was observed in patients with mild hypertension and this finding was associated with increase in the intracellular magnesium [34].

Heterogeneity in study design, study participants and the type of magnesium supplement used could help to explain the different results in several clinical protocols

regarding the BP lowering effects of magnesium supplementation. Absorption of magnesium varies from different kinds of supplements. Hence, some forms of administered magnesium may have had lower bioavailability, which might reduce the influence on BP levels. Small studies have found that citrate and chloride forms are more absorbed than magnesium oxide and sulfate. Chelated magnesium is more likely to survive the passage from the stomach to the small intestines intact. Therefore, the chelated magnesium bioavailability seems to be higher than other preparations [35].

Magnesium is involved in the physiological pathways that regulate glucose and lipid metabolism. A growing body of evidence derived from clinical trials shows that oral magnesium supplements improve insulin sensitivity and dyslipidemia in diabetic and non-diabetic individuals [7, 31, 36]. Insulin resistance is inversely correlated with HDL-cholesterol levels and atherogenic changes characterized by low HDL-cholesterol are mainly seen in insulin-resistant subjects [37]. Magnesium deficiency may be associated to low HDL-cholesterol in the prediabetic state and chronic magnesium supplementation can contribute to an improvement in islet b-cell response and insulin action [38], and is useful in the treatment of hyperlipidemia [39] increasing the HDL-cholesterol levels [7, 40]. Serum magnesium levels have been correlated with total and HDL-cholesterol in a large adult study population [41]. Interestingly, the relationship between magnesium status and lipids in healthy individuals may be different from that in patients with chronic conditions, such as obesity, diabetes, and hypertension [42, 43]. In this study, patients in the magnesium group demonstrated a beneficial effect with a significant decrease in total cholesterol, and a non-significant increase in HDL-cholesterol and reduction in LDL-cholesterol compared to no significant changes in the lipid profile in placebo group.

There was a small but significant reduction in serum sodium and intracellular sodium in the placebo group, with a negative correlation between intracellular magnesium and intracellular sodium. These findings could indicate a protective effect of magnesium on hyponatremia induced by diuretic treatment. Although the mechanisms were not investigated in the present study, the Na+/Mg2+ antiport has been previously confirmed in mammalian cells [44], and changes in intracellular magnesium could also modify transmembrane calcium movements [45].

Low urinary magnesium excretion has been previously associated with an increased risk of ischemic heart disease [46]. In addition, urinary magnesium excretion can estimate the amount of absorbed magnesium, considering that renal elimination is highly relevant for an adequate magnesium homeostasis. Chronic intervention studies are usually associated to lost of follow-up and low treatment compliance. In the present study, both groups presented normal renal function indicating no influence on magnesium excretion. Therefore, the increase in intracellular and urinary magnesium in the group receiving 6-month supplementation points out the good compliance by these patients. Indeed, the increase in the intracellular ion concentrations has already been associated with BP lowering effect of oral magnesium supplementation [34].

In this study, magnesium supplementation was not able to change arterial stiffness and wave reflection parameters. In contrast, we recently demonstrated that low intracellular magnesium was associated with more intense wave reflection [47]. Likewise, it has been previously demonstrated that serum magnesium levels were associated with augmentation index although not with PWV. However, the cross-sectional design and the oscilometric method used in that study to evaluate arterial stiffness and pulse wave reflection may be considered as limitations of those findings [48].

Endothelial dysfunction has been suggested to represent an early indicator of atherosclerosis, associated with an increased incidence of vascular diseases. Past studies have demonstrated that low magnesium promotes endothelial cell dysfunction [49] and endothelium-dependent relaxation induced by extracellular magnesium is mediated by NO release by the endothelium [50]. The results of the present study demonstrated the beneficial effects of oral magnesium supplementation on endothelial function, including the positive correlation between variation of intracellular magnesium and brachial FMD. One of the possible mechanisms is that magnesium affects calcium ion concentrations and its availability at critical sites, acting as a physiologic calcium channel blocker [51]. Although not directly involved in the biochemical process of contraction, magnesium influences vascular tone, baseline tension and vascular responsiveness to vasoconstrictor agents, both via endothelium independent and endothelium dependent pathways [52, 53]. Our results are in accordance with previous report that oral magnesium supplementation resulted in a significant improvement in brachial artery endothelial function in elderly diabetic patients [12] and in patients with coronary artery disease [54].

Magnesium deficiency has been introduced as a cardiovascular risk factor, and some studies have shown that hypomagnesemia is associated with atherosclerosis [55, 56]. Our data demonstrated an increase in carotid IMT in the placebo group while hypertensive women receiving magnesium supplementation presented no difference. This result might be attributed to higher blood pressure levels in the placebo group. In accordance with these findings, a recent study in hemodialysis patients showed that magnesium oxide for 6 months was able to decrease carotid IMT with an increase in the placebo group [57]. Indeed, the role of magnesium in attenuating the progression of atherosclerosis has been previously demonstrated in hemodialysis patients [58].

Some limitations warrant consideration in the present study. The beneficial effect of magnesium on endothelial function may be considered the main finding but we did not study all biochemical endothelial markers. Moreover, there was no concomitant assessment of endothelium-independent dilation. On the other hand, in our experimental model, all the results were compared to a control, not supplemented group, and there was no improvement in brachial FMD in these patients receiving placebo. Lastly, the single investigator performing the exams was blinded to the treatment assigned.

The significant magnesium effects in our study may relate, in part, to the fact that, unlike other studies, we investigated only female patients on thiazide diuretics, who are at particular risk of magnesium wasting. In fact, the recommended dietary allowance (RDA) for magnesium is different for middle-aged men (420mg/day) and women (320mg/day). Considering the study design as a randomized, double blind, clinical trial, we enrolled only hypertensive women in order to avoid this gender difference as a confounding factor. Hence, this patient cohort may indeed benefit from magnesium supplementation with respect to blood pressure control and vascular protection. The effects of sex steroid hormones on magnesium metabolism and actions are not clear yet. Nevertheless, this concern is not relevant in this study since almost all patients were postmenopausal women.

In conclusion, 6-month magnesium supplementation was able to lower BP, attenuate subclinical atherosclerosis and improve endothelial function in hypertensive women on thiazide treatment. The beneficial effects of magnesium supplementation may be more evident in patients with a negative magnesium balance. Since magnesium is an inexpensive, natural, and relatively safe element, its possible role as adjuvant therapy in those subjects who are at high risk of magnesium deficiency should be considered. There is still considerable uncertainty on the clinical utility of magnesium

supplements. Therefore, further studies with larger populations are needed to confirm our findings.

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Table 1. Clinical characteristics of the study groups.

Parameters	**Placebo (n=18)**	**Magnesium (n=17)**	***P* value**
Age, years	57 ± 5	54 ± 7	0.571
Menopause, n (%)	16 (88.9)	17 (100)	0.357
Current smoker, n (%)	1 (5.5)	1 (5.9)	1.000
BMI, kg/m^2	26.8 ± 3.9	29.7 ± 4.1	0.311
Waist, cm	92.2 ± 10.9	98.3 ± 9.9	0.259
WHR, units	0.84 ± 0.39	0.84 ± 0.63	0.991
General CV risk, %	14.5 ± 7.3	14.2 ± 8.5	0.228
Creatinine, mg/dl	0.78 ± 0.16	0.76 ± 0.12	0.946
AST, U/l	19.8 ± 0.7	19.0 ± 1.5	0.809
ALT, U/l	18.6 ± 1.3	19.4 ± 1.4	0.820

The values are expressed as mean ± SD. BMI, body mass index; WHR, waist-to-hip ratio; CV, cardiovascular; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table 2. Office and ambulatory blood pressure levels in placebo and magnesium supplementation groups.

Parameters	Placebo (n=18)			Magnesium (n=17)			Between-group comparison
	Baseline	*Final*	*P* value	*Baseline*	*Final*	*P* value	*P* value *
Office SBP, mmHg	143 ± 16	142 ± 18	0.635	144 ± 17	134 ± 14	0.036	0.240
Office DBP, mmHg	86 ± 10	86 ± 9	0.760	88 ± 9	81 ± 8	0.005	0.172
SBP-24h, mmHg	134 ± 11	135 ± 13	0.168	135 ± 11	129 ± 11	0.074	0.322
DBP-24h, mmHg	86 ± 8	84 ± 7	0.076	86 ± 6	81 ± 10	0.111	0.472
PP-24h, mmHg	48 ± 9	51 ± 9	0.548	49 ± 9	50 ± 12	0.871	0.811
Systolic load, %	54 ± 27	53 ± 29	0.191	57 ± 32	47 ± 34	0.190	0.678
Diastolic load, %	65 ± 23	57 ± 23	0.221	68 ± 20	50 ± 32	0.109	0.597
Daytime SBP, mmHg	140 ± 11	137 ± 15	0.148	139 ± 11	133 ± 11	0.097	0.466
Daytime DBP, mmHg	91 ± 9	87 ± 8	0.051	90 ± 7	85 ± 10	0.087	0.557
Night-time SBP, mmHg	131 ± 18	130 ± 11	0.383	126 ± 14	123 ± 12	0.195	0.258
Night-time DBP, mmHg	80 ± 9	77 ± 7	0.299	77 ± 8	75 ± 9	0.231	0.605
SBP nocturnal fall, %	6.3 ± 7.1	5.2 ± 7.3	0.878	10.1 ± 5.4	7.3 ± 5.6	0.487	0.558
DBP nocturnal fall, %	11.8 ± 7.8	11.7 ± 6.4	0.998	14.5 ± 7.1	11.5 ± 5.7	0.430	0.951

The values are expressed as mean ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure. * Student t-test for between groups comparison at the end of the study.

Table 3. Biochemical profile at baseline and study-end in the placebo and magnesium supplementation groups.

Parameters	Placebo (n=18)			Magnesium (n=17)			Between-group comparison *P* value
	Baseline	***Final***	***P* value**	***Baseline***	***Final***	***P* value**	
Glucose, mg/dl	90 ± 12	96 ± 11	0.428	93 ± 8	91 ± 10	0.270	0.346
Insulin, mcU/ml	12.8 ± 8.9	12.8 ± 11.1	0.949	11.2 ± 3.9	10.7 ± 3.8	0.733	0.596
HOMA-IR	2.9 ± 2.2	2.8 ± 2.6	0.832	2.6 ± 1.1	2.4 ± 0.9	0.719	0.675
HOMA-beta	176 ± 106	139 ± 161	0.289	141 ± 48	184 ± 168	0.361	0.586
Total cholesterol, mg/dl	223 ± 36	220 ± 38	0.244	241 ± 56	216 ± 32	0.038	0.816
HDL-cholesterol, mg/dl	54 ± 14	50 ± 13	0.052	58 ± 17	62 ± 15	0.720	0.126
LDL-cholesterol, mg/dl	146 ± 32	144 ± 32	0.541	155 ± 39	126 ± 31	0.058	0.253
Triglycerides, mg/dl	117 ± 57	129 ± 60	0.664	111 ± 60	114 ± 55	0.102	0.582
Serum Na, mEq/l	142 ± 2	140 ± 2	0.046	140 ± 3	140 ± 3	0.456	0.536
Serum K, mEq/l	4.2 ± 0.3	3.9 ± 0.3	0.384	4.2 ± 0.4	4.1 ± 0.3	0.601	0.410
Serum Ca, mg/dl	9.3 ± 0.5	9.6 ± 0.4	0.516	9.7 ± 0.9	9.4 ± 0.5	0.317	0.253
Serum Mg, mg/dl	2.1 ± 0.2	1.9 ± 0.2	0.048	2.1 ± 0.2	2.1 ± 0.2	0.756	0.029
UMCR, g/g	0.05 ± 0.02	0.05 ± 0.02	0.620	0.05 ± 0.02	0.12 ± 0.07	0.057	0.018
ic Mg, mg/dl eryt	4.27 ± 0.64	4.06 ± 0.52	0.101	4.05 ± 0.61	4.75 ± 0.56	0.001	0.005
ic Na, mg/dl eryt	23 ± 9	18 ± 6	0.504	21 ± 9	20 ± 3	0.343	0.585

The values are expressed as mean ± SD. * Paired t-test for intra-group analysis. ** Student t-test for independent analysis between the groups. HOMA IR, Homeostatic Model Assessment - Insulin Resistance, HOMA Beta, Homeostatic Model Assessment Beta Cell Function; HDL, high density lipoprotein, LDL, low density lipoprotein; UMCR, urinary magnesium creatinine ratio; ic, intracellular; eryt, erythrocytes.

Table 4. Comparison of vascular tests between the groups.

Parameters	Placebo (n=18)			Magnesium (n=17)			Between-group comparison *P* value *
	Baseline	***Final***	***P value***	***Baseline***	***Final***	***P value***	
Brachial FMD, %	8.3 ± 5.3	5.8 ± 3.7	0.064	8.3 ± 5.6	11.9 ± 6	0.109	0.008
RHI, units	1.96 ± 0.36	1.85 ± 0.56	0.708	1.93 ± 0.30	2.09 ± 0.49	0.374	0.326
PWV, m/s	8.24 ± 1.28	8.16 ± 0.88	0.781	8.24 ± 1.12	8.08 ± 1.04	0.605	0.801
PWVnorm, m/s	7.92 ± 1.28	8.16 ± 1.12	0.610	7.76 ± 1.20	7.68 ± 1.04	0.819	0.321
AP, mmHg	19 ± 6	19 ± 4	0.960	18 ± 5	18 ± 7	0.920	0.597
AIx, %	38 ± 9	39 ± 2	0.721	38 ± 4	36 ± 7	0.260	0.158
AIx@75, %	36 ± 9	35 ± 3	0.798	33 ± 3	32 ± 6	0.595	0.263
Aortic SBP, mmHg	131 ± 15	130 ± 16	0.756	130 ± 14	129 ± 13	0.897	0.915
Aortic PP, mmHg	46 ± 11	47 ± 10	0.577	46 ± 11	45 ± 14	0.878	0.503
Mean carotid IMT, mm	0.78 ± 0.13	0.89 ± 0.14	0.033	0.79 ± 0.16	0.79 ± 0.19	0.716	0.282
Max carotid IMT, mm	0.99 ± 0.35	1.13 ± 0.30	0.156	0.92 ± 0.19	0.93 ± 0.24	0.681	0.169
Carotid media-lumen ratio, mm	0.11 ± 0.01	0.13 ± 0.02	0.011	0.11 ± 0.02	0.11 ± 0.03	0.917	0.036

All values are expressed as mean±SD. FMD, flow-mediated dilation; RHI, reactive hyperemia index; PWV, pulse wave velocity; PWVnorm, pulse wave velocity normalized for mean arterial pressure; AP, augmentation pressure; AIx, augmentation index; AIx@75, augmentation index corrected for heart rate of 75 bpm; SBP systolic blood pressure; PP, pulse pressure, IMT, intima-media thickness. * Paired t-test for intra-group analysis. ** *t* test for inter-group analysis.

Figure legends

Figure 1. Changes in serum (A), urinary (B) and intracellular (C) magnesium in the study groups. Correlation between intracellular (ic) magnesium and sodium (D).

Figure 2. Changes in brachial flow-mediated dilation (FMD) (A) and in reactive hyperemia index (RHI) by peripheral artery tonometry (B) in both groups. Correlation of changes in intracellular (ic) magnesium with study-end values of brachial FMD (C) and with changes in brachial FMD (D) after 6 months of study.

Figure 3. Changes in carotid intima-media thickness (IMT) (A) and in carotid media-lumen ratio (B) in the placebo and magnesium groups. Correlation of serum magnesium with mean carotid IMT (C) and with maximum carotid IMT (D).

Figure

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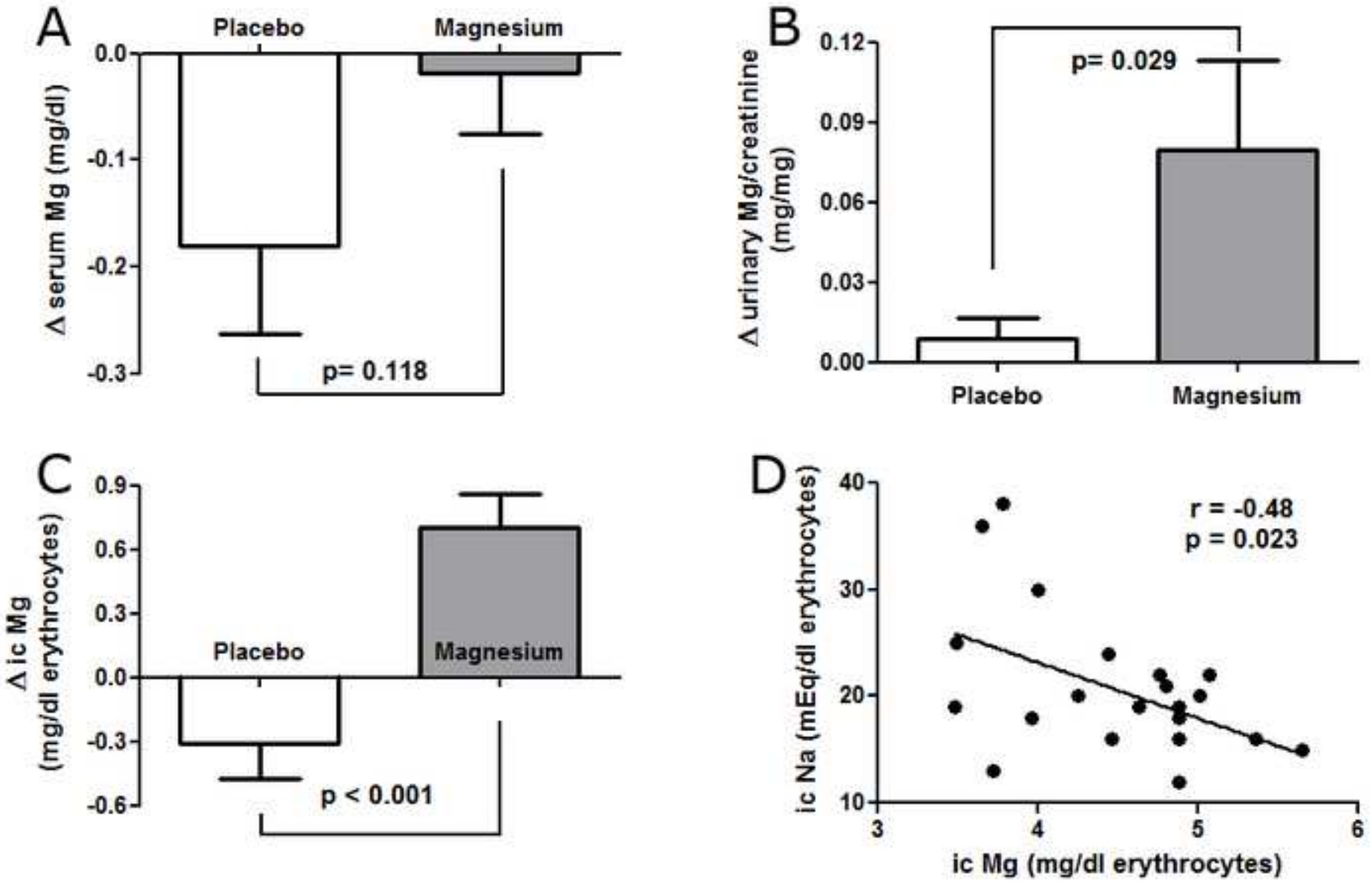

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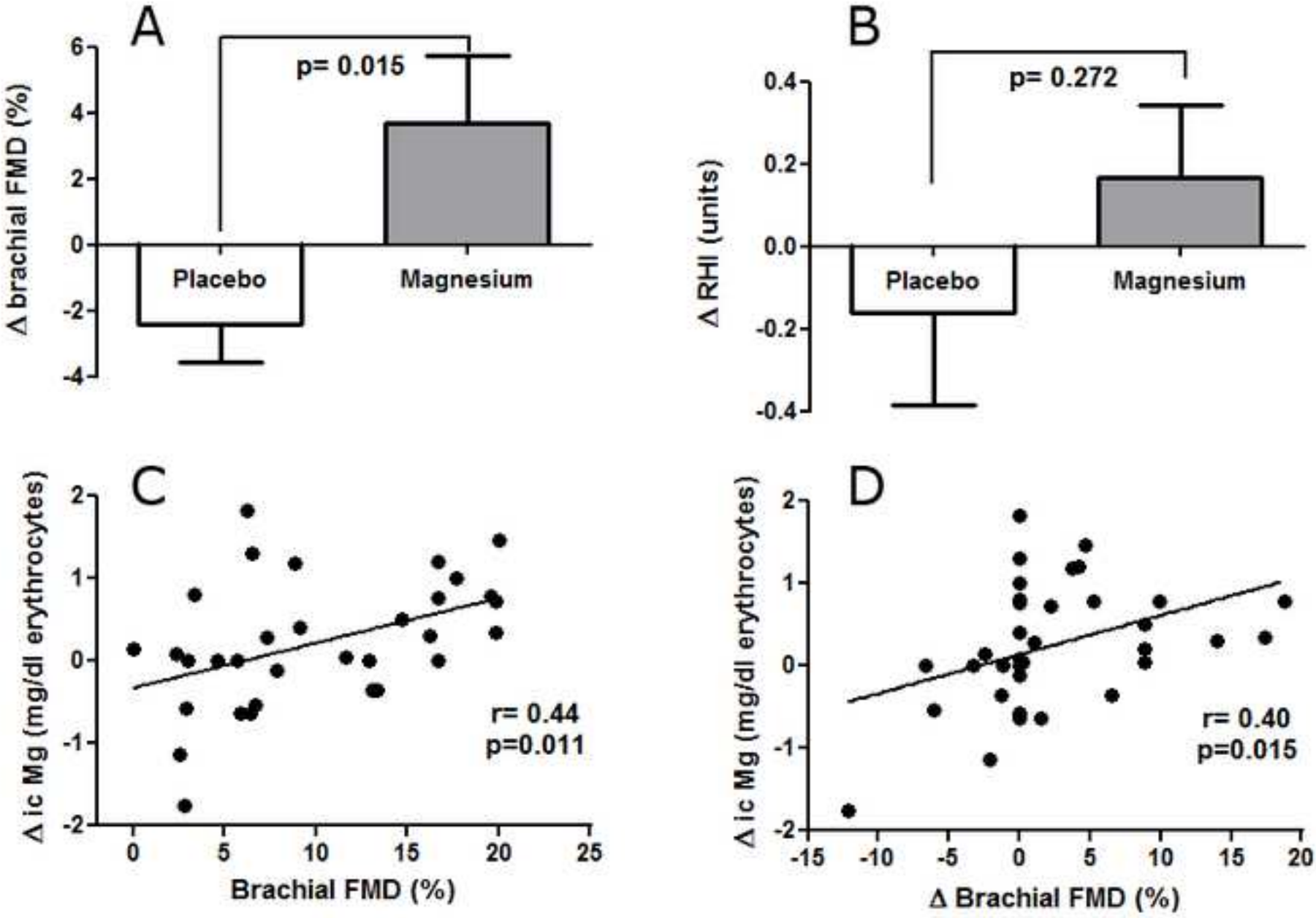

Figure

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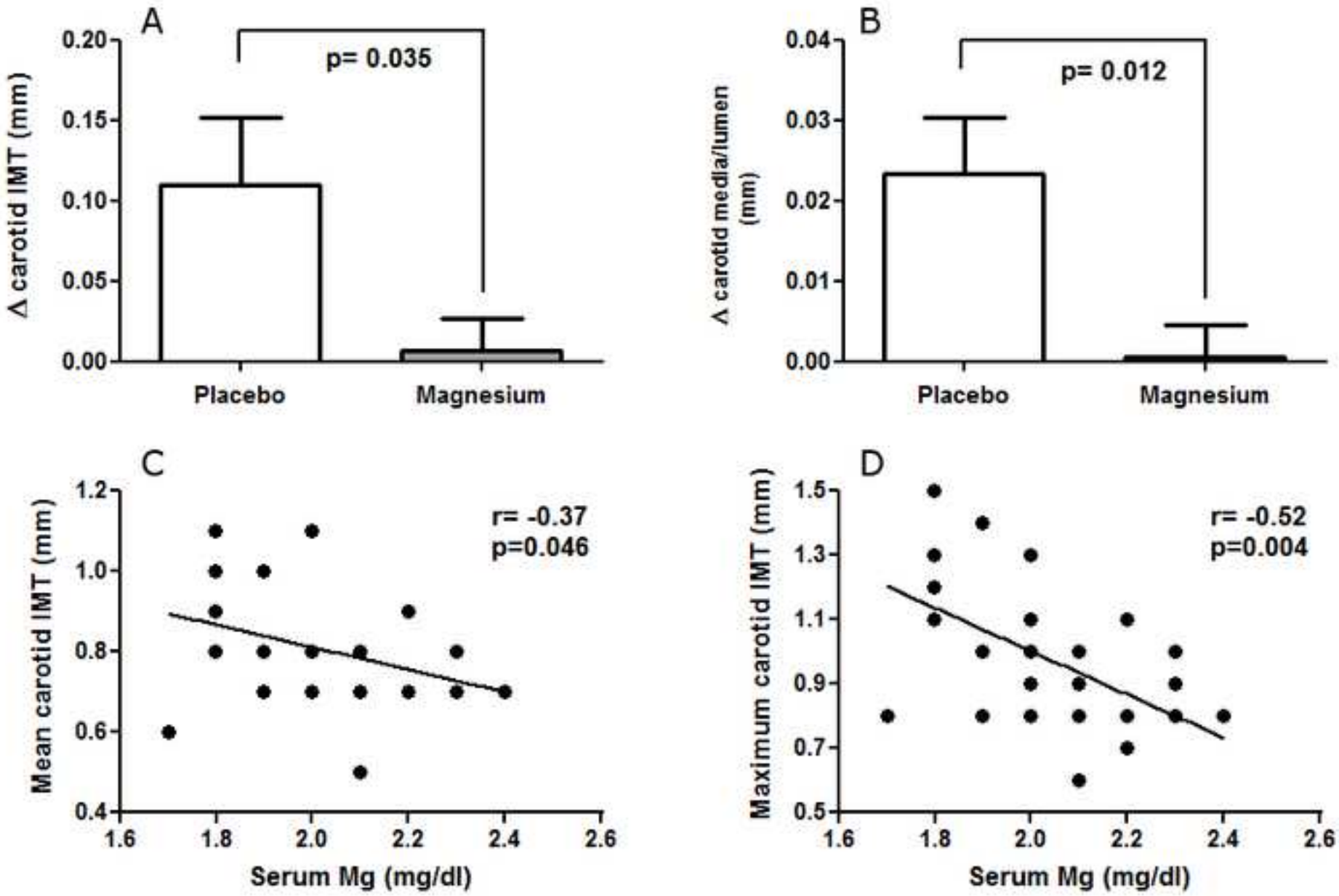

March 24th, 2016.

To Prof. Guido Grassi
Executive Editor
Journal of Hypertension

Ms. Ref. No.: JH-D-15-01145

Title: Oral magnesium supplementation improves endothelial function and attenuates subclinical atherosclerosis in thiazide-treated hypertensive women

Dear Dr. Grassi,

Please find enclosed the response to the referees and the revised version of our manuscript that has been changed according to the referees` suggestions. We are very grateful for the valuable criticisms.

We hope that the present version may be now suitable for publication in the Journal of Hypertension.

We look forward to your editorial decision.

Yours sincerely,

Mario Fritsch Neves, MD, PhD

Manuscript

"Oral magnesium supplementation improves endothelial function and attenuates subclinical atherosclerosis in thiazide-treated hypertensive women**"**

Response to Reviewers

We thank the reviewers for their thoughtful review. We have revised the manuscript in accordance to all the points raised by the criticisms of the reviewers. We believe that this has resulted in a significant improvement of the manuscript and the clarity with which it is presented.

Reviewer 1:

This is an interesting study, showing a significant correlation between the intracellular magnesium variation and FMD (r=0.44, p=0.011) in hypertensive women. Magnesium supplementation was associated with better BP control, improved endothelial function and amelioration of IMT.

Q1. The manuscript is well written, pending on the lacked information on renal and liver functions. Both can deeply influence the rate of magnesium absorption/excretion. Thus, it is necessary to show data on liver and renal function in the appropriate table (n.1).

A1: **We completely agree with this referee's observation. Indeed, serum creatinine values were reported in the second paragraph of "Results" indicating normal renal function in both groups. As the study population consisted of middle-aged women and serum creatinine levels were normal, it was not necessary to estimate GFR to show normal renal function. All the patients presented normal liver function. Considering the relevance of this comment, we included the values of creatinine, AST and ALT in Table 1. We also included a comment about this issue in Discussion: "In the present study, both groups presented normal renal function indicating no influence on magnesium excretion."**

Reviewer 2

In this randomized, double-blind study, the authors assessed the effect of 6-month magnesium supplementation on blood pressure (BP) and vascular function in thiazide-treated hypertensive women. Magnesium supply reduced office BP, ameliorated FMD and lipid profile, while unaffecting plasma glucose, arterial stiffness or IMT parameters. In contrast, the placebo-group showed a significant increase in carotid IMT. The authors concluded that magnesium supplementation was associated with better BP control, improved endothelial function and amelioration of atherosclerosis in the thiazide-treated hypertensive women.

Q1. It is not clear the cardiovascular risk profile of the women recruited. The smoking history, as well as the estimated GFR are missing. The latter is crucial, when considering that magnesium is eliminated by urine.

A1. We agree with the referee's point of view. Indeed, cardiovascular risk is shown in table 1 pointing out an intermediate profile. There was only 1 smoker in each group. We added this information in Table 1. Serum creatinine values were reported in the second paragraph of "Results" indicating normal renal function in both groups. Considering the relevance of this comment, we included the values of creatinine in Table 1. As the study population consisted of middle-aged women and serum creatinine levels were normal, it was not necessary to estimate GFR to show normal renal function. We also included a comment about this issue in Discussion: "In the present study, both groups presented normal renal function indicating no influence on magnesium excretion."

Q2. 2. The authors recognize the recruitment of women as a limitation of the present study. It is not clear why the authors excluded men. It is because women, but not men, undergo to hypomagnesemia under diuretic intake?

A2. The point raised by the referee is completely appropriate. In fact, women and men are candidates to hypomagnesemia under diuretic therapy. However, the recommended dietary allowance (RDA) for magnesium is different for middle-aged men (420mg/day) and women (320mg/day). As the study design was a randomized, double blind, clinical trial, we enrolled only hypertensive women in order to avoid this gender difference as a confounding factor. We recognized this consideration and we added this comment in Discussion, one before the last paragraph.

Q3. It is interesting that magnesium supply does not affect vascular atherosclerosis. However, it is quite surprising that 6 months of placebo might induce a significantly progression of IMT in middle-aged women. Is this the unique effect of uncontrolled BP? The authors should better clarify this aspect.

A3. We agree with the referee's observation. Indeed, we believe that this finding was related to higher blood pressure levels in the placebo group. Thus, we added this comment in Discussion: "Our data demonstrated an increase in carotid IMT in the placebo group while hypertensive women receiving magnesium supplementation presented no difference. This result might be attributed to higher blood pressure

levels in the placebo group. In accordance with these findings, a recent study in hemodialysis patients showed that magnesium oxide for 6 months was able to decrease carotid IMT with an increase in the placebo group."

Q4. Have the authors any idea on the possibility the estrogen/progestin might play a role on the magnesium-induced impact on these parameters?

A4. This is an interesting question raised by the referee and we agree with this concern. "The effects of sex steroid hormones on magnesium metabolism and actions are not clear yet. Nevertheless, this concern is not relevant in this study since almost all patients were postmenopausal women." We added these sentences in Discussion, one before the last paragraph.