

Magnesium supplementation reduces inflammation in rats with induced chronic kidney disease

Running Title: Magnesium reduces inflammation in CKD

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ABSTRACT

Background: Inflammation is a common feature in chronic kidney disease (CKD) that appears specifically associated with cardiovascular derangements in CKD patients. Observational studies have revealed a link between low Mg levels and inflammation. In this study, we hypothesize that Mg might have a modulatory effect on the inflammation induced under the uremic milieu.

Methods: *In vivo* studies were performed in a 5/6 nephrectomized rat model of CKD. Furthermore, a possible direct effect of Mg was addressed through *in vitro* studies with vascular smooth muscle cells (VSMCs).

Results: Uremic rats fed a normal (0.1%) Mg diet showed a systemic inflammatory response evidenced by the elevation in plasma of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6, and GPx activity, a marker of oxidative stress. Importantly, an increased expression of these cytokines in the aortic tissue was also observed. In contrast, a dietary Mg supplementation (0.6%), greatly prevented the oxidative stress and the pro-inflammatory response. *In vitro*, in VSMCs cultured in a pro-inflammatory high-phosphate medium, incubation with Mg 1.6 mM inhibited the increase in the production of ROS, the rise in the expression of TNF- α , IL-1 β , IL-6 and IL-8 and the activation of NF- κ B signaling that was observed in cells incubated with a normal (0.8 mM) Mg.

Conclusion: Mg supplementation reduced inflammation associated to CKD; exerting a direct effect on vascular cells. These findings support a possible beneficial effect of Mg supplementation along the clinical management of CKD patients.

Key words: Chronic kidney disease, Inflammation, Magnesium, Phosphate, Uremia, Vascular smooth muscle cells.

ABBREVIATIONS

Ca, calcium;

CKD, Chronic kidney disease;

CTR, Calcitriol;

CVD, Cardiovascular disease;

DMEM, Dulbecco's Modified Eagle Medium;

DTT: Dithiothreitol;

eGFR, estimated Glomerular filtration rate;

FBS, Fetal bovine serum;

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase;

GPx: Glutathione peroxidase;

H₂DCFDA, 2',7'-Dichlorofluorescein diacetate;

HP, high phosphate;

IMIBIC, Instituto Maimónides de Investigación Biomédica de Córdoba;

IL-1 β , IL-6, IL-8, interleukins 1 β , 6, 8, respectively;

NF- κ B p65, Nuclear factor kappa-light-chain-enhancer of activated B cells protein 65; NF-

κ B, Nuclear factor kappa-light-chain-enhancer of activated B cells;

Nx, nephrectomy/ nephrectomized;

P, phosphate;

PMSF: Phenylmethylsulfonyl fluoride;

ROS, reactive oxygen/nitrogen species;

TFIIB, Transcription Factor II B;

TNF- α , Tumor necrosis factor alpha;

VSMCs, vascular smooth muscle cells;

INTRODUCTION

Observational studies have revealed a link between low Mg levels, inflammation and a number of pathological derangements (1-5). Experimental animals with Mg deficiency showed systemic inflammation with increased levels of inflammatory markers as cytokines and acute phase proteins (6). Conversely, Mg repletion reduced inflammatory markers (7). Oxidative stress, has been also constantly reported in Mg-deficient animals (8). In fact, there has been established a mechanistic relationship between inflammation and oxidative stress. Overproduction of reactive oxygen species (ROS) may up-regulates pro-inflammatory mediators, such as NF- κ B (9), whereas, inflammatory cytokines may also induce oxidative stress, which in turn enhances the inflammatory process (10,11). There are many epidemiologic, clinical and experimental data supporting a key role of Mg in cardiovascular pathology (1,3,5,12,13), which might largely take place through the inflammatory process (1,2,14,15). Importantly, Mg appears to exert a direct modulation of the vascular function by acting on both, endothelial and VSMCs. Hypomagnesemia has been associated with endothelial dysfunction (16) and vascular calcification (4), which contributes to atherosclerosis by favoring the establishment of a pro-inflammatory and pro-atherogenic setting (17); while high dietary Mg and moderate hypermagnesemia has a protective effect (12,18,19).

Chronic systemic inflammation and oxidative stress are common features in CKD (20-22) and have also been associated specifically to the progression of cardiovascular disease, the major cause of morbidity and mortality in CKD patients (23,24). Of note, low Mg appeared independently associated with incidence and progression of CKD (25) and estimated glomerular filtration rate (eGFR) decline in the general population (26), and was shown to promote vascular calcification (27,28). Our previous study demonstrated that dietary Mg supplementation reduced both vascular calcification and mortality in uremic rats in a way independent from its phosphate binder properties (29). We also showed that treatment of human umbilical vein endothelial cells (HUVECs) with Mg prevented the increased expression of BMP-2 and p65-NF- κ B induced by TNF- α , providing a mechanism whereby Mg may protect against inflammation and vascular calcification.

In this study, we tested the hypothesis that the protective effect of Mg supplementation under the uremic milieu might be related to its anti-inflammatory properties. Thus, the specific objectives were: (a) to evaluate whether a dietary Mg supplementation would be able to reduce the systemic inflammation induced along the uremia in rats; and, (b) to investigate a possible direct effect of Mg on the pro-inflammatory response by VSMCs, the other key cell type present at the vascular wall.

MATERIALS AND METHODS

In vivo studies

Animals

Experimental work with animals was carried out at IMIBIC animal research facility. Nine to 10-week-old male healthy Wistar rats weighing 250–300 g, from Charles River (Wilmington, Massachusetts, USA) were used. Before entering the study, rats were given *ad libitum* access to a standard semi-purified diet based on the C1031 diet (Altromin, GmbH, Lage, Germany) prepared to contain Ca 0.8%, P 0.6% and Mg 0.1%. Euthanasia was performed by aortic puncture and exsanguination under general anaesthesia (20 mg/kg thiopental sodium ip). All experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the University of Cordoba, and all rats received humane care in compliance with the National Society for Medical Research and conform to Directive 2010/63/EU.

Experimental design

CKD was induced by 5/6 nephrectomy (5/6 Nx) performed through a two-step procedure as previously described (29). One day after the second kidney surgery, rats were fed a high P diet (0.6% Ca and 1.2 % P) and received calcitriol, 20 ng/48h/rat ip, (Calcijex, Abbott, Madrid, Spain) to control secondary hyperparathyroidism. Rats were randomly divided into groups (n=8-10) which were fed diets with 0.1% or 0.6% Mg content for 14 days. Sham-operated rats receiving a normal diet containing 0.1% Mg were used as controls. All diets were purchased from Altromin (Altromin GmbH, Lage, Germany). After two weeks all rats were sacrificed.

Biochemical Determinations

Blood for biochemical analyses was obtained from the abdominal aorta at the time of sacrifice. Blood for measurements of ionized calcium (iCa) levels was collected in heparinized syringes and measured immediately in a Ciba- Corning 634 ISE Ca²⁺/pH

analyzer (Ciba- Corning, Essex, UK). Plasma was separated by centrifugation and stored at -20° C until further analysis. Plasma creatinine, magnesium and phosphate were measured by spectrophotometry (BioSystems SA, Barcelona, Spain).

Determination of cytokines

Plasma cytokines (IL-1 β , IL-6 and TNF- α) were measured by using the Bio-Plex Pro™ Rat Single-plex Kits (Ref. 171L1008M, 171L1012M, 171L1025M, respectively; BioRad Laboratories, Hercules, CA, USA), following the manufacturer's instructions. Data analysis was performed using the Bio-Plex® 200 system.

Histological analysis

Microscopic evaluation of the kidneys and thoracic aorta was performed in tissue sections (3 μ m) fixed with 4% paraformaldehyde solution and embedded in paraffin. Renal sections were stained with H&E, Masson Trichrome and periodic acid Schiff (PAS). For aorta sections, hematoxylin-eosin staining was performed. Inflammation, fibrosis, necrosis, calcification and vascular damage were evaluated by an anatomic pathologist and a semiquantitative assessment was performed using a scale range of intensity from 0 to 4, being 0: normal; 1: low; 2: mild; 3: moderate and 4: severe.

Real-Time polymerase chain reaction (RT-PCR)

Total RNA was extracted with TRIzol reagent (Sigma–Aldrich, St. Louis, MO, U.S.A.) and treated with DNase (DNase kit, St. Louis, MO, U.S.A.). cDNA was synthesized with iScript Retrotranscription Kit (Bio–Rad, Hercules, CA, U.S.A.). Real time-quantitative PCR was performed with SensiFast™ No-ROX SYBR kit (Bioline Reagents, Taunton, MA, U.S.A.) according to the manufacturer's protocol in a Light cycler 480 thermal system (LC480, Roche Diagnostic, Indianapolis, IN, U.S.A.). The expression of target genes was normalized to the expression of glyceraldehyde-3-phosphate

dehydrogenase (GAPDH). Each data point was normalized to corresponding control values in data presentation. The primers for PCR amplification are indicated in Table 1.

Evaluation of Glutathione peroxidase (GPx) activity in plasma

It was measured according to the method by Flohé and Gunzler (1984) (30), which is based on the capacity of glutathione peroxidase to block free radical generation by *tert*-butylhydroperoxide.

In vitro studies

VSMCs culture and experimental design

Human aortic smooth muscle cells were obtained from Clonetics (Lonza Walkersville, Inc., USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS) (20%) (Bio Whittaker; Verviers, Belgium), Na pyruvate (1 mM), glutamine (4.5 g/L), penicillin (100 U/mL), streptomycin (100 mg/mL), and HEPES (20 mM) (all reagents from Sigma Aldrich Inc; MO, USA) at 37°C in a humidified atmosphere with 5% CO₂. Cells were used after the 5th passage. After reaching 80-90% confluence, cells were incubated in a high phosphate medium that contained Na₂HPO₄³⁻ and NaH₂PO₄³⁻ salts 1:2 to obtain a final phosphate concentration of 3.3 mM and a normal (0.8 mM) Mg concentration or supplemented with Mg 1.6 mM by the addition of MgCl₂. Cells incubated in normal phosphate (0.9 mM) and Mg (0.8 mM) medium were used as controls. After 9 days of culture and treatment, total RNA from VSMCs was extracted as described earlier.

Determination of cytokines

Cytokines released by VSMCs were evaluated in the culture medium. After the experimental period (9 days) was finished, the medium was recovered and stored at -80°C until measurement by using the Bio-Plex Pro Human Cytokine 4-Plex system (BioRad Laboratories, Hercules, CA, USA), following the manufacturer's instructions.

Western blot analysis

To obtain cytosolic and nuclear fractions, treated cells were incubated for 15 min at 4°C in low ionic strength lysis buffer A, pH 7.9, containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 70 mg/ml protease inhibitor cocktail, 0.5% Igepal CA-630 (Sigma–Aldrich, St. Louis, MO, USA). The suspension was centrifuged at 13000×g for 3 min at 4°C and supernatant (cytosolic fraction) was stored. Nuclear extracts were obtained by incubating the remaining nuclear pellets on ice in high ionic strength lysis buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 M DTT, 1 mM PMSF, 46 mg/ml protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA) for 15 min, with repeated vortexing and centrifuged at 14000×g for 5 min at 4°C, and then supernatants (nuclear fractions) were collected and stored at –80°C until use. Equal amounts of protein from nuclear extracts were loaded and electrophoresed on SDS/PAGE (10% gel) and subsequently transferred to a nitrocellulose membrane (Invitrogen, CA, U.S.A.). The membranes were blocked in TTBS with 5% non-fat dry milk powder (Bio–Rad) 1 h at room temperature and incubated overnight at 4°C with p65-NF-κβ antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and a transcription factor II B (TFIIB) antibody (1:1000, Cell Signalling, Danvers, MA, USA). Blots were immunolabeled using a horseradish peroxidase-conjugated secondary antibody and developed on autoradiographic film using the ECL Advance Western blotting detection system (Amersham biosciences, UK, Ltd., Little Chalfont, England). Specific bands were quantified by densitometric analyses (by measurement of the integrated optical) with Image J software (National Institutes of Health, Bethesda, MA, USA). The band density for the target protein in each sample was normalized to TFIIB levels.

Confocal microscopy analysis

Cells were seeded on coverslips, and after reaching 90% confluence, they received the different treatments for 24 h. Then, they were rinsed in PBS, fixed and permeated in cold 50% methanol for 2 min, cold 100% methanol for 20 min, and cold 50% methanol for 2 min. Then, cells were washed in PBS (3x5 min) and incubated for 1 h with anti-p65-

NF κ B (1:150, Ref. SC-8008, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking solution (1% BSA) at room temperature. After being washed with PBS (3x5 min), cells were incubated for 1 h with Alexa Fluor 488 F(ab')₂ fragment of rabbit anti-mouse IgG (1:5000; Invitrogen, Ref A-21204, Paisley, UK) in PBS containing 1% BSA. After a final wash with PBS (3x5 min), DAPI (Invitrogen, CA, USA) was used for nuclear stain. Finally, the glass coverslips were mounted on slides to examine fluorescence. Pictures were obtained at 40x in Axio Observer Z1 (LSM710 Exciter Zeiss). Image J software (NIH, USA) was used to analyze confocal immunofluorescence staining. A ROI analysis was performed to quantifying fluorescence intensity in the nuclei, being compared with the blue-DAPI pixels identifying the nuclear region.

Analysis of ROS production, proliferation and cell death

VSMCs were plated at a density of 10000 cells/cm² in black 96-well plates with clear bottom. When the cell confluence reached 90%, they received the corresponding experimental treatments for 24 hours. The production of intracellular ROS was determined with the probe 2-,7-dichlorodihydrofluorescein diacetate (H₂DCFDA) at a concentration of 10 μ M. This non-fluorescent probe becomes fluorescent in the presence of a variety of ROS. After treatments, the medium was removed and the cells were washed twice with serum-free DMEM. The probe was diluted in serum-free DMEM and added to cells for 30 min. Cells were then washed with saline and HBSS. Finally, 100 μ l of HBSS with 2% FBS was added to the cells. Fluorescence was measured immediately at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using infinite F200PRO Tecan Microplate Reader using i-control software for data acquisition (Tecan Trading AG, Männedorf, Switzerland). In order to obtain a representative signal into the well, the reading of the plate in the fluorimeter was performed in an 'acquisition mode 3 \times 3', covering a wider surface of each well of M96 plate. Fluorescence units (F.U.) were normalized based on protein amount measured by the Bradford assay. Proliferation rate was measured through the WST-1 assay (Roche Diagnostic GmbH, Mannheim, Germany) following the manufacturer instructions. After treatments, WST-1 reagent was added to each well and after 1h of incubation, the absorbance was measured at 450 nm using the microplate (ELISA) reader. Cell death was evaluated by using a LDH cytotoxicity assay kit

(Canvax Biotech, Córdoba, Spain) following the manufacturer instructions. Briefly, after treatments, 50µl of culture supernatant from each well was transferred to a new 96-well plate and 50µl of reaction mixture from kit was added. The plate was incubated in the dark at room temperature for 30 min. Then, 50µl of stop solution was added and the absorbance was measured at 450 nm using the microplate (ELISA) reader.

Statistical analysis

Results are expressed as mean \pm SEM. The difference between means was assessed by One-way analysis of variance (ANOVA) followed by *post hoc* Duncan analysis. A two-sided P-value of less than 0.05 was considered statistically significant. These analyses were performed with SPSS 20.0 software (Chicago, IL, USA).

Reporting of the study conforms to broad EQUATOR guidelines.

RESULTS

In vivo experiments

Blood chemistry of experimental groups is shown in Table 2. As expected, there was a marked increase of creatinine in the Nx rats fed a normal-Mg diet, in comparison with the control (sham-operated) rats; however, this increase was partially prevented in the rats receiving Mg 0.6%. Similarly, nephrectomized rats had higher serum P level; but those fed Mg 0.6% showed lower values than the Nx-Mg 0.1% group. The serum Mg was increased in the Nx rats but it was significantly greater in the group receiving Mg 0.6% than in the Nx-Mg 0.1%. The serum ionized calcium was reduced in Nx rats but the levels were lower in rats fed the 0.1% Mg diet.

Histological examination of kidneys (Figure 1) revealed that Nx-Mg 0.1% animals showed both renal tubular and glomerular lesions, inflammation and increased interstitial cellularity. Tubular cell toxicity, inflammatory reaction, fibrosis and calcification were also more prominent in the Nx-Mg 0.1% than in the Nx-Mg 0.6% group. In the Nx-Mg 0.1% rats, inflammatory findings were present, mainly at the periglomerular level and the intertubular zone, where mixed inflammation (lymphocytes and neutrophils) could be observed. This inflammation appeared reduced in Nx-Mg 0.6% rats compared to the Nx-Mg 0.1% ones, which is probably related to the greater severity of the tubular necrosis and calcification changes found in this last group. Similarly, necrosis and vascular damage were also reduced after dietary Mg supplementation.

Dietary Mg supplementation reduced pro-inflammatory cytokine production and oxidative stress induced in rats with CKD

As shown in Figure 2, plasma level of pro-inflammatory cytokines, namely TNF- α , IL-1 β and IL-6, was markedly increased in the nephrectomized rats fed the normal Mg 0.1% diet in comparison with the control (sham-operated) rats, as assessed by bio-plex analysis. However, though an increase was also observed in rats receiving the Mg 0.6% diet, it was significantly lower than that of the Nx- Mg 0.1% rats. Since oxidative stress is related to CKD (22,31), we also evaluated this process in the study groups. It was performed by measuring in plasma the activity of the antioxidant enzyme GPx, which is

well-known to be induced to counteract the increase in the levels of ROS. As shown in Figure 2D, GPx activity was greatly increased in Nx rats on the Mg 0.1%, as compared to Sham-operated rats. However, this increase was largely prevented in Nx rats on the Mg 0.6%. Therefore, Mg supplementation appeared to alleviate the increase of pro-inflammatory cytokines as well as the induction of oxidative stress related to the development of the renal insufficiency.

Dietary Mg supplementation reduced pro-inflammatory cytokine expression in the aortic tissue and the histological injury induced in rats with CKD.

Next, we looked specifically at the aortic tissue (Figure 3). The pattern of expression of all the studied cytokines at the mRNA level mirrored that observed at the protein level in plasma (Figure 3A). Furthermore, it was histologically showed that dietary Mg supplementation (Mg 0,6%) reduced significantly aorta inflammation, vascular damage and dilatation observed in the Nx- Mg 0.1% rats (Figure 3B-F). In aortas from the Sham group a conserved parenchymal structure without inflammation, necrosis or fibrosis was observed. As we had previously demonstrated (29), a reduction in vascular calcification was also observed in the Nx- Mg 0.6% rats compared to the Nx- Mg 0.1% group. Therefore, Mg supplementation reduced specifically the expression of pro-inflammatory cytokines and vascular damage in aortic tissues.

In vitro experiments

Treatment with Mg prevented HP-induced increase in pro-inflammatory cytokines in VSMCs

Experiments were performed with a model of VSMCs cultured in a medium with high phosphate (HP), which is as a direct uremia-related pro-inflammatory factor (32). As expected, cells cultured in a high phosphate (HP) concentration (3.3 mM) with a normal Mg concentration (0.8 mM) showed an increased release of inflammatory mediators such as IL-1 β , IL-6, IL-8 and TNF- α as compared to controls (Figure 4A). The addition of Mg 1.6 mM to the HP medium totally prevented the increase in the cytokine production,

resulting in values similar to the control. The expression of cytokines at the mRNA level showed a similar pattern to that described for the protein level: in the HP-Mg 0.8 mM medium there was a marked increase of all cytokines but this was completely inhibited by a Mg of 1.6 mM in the HP- medium (Figure 4B).

Treatment with Mg prevented HP-induced activation of NF- κ B signaling pathway in VSMCs

Since we had previously shown in VSMCs that a HP medium was able to induce the secretion of inflammatory cytokines through the NF- κ B signaling system (32), here we evaluated whether it would be modulated by Mg. Western blotting analysis revealed that, as compared to control cells, in cells cultured in the HP-Mg 0.8 mM medium there was an increase in the nuclear content of p65-NF- κ B medium (Figure 5A). However, the addition of Mg 1.6 mM to the HP medium prevented the nuclear translocation of p65-NF- κ B. These results were confirmed through immunofluorescence staining. As shown in Figure 5B, control cells had scattered signal of p65-NF- κ B in the cytoplasm while in HP-Mg 0.8 mM-treated cells there was an enhanced nuclear staining of p65-NF- κ B. Analysis of green fluorescence intensity in all samples also corroborated the inhibition of the nuclear translocation of p65-NF- κ B induced by the addition of Mg 1.6 mM to the HP medium.

Treatment with Mg prevented HP-induced increase in ROS production, proliferation and cell death in VSMCs

ROS production was measured through fluorescence by using the H₂DCFDA probe. As shown in Figure 6A, fluorescence intensity was significantly increased in cells cultured in in the HP-Mg 0.8 mM medium as compared with control cells. Conversely, when the cells were cultured in the HP+Mg 1.6 mM medium, the high level of H₂DCFDA fluorescence induced by high phosphate was significantly reduced. In the cells cultivated in the HP with a normal (0.8 mM) Mg concentration there was also a significant increase in the proliferation rate measured through the WST-1 assay, which was prevented when Mg 1.6 mM was added to the cultures (Figure 6B). Furthermore, as demonstrated by the LDH

assay, cell death was induced in cells cultured in in the HP-Mg 0.8 mM medium but it was significantly inhibited in the cells cultured in the medium containing Mg 1.6 mM (Figure 6C).

DISCUSSION

This study aimed at assessing a possible modulatory effect of Mg on the inflammatory processes associated with CKD. The *in vivo* studies in uremic rats showed certainly that a dietary Mg supplementation reduced the systemic inflammation. To assess a direct effect of Mg, additional *in vitro* studies were performed where the addition of Mg inhibited the pro-inflammatory response induced by high phosphate in VSMCs, acting on the NF- κ B signaling pathway.

As in our previous work (29), here we used an *in vivo* model of renal insufficiency (a 5/6 Nx plus a high phosphate diet) that resembles the mineral abnormalities associated with CKD, and rats received a diet containing either a normal (0.1%) or a high (0.6%) Mg content. Though a content of 0.05% has been extensively used as normal in rat experimental studies, a slightly higher dietary concentration might be required if the experimental conditions might affect the absorption of Mg. A high dietary phosphate content can reduce the absorption and/or availability of Mg and a decreased magnesium absorption is often found in renal disease (33). Similarly to our conditions, contents of 0.1% or around has been also established as the normal levels in many studies addressing dietary Mg supplementation, in which a 0.6% Mg was also given as a high-Mg diet (34-37). Our results showed that in the uremic rats fed a normal Mg (0.1%) a systemic inflammatory response was induced, as deduced from the elevation in the plasma of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6, which are related to acute phase responses and inflammation. Furthermore, this was also accompanied by increased plasma GPx activity, a well-known marker of the occurrence of oxidative stress.

Magnesium is absorbed in the gut and excess magnesium is excreted by the kidneys and the feces. The kidney plays a principal role in Mg balance, as serum magnesium levels are mostly controlled by its excretion in urine. In the uremic setting, as renal function further deteriorates, the renal capacity for Mg elimination is progressively reduced to reach a point in that it may be insufficient to balance intestinal Mg absorption (38,39) and dietary Mg intake becomes a major determinant of the serum levels (39). In our study the dietary Mg supplementation (0.6%)-produced a mild but statistically significant increase in the serum levels of Mg in comparison to that in the animals receiving the normal 0.1% Mg diet.

And importantly, Mg supplementation greatly prevented the induction of oxidative stress and the pro-inflammatory response elicited under the uremic conditions. Interestingly, some physiological alterations observed in CKD, such as the increased ROS production and the release of inflammatory cytokines, resembles those related to Mg deficiency. Indeed, the same array of pro-inflammatory cytokines that we observed were elevated in normal rats under Mg deficiency (6), while a Mg repletion therapy promoted an anti-inflammatory response and alleviated endothelial dysfunction and inflammation (7). Our data also showed that the elevation in the plasma levels of the pro-inflammatory cytokines was associated with its increased expression at the aortic tissue. And, notably, it was also down-regulated by the Mg dietary supplementation. Though many organs and cells may have a role in the induction of a systemic inflammatory response, these findings point to the vascular wall as a key actor. Moreover, it is likely that this specific vascular inflammatory pattern may have a specific role in the development of the vascular alteration related to renal insufficiency. Indeed, our histological evaluation showed that Mg supplementation greatly reduced the vascular damage observed in the aortic tissues of the Nx- Mg 0.1% rats. In the same *in vivo* model of CKD we had demonstrated that a 0.6% Mg dietary supplementation prevented the development of vascular calcification in a phosphate binder-independent way (29).

In our nephrectomized rats, dietary supplementation of Mg resulted the amelioration of uremia, which was also associated with a marked amelioration in the histological signs of kidney injury. This protective effect of Mg had been already observed by us in the same model (29). A moderate hypermagnesemia seems to have beneficial effects on mortality in CKD patients (40), whereas low Mg is independently associated with incidence and progression of CKD (25) and with eGFR decline in the general population (26). Indeed, Mg may act specifically to protect against high phosphate-induced kidney injury (25). Interestingly, increasing Mg concentrations directly suppressed cell damage and a pro-inflammatory response in tubular epithelial cells incubated in a high phosphate medium (25). Moreover, Mg has been proved as an active phosphate binder (41), so that dietary Mg may halt the progression of renal disease through a reduction in serum phosphorus levels. Reduction in phosphate also associates to increase in serum calcium by favoring availability and absorption of calcium and in by increasing the calcemic effect of PTH (42).

In addition, Mg might also improve the uremia by preventing the high phosphate-induced vascular and soft-tissue calcification (28, 29) that likely contribute to worsening of renal failure. Therefore, it cannot be excluded the possibility that in our study the partial protection of the kidney function would account, to some extent, for the reduction of inflammation. However, even assuming this association, and since there is a trade-off model relationship between inflammation and renal function, it is difficult to discern which of these processes might have been affected by supplementary Mg the first. While future studies are required in order to elucidate this point, in this study we further investigated whether the observed anti-inflammatory effect of Mg might be directly related to the modulation of inflammation at the vascular setting. It was addressed through the *in vitro* studies

The modulation of the endothelial inflammatory response by Mg has been largely addressed already (1,7,43,44). Indeed, in a previous study we had shown that treatment with Mg prevented the increased expression of BMP-2 and p65-NF- κ B induced by TNF- α in HUVECs, providing a mechanism whereby Mg may protect against inflammation and vascular calcification (29). On the other hand, different stimuli can lead VSMCs to increase the production of pro-inflammatory cytokines and ROS (45,46), which prompted us to explore a possible independent effect of Mg on this other key cell type present at the vascular wall. Since our *in vivo* model of CKD included a high phosphate (HP) intake, which led to a high serum phosphate (an undoubted hallmark of uremia) and we had previously found that a HP has an independent direct effect as a pro-inflammatory factor in an *in vitro* model of VSMCs (32), in the present work we used this same model to address the role of Mg. The results showed that, as compared to cells incubated in a normal (0.8 mM) Mg concentration, the addition to cultures of Mg 1.6 mM totally abolished the HP-induced increase in the production of ROS and the rise of the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 and IL-8, as measured at both the protein and mRNA level.

NF- κ B plays a major role in inducing the expression of inflammatory cytokines in VSMCs (32,47) and Mg inhibited endotoxin-induced up-regulation of inflammatory molecules and NF- κ B activation in activated RAW264.7 cells (48). Here, we also showed that the preventive effect of Mg 1.6 mM against inflammation was associated with the

down-regulation of NF- κ B signaling through inhibiting the nuclear translocation of p65-NF- κ B. Since we had previously stated in the same model that the inflammatory response by VSMCs was up-stream mediated by the ROS-induced activation of NF- κ B (32), it might likely be assumed that the inhibitory effect exerted by Mg 1.6 mM on the oxidative stress could also underlie its modulatory action on the NF- κ B-dependent inflammatory response. Previous studies aimed at addressing mechanistic insights on the protective effect of Mg on vascular cells gave relevance to the properties of Mg as a calcium antagonist (1), so that calcium action is susceptible of being modified by the Mg supplement. A rise in the intracellular calcium can activate transduction pathways leading to an increase in oxidative stress, the activation of the NF- κ B signaling and the production of pro-inflammatory cytokines (49,50). Thus, as a physiological inhibitor of calcium channels, Mg might prevent calcium flux and therefore, the inflammatory response.

VSMCs can display pathological phenotypes, allowing them to dedifferentiate, proliferate and migrate. Uremia is able to induce VSMCs apoptosis and an osteoblastic-like phenotype leading to vascular calcification (51). Oxidative stress, inflammation and increased phosphate levels are major contributors to this phenotypic switch through the Wnt-Beta catenin and NF- κ B signaling (45,51,52). Notably, in the same *in vitro* model used in this study, we had previously demonstrated that high phosphate induces Wnt-Beta catenin-dependent calcification and a ROS/NF- κ B -dependent production of pro-calcific cytokines (28, 32,53). Now, we show that this is accompanied by the induction of proliferation and cell death and both were inhibited by the addition of higher amounts of Mg. Cell proliferation is a major hallmark of VSMC phenotypic switch, with ROS being key promoting factors (51,54). Thus, it is not surprising to find such an induction in our cells exposed to the HP medium and its prevention by Mg is likely to occur through the inhibition of ROS and NF- κ B activation. Furthermore, the Wnt-Beta catenin could also play a role, since we had demonstrated in the same model that Mg inhibits Wnt-Beta catenin activity and reverses the osteogenic transformation (28). Moreover, ROS and pro-inflammatory cytokines were shown to induce VSMCs apoptosis (51,52, 54), which may explain our finding of increased cell death in the HP medium and the ameliorating effect of the addition of high Mg. In agreement with our results, simultaneous activation of apoptosis and proliferation signaling was observed in VSMCs following high phosphate stimulation

(55). Similarly, a recent study in high fructose treated VSMCs showed increased levels of inflammation, oxidative stress, proliferation and apoptosis mediated by the activation of NF- κ B (56).

Our results support previous findings on the beneficial effects of Mg in the modulation of a systemic inflammatory response, and specifically extend it to that related to renal insufficiency. But they go even beyond by showing a specific direct effect on the vascular wall. This is of special interest since the vasculature appears as a preponderant inflammatory target under the uremic setting, which might be tightly linked to the development of vascular pathologies associated with CKD as atherosclerosis. Accumulating evidence indicates a significant relationship between Mg and the establishment of atherosclerosis and other CVD (5,15,57) and Mg supplementation was associated with their suppression or prevention (5,16,7,58); a moderate hypermagnesemia having a protective effect (12,18,19). Prior studies highlighted the involvement of Mg in the endothelial dysfunction (15,16,7,43). Here we show an important contribution of the VSMCs as direct targets of Mg to protect the vascular system from inflammation. Thus, the acknowledged protective role of Mg at the vascular level might be at least partially explained by its anti-inflammatory effect exerted on the VSMCs. This also supports the opportuneness of doing the necessary efforts to restore serum Mg levels in patients with hypomagnesemia in order to correct and or prevent the panoply of possible derived pathologies. In particular, it has been claimed the benefit of Mg repletion in patients suffering from cardiovascular diseases (16,18). Therefore, beyond its phosphate-binder action, and even though it must be kept in mind that too much magnesium could have adverse effects, e.g. on bone metabolism (59), Mg repletion and/or supplementation may arise as a valuable therapeutic option to reduce the cardiovascular alterations in CKD patients.

Growing evidence suggests that the Ca/Mg intake ratio can modify significantly the individual associations of dietary Ca and Mg with the risk of total mortality and that due to CVD and cancer, so that a higher Mg could even associate with an increased risk (60-64). Thus, a Ca/Mg ratio between 1.70 and 2.6 may be required for high intakes of Ca and Mg to be protective (62). Interestingly, a recent *in vitro* study using the CT26 colon cancer cell

line showed that with Ca/Mg ratios >1 , Mg deficiency resulted in oxidative stress and NF- κ B p65 activation, while this was not observed with a Ca/Mg ratio of 1 (50). Though a possible modifying effect of the Ca/Mg intake ratio cannot be totally discarded in our *in vivo* model, it would not likely be much relevant as it is usually important when magnesium or calcium are provided in deficient or barely adequate amounts, which is not the case. If so, further studies would be deserved to elucidate it.

In summary, our *in vivo* studies showed that a dietary Mg supplementation greatly ameliorated the systemic inflammation and oxidative stress induced in a rat model of CKD and specifically reduced the expression of inflammatory cytokines at the vascular wall. Furthermore, in cultured VSMCs, the addition of Mg 1.6 mM inhibited the high phosphate-induced increase in oxidative stress, the production of inflammatory cytokines and the activation of NF- κ B signaling that was observed in cells incubated with a normal (0.8 mM) Mg concentration. These findings deliver novel mechanistic insights into the specific cell mediated processes underlying the inflammation associated to CKD and the protective role of Mg

In conclusion, Mg supplementation reduced inflammation associated to CKD; exerting a direct effect on vascular cells. Our data suggests an important contribution of the VSMCs as direct targets of Mg to protect the vascular system from inflammation under the uremic setting. These findings sustain the importance of controlling Mg levels and support a possible beneficial effect of Mg supplementation along the clinical management of CKD patients, which show a poor cardiovascular outcome.

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CONFLICT OF INTEREST

MR has received honoraria for lectures from Amgen, Kiowa Kirin, Sanofi and Viphor. All the other authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS

M. Rodríguez, A. Canalejo, J. R. Muñoz-Castañeda and Y. Almadén equally contributed to the study concept, design and the interpretation of the data; R. López-Baltanás, M. E. Rodríguez-Ortiz, C. Herencia, F. Leiva-Cepas, J. M. Díaz-Tocados, J. D. Torres-Peña and A. Ortiz-Morales performed research, collected and compiled data; R. López-Baltanás, M. E. Rodríguez-Ortiz, A. Canalejo and Y. Almadén analyzed and interpreted the data; A. Canalejo and Y.A. wrote the paper; M. Rodríguez and J. M. Muñoz-Castañeda reviewed the paper; M. Rodríguez, J.M. Muñoz-Castañeda, A. Canalejo and Y. Almadén had primary responsibility for final content. All authors read and approved the final manuscript.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Mazur A, Maier JA, Rock E, Gueux E, Nowacki W, Rayssiguier Y. Magnesium and the inflammatory response: potential physiopathological implications. *Arch Biochem Biophys*. 2007;458:48-56.
2. Rodriguez-Morán M, Guerrero-Romero F. Elevated concentrations of TNF-alpha are related to low serum magnesium levels in obese subjects. *Magnes Res*. 2004;17:189-196.
3. Ma J, Folsom AR, Melnick SL, et al. Associations of serum and dietary magnesium with cardiovascular disease, hypertension, diabetes, insulin, and carotid arterial wall thickness: the ARIC study. Atherosclerosis Risk in Communities Study. *J Clin Epidemiol*. 1995;48:927-940.
4. Ishimura E, Okuno S, Kitatani K, et al. Significant association between the presence of peripheral vascular calcification and lower serum magnesium in hemodialysis patients. *Clin Nephrol*. 2007;68:222-227.
5. Altura BM, Altura BT. Magnesium and cardiovascular biology: an important link between cardiovascular risk factors and atherogenesis. *Cell Mol Biol Res*. 1995;41:347-359.
6. Malpuech-Brugère C, Nowacki W, Daveau M, et al. Inflammatory response following acute magnesium deficiency in the rat. *Biochim Biophys Acta*. 2000;1501:91-98.
7. Kharitonova M, Iezhitsa I, Zheltova A, Ozerov A, Spasov A, Skalny A. Comparative angioprotective effects of magnesium compounds. *J Trace Elem Med Biol*. 2015;29:227-234.

8. Zheltova AA, Kharitonova MV, Iezhitsa IN, Spasov AA. Magnesium deficiency and oxidative stress: an update. *Biomedicine (Taipei)*. 2016;6:20.
9. Pashkow FJ, Watumull DG, Campbell CL. Astaxanthin: a novel potential treatment for oxidative stress and inflammation in cardiovascular disease. *Am J Cardiol*. 2008;101:58D-68D.
10. Sprague AH, Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochem Pharmacol*. 2009;78:539-552.
11. Schleicher E, Friess U. Oxidative stress, AGE, and atherosclerosis. *Kidney Int Suppl*. 2007;(106):S17-S26.
12. Helfand M, Buckley DI, Freeman M, et al. Emerging risk factors for coronary heart disease: a summary of systematic reviews conducted for the U.S. Preventive Services Task Force. *Ann Intern Med*. 2009;151:496-507.
13. Qu X, Jin F, Hao Y, et al. Magnesium and the risk of cardiovascular events: a meta-analysis of prospective cohort studies. *PLoS One*. 2013;8:e57720.
14. Peacock JM, Ohira T, Post W, Sotoodehnia N, Rosamond W, Folsom AR. Serum magnesium and risk of sudden cardiac death in the Atherosclerosis Risk in Communities (ARIC) Study. *Am Heart J*. 2010;160:464-470.
15. Maier JA. Low magnesium and atherosclerosis: an evidence-based link. *Mol Aspects Med*. 2003;24:137-146.
16. Del Gobbo LC, Imamura F, Wu JH, de Oliveira Otto MC, Chiuve SE, Mozaffarian D. Circulating and dietary magnesium and risk of cardiovascular disease: a systematic review and meta-analysis of prospective studies. *Am J Clin Nutr*. 2013;98:160-173.
17. Van Laecke S, Van Biesen W, Vanholder R. Hypomagnesaemia, the kidney and the vessels. *Nephrol Dial Transplant*. 2012;27:4003-4010.

18. DiNicolantonio JJ, Liu J, O'Keefe JH. Magnesium for the prevention and treatment of cardiovascular disease. *Open Heart*. 2018;5:e000775.
19. Mortazavi M, Moeinzadeh F, Saadatnia M, Shahidi S, McGee JC, Minagar A. Effect of magnesium supplementation on carotid intima-media thickness and flow-mediated dilatation among hemodialysis patients: a double-blind, randomized, placebo-controlled trial. *Eur Neurol*. 2013;69:309-316.
20. Stenvinkel P, Ketteler M, Johnson RJ, et al. IL-10, IL-6, and TNF-alpha: central factors in the altered cytokine network of uremia--the good, the bad, and the ugly. *Kidney Int*. 2005;67:1216-1233.
21. Ward RA, McLeish KR. Polymorphonuclear leukocyte oxidative burst is enhanced in patients with chronic renal insufficiency. *J Am Soc Nephrol*. 1995;5:1697-1702.
22. Agharazii M, St-Louis R, Gautier-Bastien A, et al. Inflammatory cytokines and reactive oxygen species as mediators of chronic kidney disease-related vascular calcification. *Am J Hypertens*. 2015;28:746-755.
23. Silverstein DM. Inflammation in chronic kidney disease: role in the progression of renal and cardiovascular disease. *Pediatr Nephrol*. 2009;24:1445-1452.
24. Navarro-González JF, Mora-Fernández C, Muros M, Herrera H, García J. Mineral metabolism and inflammation in chronic kidney disease patients: a cross-sectional study. *Clin J Am Soc Nephrol*. 2009;4:1646-1654.
25. Sakaguchi Y, Iwatani H, Hamano T, et al. Magnesium modifies the association between serum phosphate and the risk of progression to end-stage kidney disease in patients with non-diabetic chronic kidney disease. *Kidney Int*. 2015;88:833-842.

26. Ferrè S, Li X, Adams-Huet B, et al. Low serum magnesium is associated with faster decline in kidney function: the Dallas Heart Study experience. *J Investig Med*. 2019;67:987-994.
27. Montezano AC, Zimmerman D, Yusuf H, et al. Vascular smooth muscle cell differentiation to an osteogenic phenotype involves TRPM7 modulation by magnesium. *Hypertension*. 2010;56:453-462.
28. Montes de Oca A, Guerrero F, Martinez-Moreno JM, et al. Magnesium inhibits Wnt/ β -catenin activity and reverses the osteogenic transformation of vascular smooth muscle cells. *PLoS One*. 2014;9:e89525.
29. Diaz-Tocados JM, Peralta-Ramirez A, Rodríguez-Ortiz ME, et al. Dietary magnesium supplementation prevents and reverses vascular and soft tissue calcifications in uremic rats. *Kidney Int*. 2017;92:1084-1099.
30. Flohé L, Günzler WA. Assays of glutathione peroxidase. *Methods Enzymol*. 1984;105:114-121.
31. Dalfino G, Simone S, Porreca S, et al. Bone morphogenetic protein-2 may represent the molecular link between oxidative stress and vascular stiffness in chronic kidney disease. *Atherosclerosis*. 2010;211:418-423.
32. Martínez-Moreno JM, Herencia C, de Oca AM, et al. High phosphate induces a pro-inflammatory response by vascular smooth muscle cells and modulation by vitamin D derivatives. *Clin Sci (Lond)*. 2017;131:1449-1463.
33. Brannan PG, Vergne-Marini P, Pak CY, Hull AR, Fordtran JS. Magnesium absorption in the human small intestine. Results in normal subjects, patients with chronic renal disease, and patients with absorptive hypercalciuria. *J Clin Invest*. 1976;57:1412-1418.

34. Laurant P, Hayoz D, Brunner H, Berthelot A. Dietary magnesium intake can affect mechanical properties of rat carotid artery. *Br J Nutr.* 2000;84:757-764
35. Finckenberg P, Merasto S, Louhelainen M, et al. Magnesium supplementation prevents angiotensin II-induced myocardial damage and CTGF overexpression. *J Hypertens.* 2005;23:375-380.
36. ElZohary L, Weglicki WB, Chmielinska JJ, Kramer JH, Mak IT. Mg-supplementation attenuated lipogenic and oxidative/nitrosative gene expression caused by Combination Antiretroviral Therapy (cART) in HIV-1-transgenic rats. *PLoS One.* 2019;14:e0210107.
37. García-Legorreta A, Soriano-Pérez LA, Flores-Buendía AM, et al. Effect of Dietary Magnesium Content on Intestinal Microbiota of Rats. *Nutrients.* 2020;12:2889.
38. Cunningham J, Rodríguez M, Messa P. Magnesium in chronic kidney disease Stages 3 and 4 and in dialysis patients. *Clin Kidney J.* 2012;5(Suppl 1):i39-i51.
39. Navarro-González JF, Mora-Fernández C, García-Pérez J. Clinical implications of disordered magnesium homeostasis in chronic renal failure and dialysis. *Semin Dial.* 2009;22:37-44.
40. Lacson E Jr, Wang W, Ma L, Passlick-Deetjen J. Serum Magnesium and Mortality in Hemodialysis Patients in the United States: A Cohort Study. *Am J Kidney Dis.* 2015;66:1056-1066.
41. De Francisco AL, Leidig M, Covic AC, et al. Evaluation of calcium acetate/magnesium carbonate as a phosphate binder compared with sevelamer hydrochloride in haemodialysis patients: a controlled randomized study (CALMAG study) assessing efficacy and tolerability. *Nephrol Dial Transplant.* 2010;25:3707–3717.

42. Rodriguez M, Martin-Malo A, Martinez ME, et al. Calcemic response to parathyroid hormone in renal failure: Role of phosphorus and its effect on calcitriol. *Kidney Int.* 1991;40:1055–1062.
43. Kawano H, Yokoyama S, Smith TL, Nishida HI, Taguchi T, Kummerow FA. Effect of magnesium on secretion of platelet-derived growth factor by cultured human umbilical arterial endothelial cells. *Magnes Res.* 1995;8:137-144.
44. Ferrè S, Baldoli E, Leidi M, Maier JA. Magnesium deficiency promotes a pro-atherogenic phenotype in cultured human endothelial cells via activation of NFκB. *Biochim Biophys Acta.* 2010;1802:952-958.
45. Shao JS, Aly ZA, Lai CF, et al. Vascular Bmp Msx2 Wnt signaling and oxidative stress in arterial calcification. *Ann N Y Acad Sci.* 2007;1117:40-50. d
46. Kiyani Y, Tkachuk S, Hilfiker-Kleiner D, Haller H, Fuhrman B, Dumler I. oxLDL induces inflammatory responses in vascular smooth muscle cells via urokinase receptor association with CD36 and TLR4. *J Mol Cell Cardiol.* 2014;66:72-82.
47. Yang X, Coriolan D, Murthy V, Schultz K, Golenbock DT, Beasley D. Proinflammatory phenotype of vascular smooth muscle cells: role of efficient Toll-like receptor 4 signaling. *Am J Physiol Heart Circ Physiol.* 2005;289:H1069-H1076.
48. Lin CY, Tsai PS, Hung YC, Huang CJ. L-type calcium channels are involved in mediating the anti-inflammatory effects of magnesium sulphate. *Br J Anaesth.* 2010;104:44-51.
49. Hao J, Tang J, Zhang L, Li X, Hao L. The Crosstalk between Calcium Ions and Aldosterone Contributes to Inflammation, Apoptosis, and Calcification of VSMC via the AIF-1/NF-κB Pathway in Uremia. *Oxid Med Cell Longev.* 2020; 2020:3431597.

50. Kumar G, Chatterjee PK, Madankumar S, Mehdi SF, Xue X, Metz CN. Magnesium deficiency with high calcium-to-magnesium ratio promotes a metastatic phenotype in the CT26 colon cancer cell line. *Magnes Res.* 2020;33:68-85.
51. Durham AL, Speer MY, Scatena M, Giachelli CM, Shanahan CM. Role of smooth muscle cells in vascular calcification: implications in atherosclerosis and arterial stiffness. *Cardiovasc Res.* 2018;114:590-600.
52. Grootaert MOJ, Moulis M, Roth L, et al. Vascular smooth muscle cell death, autophagy and senescence in atherosclerosis. *Cardiovasc Res.* 2018;114:622-634.
53. Martínez-Moreno JM, Muñoz-Castañeda JR, Herencia C, et al. In vascular smooth muscle cells paricalcitol prevents phosphate-induced Wnt/ β -catenin activation. *Am J Physiol Renal Physiol.* 2012;303:F1136-44.
54. Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension.* 2003;42:1075-81.
55. Xiao Y, Du YY, Gao C, Kong W. [Dynamic alteration of microRNA in high phosphorus induced calcification of vascular smooth muscle cell]. *Beijing Da Xue Xue Bao Yi Xue Ban.* 2016;48:756-765.
56. Kalra J, Mangali S, Bhat A, Jadhav K, Dhar A. Selective inhibition of PKR improves vascular inflammation and remodelling in high fructose treated primary vascular smooth muscle cells. *Biochim Biophys Acta Mol Basis Dis.* 2020;1866:165606.
57. Rodríguez-Ortiz ME, Gómez-Delgado F, Arenas de Larriva AP, et al. Serum Magnesium is associated with Carotid Atherosclerosis in patients with high cardiovascular risk (CORDIOPREV Study). *Sci Rep.* 2019;9:8013.

58. Ravn HB, Korsholm TL, Falk E. Oral magnesium supplementation induces favorable antiatherogenic changes in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol.* 2001;21:858-862.
59. Hamano N, Komaba H, Fukagawa M. Magnesium as a new player in CKD: too little is as bad as too much?. *Kidney Int.* 2017;92:1034-1036.
60. Dai Q, Shrubsole MJ, Ness RM, et al. The relation of magnesium and calcium intakes and a genetic polymorphism in the magnesium transporter to colorectal neoplasia risk. *Am J Clin Nutr* 2007;86:743–751.
61. Dai Q, Sandler R, Barry E, Summers R, Grau M, Baron J. Calcium, magnesium, and colorectal cancer. *Epidemiology.* 2012;23:504-5
62. Costello RB, Rosanoff A, Dai Q, Saldanha LG, Potischman NA. Perspective: Characterization of Dietary Supplements Containing Calcium and Magnesium and Their Respective Ratio-Is a Rising Ratio a Cause for Concern? *Adv Nutr.* 2020 Dec 26:nmaa160. doi: 10.1093/advances/nmaa160. Epub ahead of print.
63. Zhang W, Iso H, Ohira T, et al. Associations of dietary magnesium intake with mortality from cardiovascular disease: the JACC study. *Atherosclerosis* 2012;221:587–595.
64. Dai Q, Shu XO, Deng X, et al. Modifying effect of calcium/magnesium intake ratio and mortality: a population-based cohort study. *BMJ Open.* 2013;3:e002111.

TABLES

Table 1. Primers used for quantitative real-time PCR

Gene	Forward primer	Reverse primer
GAPDH	5'-TGATGACATCAAGAAGGTGGTGAAG-3'	5'-TCCTTGGAGGCCATGTGGGCCAT-3'
IL-1β	5'-CAGGATATGGAGCAACAAGTGGTG-3'	5'-GGGTAATTTTTGGGATCTACACTC-3'
IL-6	5'-GCCCCACACAGACAGCCACTCACC-3'	5'-TGCCTCTTTGCTGCTTTCACACAT-3'
IL-8	5'-GGTGCAGAGGGTTGTGGAGAAGTT-3'	5'-CATGAAGTGTTGAAGTAGATTTGC-3'
TNF-α	5'-CTTGTCCTCAGCCTCTTCTCCTTCC-3'	5'-AAGATGATCTGACTGCCTGGGCCAG-3'

Table 2. Plasma biochemical determinations in the *in vivo* study (means±SEM).

A rat model of CKD including a 5/6 nephrectomy (Nx) plus a high P diet (0.6% Ca and 1.2 % P) and a calcitriol (CTR) treatment was used. Rats were fed a normal (0.1%) Mg diet or received a dietary Mg supplementation (0.6%) and then were evaluated at day 14. Sham-operated rats fed a normal P (0.6% Ca and 0.6 % P) and Mg (0.1%) diet served as controls. Data are means±SEM (n=8/group). ^a p<0.05 vs Control; ^b p<0.05 vs Mg 0.1%.

	Control-Mg 0.1%	Nx5/6-Mg 0.1%	Nx5/6-Mg 0.6%
Creatinine (mg/dl)	0.35±0.02	1.59±0.16 ^a	0.98±0.13 ^{a,b}
Magnesium (mg/dl)	2.12±0.06	3.00±0.07 ^a	3.26±0.08 ^{ab}
Phosphate (mg/dl)	6.73±0.25	12.2±1.50 ^a	7.54±0.36 ^{a,b}
Ionized calcium (mM)	1.22±0.02	1.01±0.03 ^a	1.10±0.03 ^{a,b}

FIGURE LEGENDS

Figure 1. Dietary Mg supplementation reduced kidney injury induced in rats with CKD. A rat model of CKD including a 5/6 nephrectomy (Nx) plus a high P diet and a calcitriol treatment was used. Nx rats were fed a normal (0.1%) Mg diet or received a dietary Mg supplementation (0.6%). Sham-operated rats fed a normal P (0.6% Ca and 0.6% P) and Mg (0.1%) diet served as controls. **A)** Representative H&E, PAS and Masson's trichrome stains of histological samples of kidneys. Arrowhead: calcification; Star: tubular necrosis; Circle: vascular dilatation; Rhombus: glomerular dilatation; Cross: focal inflammation. Arrow: interstitial fibrosis. Original magnification: 100 x. **B)** Semiquantitative assessment of kidney alterations by using a scale range of intensity from 0 to 4 as described in Methods. Bars are means±SEM. ^a p<0.05 vs Control; ^b p<0.05 vs Nx-Mg 0.1%.

Figure 2. Dietary Mg supplementation reduced pro-inflammatory cytokines and glutathione peroxidase (GPx) activity (a marker of systemic oxidative stress) in plasma of rats with CKD. A rat model of CKD including a 5/6 nephrectomy (Nx) plus a high P diet and a calcitriol treatment was used. Nx rats were fed a normal (0.1%) Mg diet or received a dietary Mg supplementation (0.6%). Sham-operated rats fed a normal P and Mg diet served as controls. **A-C)** Plasma levels of TNF- α , IL-1 β and IL-6 were evaluated. **D)** Plasma GPx activity was spectrophotometrically evaluated. Bars are means±SEM (n=8/group). ^a p<0.05 vs Control; ^b p<0.05 vs 0.1% Mg.

Figure 3. Dietary Mg supplementation reduced pro-inflammatory cytokine expression in aortic tissue and the histological injury induced in rats with CKD. A rat model of CKD including a 5/6 nephrectomy (Nx) plus a high P diet and a calcitriol treatment was used. Nx rats were fed a normal (0.1%) Mg diet or received a dietary Mg supplementation (0.6%). Sham-operated rats fed a normal P and Mg diet served as controls. **A)** mRNA levels of TNF- α , IL-1 β and IL-6 were analyzed by real-time RT-PCR

Bars are means±SEM (n=8/group). **B)** Representative H&E stains of histological samples of aortas. Arrowhead: calcification; Asterisk: remodeling; Cross: Focal Inflammation. Original magnification: 100 x. **C-F)** Semiquantitative assessment of aortic alterations by using a scale range of intensity from 0 to 4 as described in Methods. Bars are means±SEM. ^a p<0.05 vs Control; ^b p<0.05 vs Nx-Mg 0.1%.

Figure 4. Mg supplementation reduced pro-inflammatory cytokine expression in VSMCs. Cells were incubated for 9 days in a high (3.3 mM) phosphate medium (HP) with a normal (0.8 mM) Mg concentration or supplemented with Mg 1.6 mM (HP+Mg). Cells incubated in normal phosphate and Mg medium were used as controls. **A)** Pro-inflammatory cytokines protein expression. Cytokines released in tissue culture media were measured. **B)** Pro-inflammatory cytokines mRNA expression. Bars are means±SEM (n=4/group, 3 experiments). ^a p<0.05 vs Control; ^b p<0.05 vs HP.

Figure 5. Mg supplementation prevented high phosphate-induced activation of NF-κB signaling pathway in VSMCs. Cells were incubated for 24 hours in a high (3.3 mM) phosphate (HP) medium with a normal (0.8 mM) Mg concentration or supplemented with Mg 1.6 mM (HP+Mg). Cells incubated in normal phosphate and Mg medium were used as controls. **A)** The p65-NF-κB (p65) protein was determined by western blotting in nuclear extracts. Each image was originally captured from the same membrane, and unused or repeated lanes were removed. Images are representative of three different experiments. Bars are means±SEM. **B)** Intracellular localization of p65-NF-κB (p65) was visualized by confocal microscopy. For each treatment, p65 staining (green immunofluorescence) was shown on the left; in the middle, the same sample was counterstained with DAPI (blue) for nuclear stain; the merged image is shown on the right. Images are representative of three different experiments. Original magnification: 400 ×. Bars are means±SEM. ^a p<0.05 vs Control; ^b p<0.05 vs HP.

Figure 6. Mg supplementation prevented HP-induced production of ROS, proliferation and cell death in VSMCs. Cells were incubated for 24 hours in a high (3.3 mM) phosphate medium (HP) with a normal (0.8 mM) Mg concentration or supplemented with Mg 1.6 mM (HP+Mg). Cells incubated in normal phosphate and Mg medium were used as controls. **A)** The levels of reactive oxygen species (ROS) were measured by using the H₂DCFDA probe. **B)** The proliferation rate was measured through the WST-1 assay. **C)** Cell death was evaluated by the LDH assay. Bars are means±SEM (n=6/group, 3 experiments). ^a p<0.01 vs Control; ^b p<0.01 vs HP.

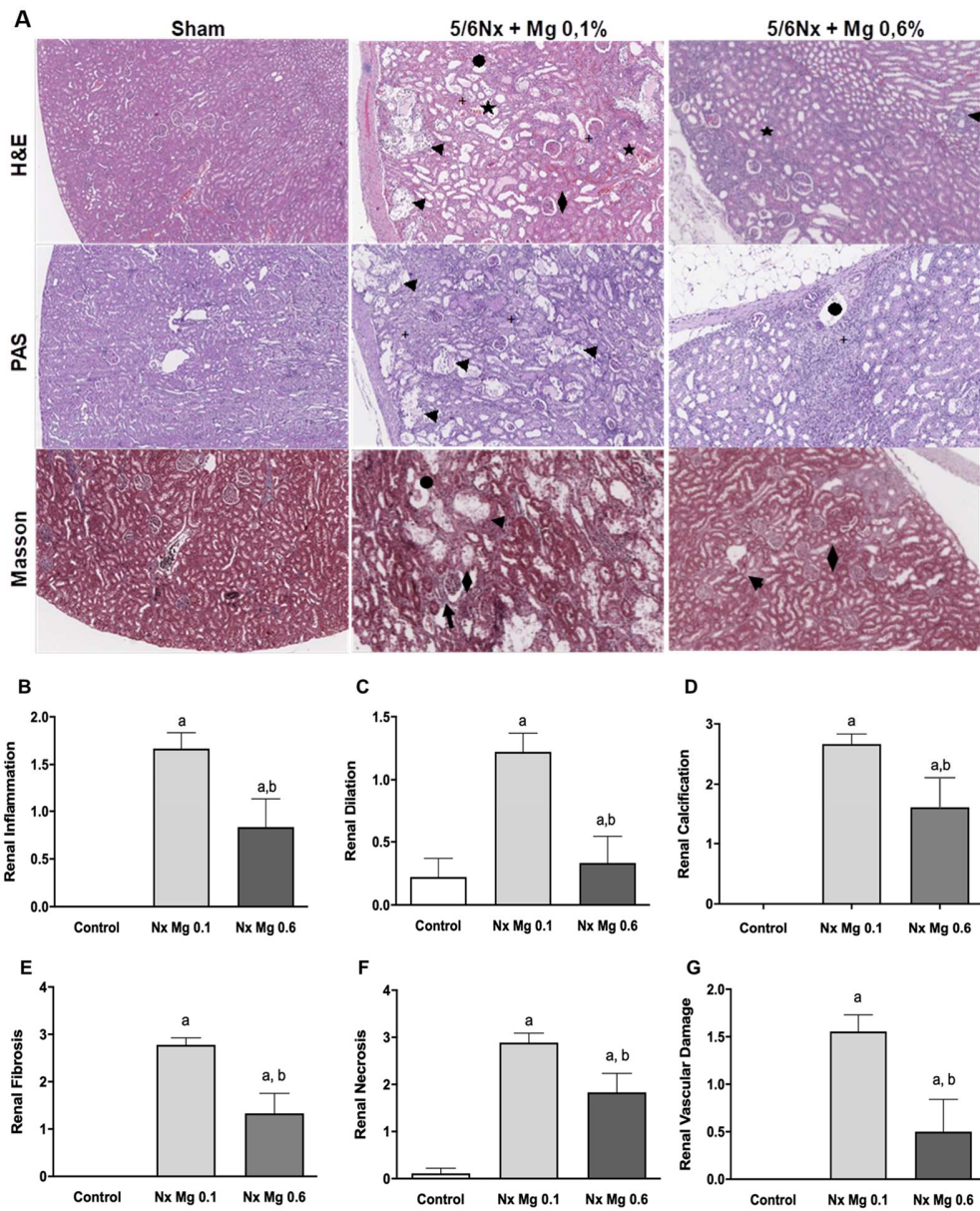


Figure 1.

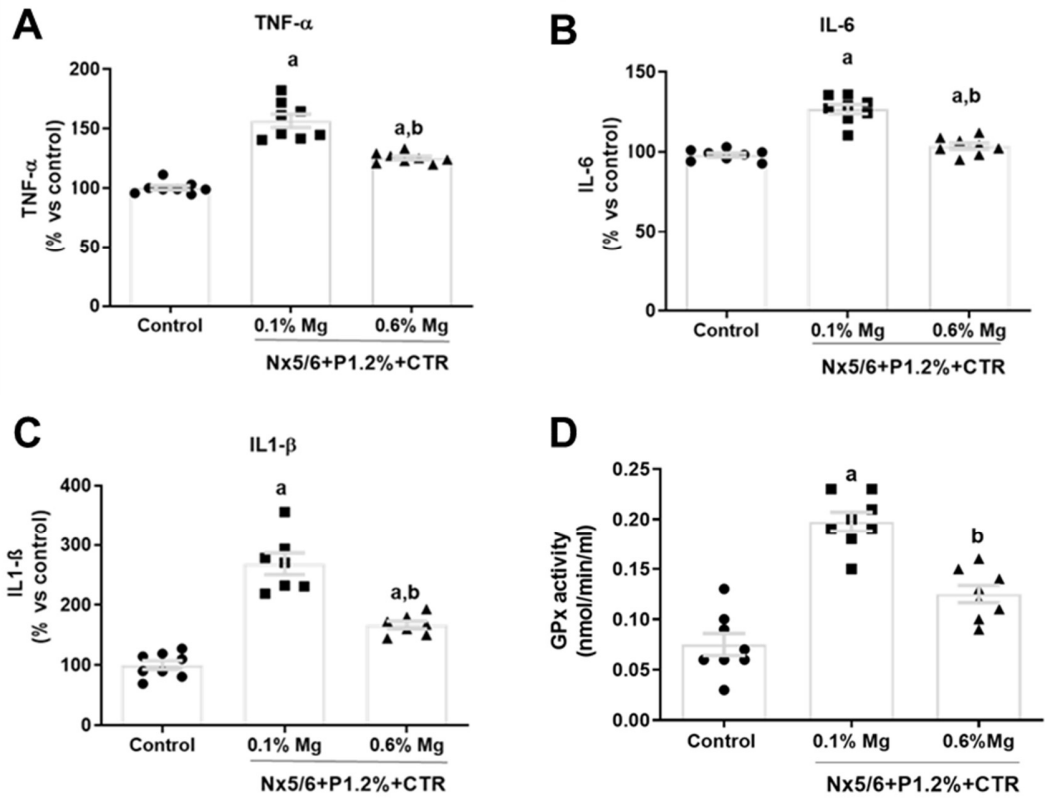


Figure 2.

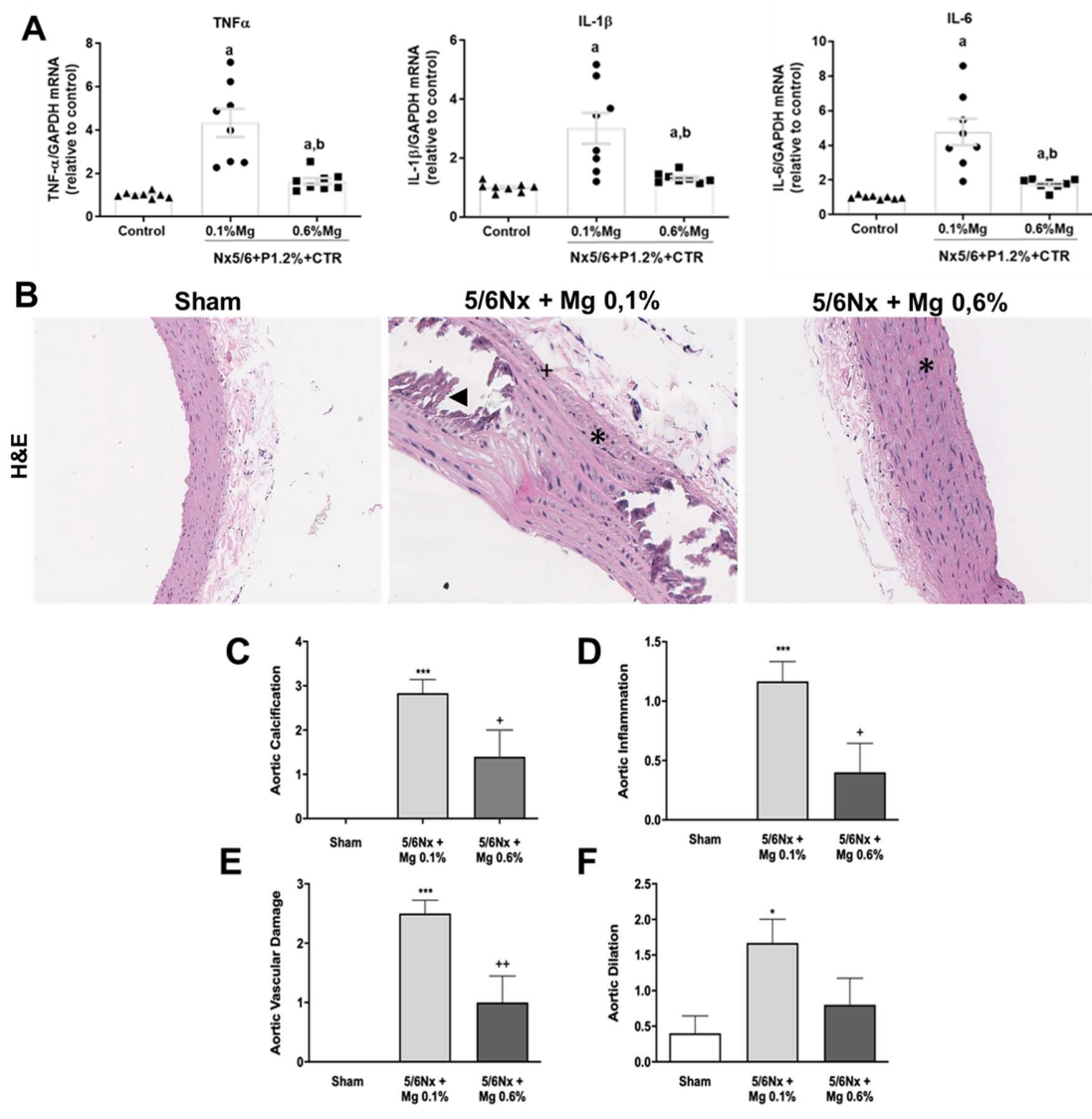


Figure 3.

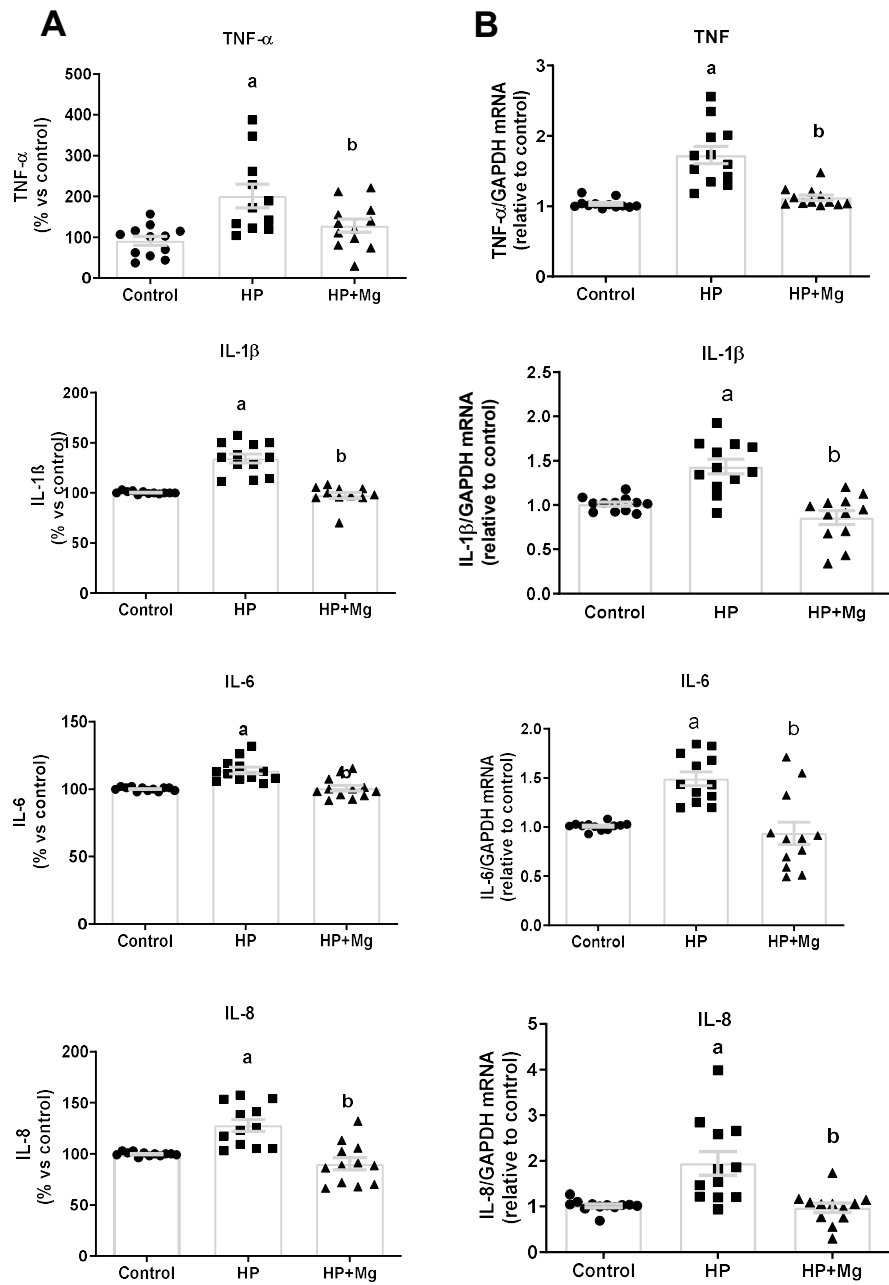
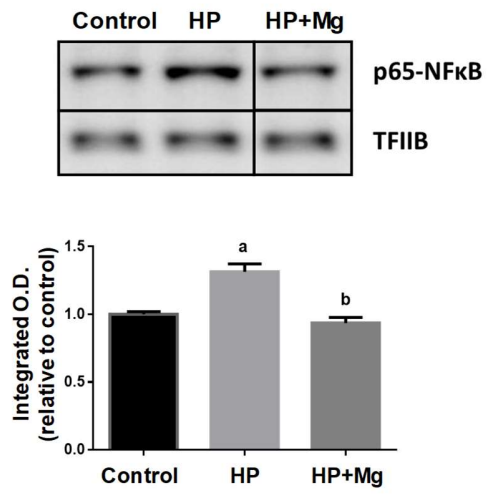


Figure 4.

A



B

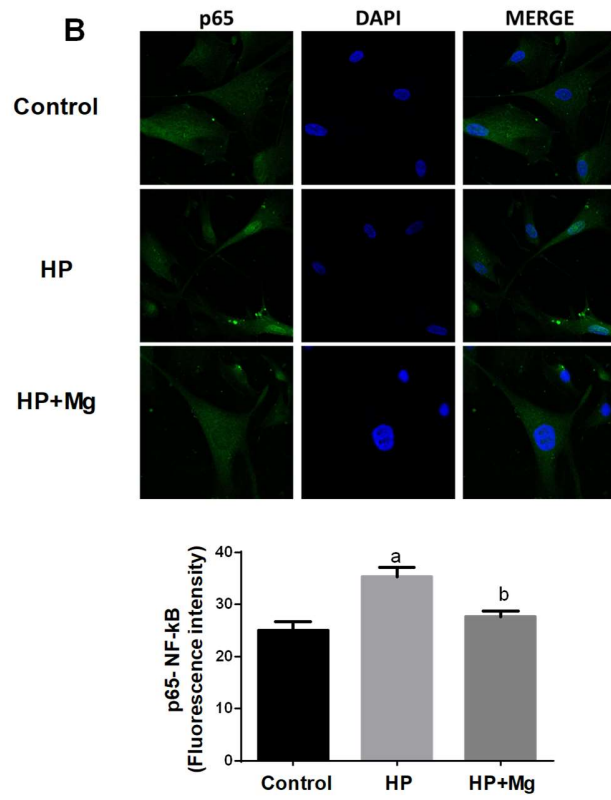


Figure 5.

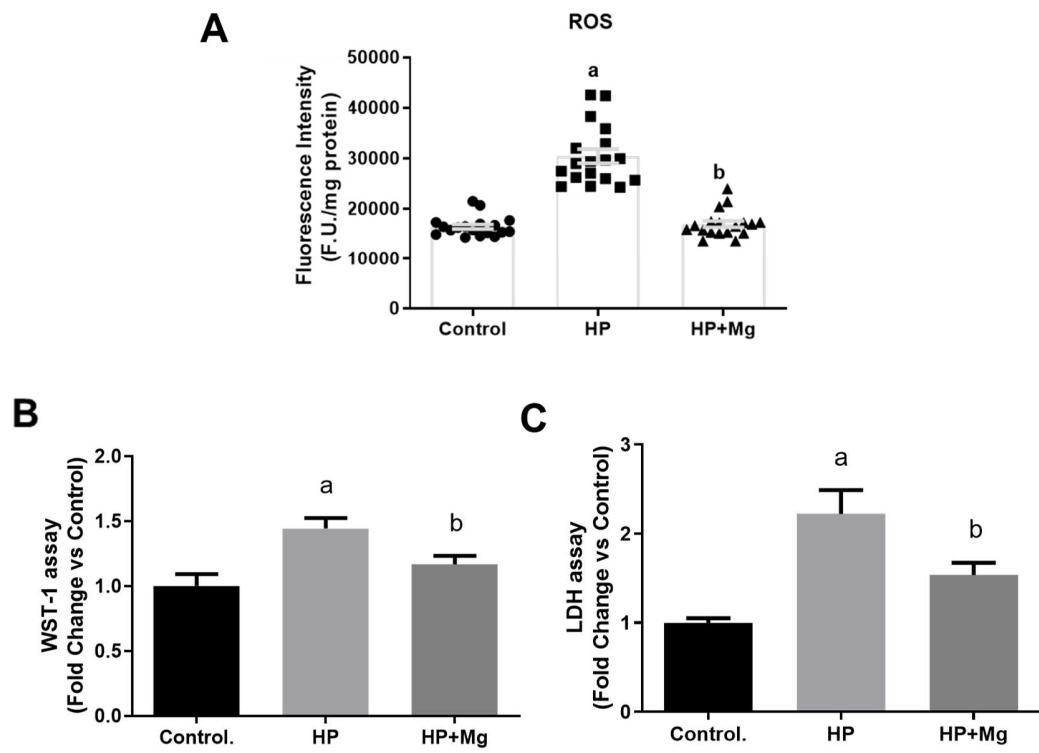


Figure 6.