



Research Paper

Magnesium prevents and rescues high phosphate induced calcification in rat aortic culture

Accepted 25 June, 2018

ABSTRACT

Min-Yu Chang^{1,6*}, Hung-Hsiang Liou^{1,2}, Shih-Yuan Hung¹, Tsun-Mei Lin³, Chih-Hsin Hung⁴, Hsi-Hao Wang¹, Yi-Che Lee¹, Li-Chun Ho¹ and Bin-Min Tsai⁵

¹Division of Nephrology, Department of Internal Medicine, E-Da Hospital/I-Shou University, Kaohsiung, Taiwan.

²Division of Nephrology, Department of Internal Medicine, Hsin-Jen Hospital, Taipei, Taiwan.

³Department of Laboratory Medical Science, E-Da Hospital/I-Shou University, Kaohsiung, Taiwan

⁴Institute of Biotechnology and Chemical Engineering, I-Shou University, Kaohsiung, Taiwan.

⁵Department of Occupational Therapy, I-Shou University, Kaohsiung, Taiwan.

⁶No.1, Yida Road, Jiaosu Village, Yanchao District, Kaohsiung City 82445, Taiwan, R.O.C.

*Corresponding author. E-mail:

ed101366@edah.org.tw,

changming0411@yahoo.com.tw. Tel: +886-7-

6150011 ext 5105. Fax: +886-7-6155138.

Vascular calcification, prevalent in patients with chronic kidney disease (CKD), is a major risk factor for cardiovascular morbidity and mortality. Magnesium is considered to have a protective effect on vascular calcification beyond phosphate control. The present study attempted to investigate the role of magnesium on inhibition and prevention of vascular calcification in an *ex vivo* study. Aortic segments of Sprague–Dawley rats were incubated in medium with high phosphate concentration for 10 or 14 days to induce calcification. Various concentrations of magnesium were introduced during different culture periods. Mineralization of aortas was assessed by von Kossa staining and quantification of calcium content. In this experiment, aortic calcification was induced by high phosphate concentration and was significantly reduced dose- and time- dependently by magnesium. Pretreatment with magnesium strongly prevented high phosphate-induced aortic calcification. Even a short time exposure to magnesium ameliorates and rescues further progression of vascular calcification. Further studies are needed to explore the mechanisms and the clinical use of magnesium to inhibit vascular calcification.

Key words: Aortic calcification, magnesium, phosphate.

INTRODUCTION

Calcification in the vascular media layer is prevalent in patients with chronic kidney disease (CKD) and is a major risk factor for cardiovascular morbidity and mortality (London et al., 2003; Jono S et al., 2008). The mechanism of medial calcification is now considered as a tightly regulated process involving molecular transition and cellular phenotypic change from vascular smooth muscle cells (VSMCs) into osteochondrogenic cells (Abedin et al., 2004; Johnson RC et al., 2006). Several factors have been demonstrated to induce this transition which included oxidant stress, parathyroid hormone fragments, vitamin D use and high phosphate level (Hruska, 2009). Among these inducers, high phosphate level is considered as the most important one. The calcification process could be blocked if the type III sodium-dependent phosphate cotransporter Pit-1, the main regulator of phosphate-induced calcification in VSMCs, is inhibited by phosphophenomic acid (PFA)

(Jono S et al., 2008).

Magnesium does not only involved in many important metabolic and enzymatic processes, but is now shown to have the potential to inhibit vascular calcification. Low magnesium level has been shown to be associated with vascular calcification and cardiovascular mortality in non-uremic populations (Adamopoulos et al., 2009) and has also been associated with the presence of common carotid artery atherosclerosis (Tzanakis et al., 2004), carotid intima-media thickness (Turgut et al., 2008) and as a significant predictor for mortality in hemodialysis patients (Ishimura et al., 2007).

Several studies have emphasized the aggressive effects of magnesium on controlling vascular calcification. While in a longitudinal study, Meema HE speculated that hypermagnesemia was associated with retardation or improvement of vascular calcification in peritoneal dialysis

patients (Meema et al., 1987). Dietary magnesium, not calcium, was found to prevent vascular calcification in a mouse model for pseudoxanthoma elasticum (Gorgels et al., 2010). From a few experiments carried out on the *in vitro* model of VSMCs, magnesium prevented β -glycerophosphate (BGP) induced calcification (Kircelli et al., 2012), negatively regulated vascular calcification induced by high phosphate through increased and restored transient receptor potential melastatin 7 (TRPM7) activity (Montezano et al., 2010), and modulated osteogenic proteins in the calcification process (Louvet et al., 2013). TRPM7 are now well accepted as important regulators of magnesium homeostasis and vascular signaling.

Magnesium has been shown to have a protective effect on vascular calcification according to the above-mentioned *in vitro* and *in vivo* studies, however, evidences from the *ex vivo* studies are yet to be elucidated. Therefore, the present study attempted to investigate the effect of magnesium on high phosphate-induced rat aortic vascular calcification in an *ex vivo* study.

MATERIALS AND METHODS

Aortic tissue culture

Thoracic aortas below the arch to the diaphragm were removed from 10-week-old male Sprague–Dawley rats. After the connective tissue had been carefully removed, the aortas were cut into several 3- to 5-mm rings and placed in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, USA), supplemented with 10% fetal bovine serum (FBS), fungizone, streptomycin and penicillin. Aortic segments were maintained at 37°C in a 5% CO₂ incubator, and the medium was changed daily. The DMEM contained 2.18 mM Ca²⁺, 1.26 mM PO₄³⁻ and 0.88 mM Mg²⁺, with a pH of 7.2. NaH₂PO₄ and MgSO₄ were added to the serum-supplemented with DMEM to create various phosphate and magnesium concentrations, respectively. Aortic segments were incubated with different time intervals according to the present study requirements. After completing the culture process, the aortic segments were washed three times in phosphate-buffered saline (PBS) and were divided into two parts for quantification of calcification and tissue analysis. An inhibitor of the TRPM7, 2-aminoethoxydiphenylborate (2-APB) (200 μ mol/L) and an inhibitor of the Pit-1 receptor PFA (1 mmol/L), were applied in this study. All experimental procedures were approved by the Institution Animal Care and Use Committee (IACUC) of E-DA Hospital, Kaohsiung, Taiwan (EDAPH 101022).

Quantification of calcification

Aortic segments were patted dry with a paper towel, placed in a 5 ml tube, and extracted by end-over-end mixing with 2

ml of 0.15 M HCl for 24 h at room temperature. The calcium content in the acid extracts of tissues were determined by the o-cresolphthalein complex-one method (OCP) (BioVision, USA). Briefly, the method is based on the purple-coloured complex formed by calcium with OCP in an alkaline medium. The optical density (OD) of the samples were measured with a spectrophotometer at 565 nm and compared with a curve calibrated with standards. The results were corrected by wet tissue weight and expressed as milligrams per gram wet weight of tissue.

Tissue analysis

For histological analyses, aortic segments were fixed with 4% paraformaldehyde PBS for 8 h and were embedded in paraffin. For Alizarin red staining, aortas were incubated with 10% neutral buffered formalin for 15 min, rinsed and incubated with a 2% aqueous Alizarin red solution (pH 4.0–4.2) for 10–15 min. After two washing steps, samples were photographed showing the presence of induced mineralization. For von Kossa staining, aortas were deparaffinized and placed in 3% Silver Nitrate Solution with a UV-light exposure for 30 min. Samples were rinsed in three changes of distilled water and placed in 5% sodium thiosulfate for 2 min. The samples were again rinsed in three changes of distilled water, and counter stain with Nuclear Fast Red for 5 min. After washing and mounting, aortas were pictured and examined.

Statistical analysis

Data were presented as mean \pm standard deviation (SD). Significance was determined by the unpaired t test or an analysis of variance (ANOVA) multigroup analysis. The non-parametric Mann-Whitney test was used as ANOVA failed to reach significance due to huge standard deviation. A *p* value of 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

In the present study, we accessed aortic rings calcification induced by phosphate after a 10-day culture. The calcium content of aortas was observed to be dose-dependently increased when phosphate content was increased in the medium. A significance was reached when phosphate concentration was up to 2.00 mM or more in culture medium (*p*<0.05) (Figure 1A). Aortic calcification significantly appeared even only after 4 days of incubation in high phosphate medium as compared with that after 10 days of incubation in normal medium (Figure 1B).

Then the magnesium concentration of the medium was

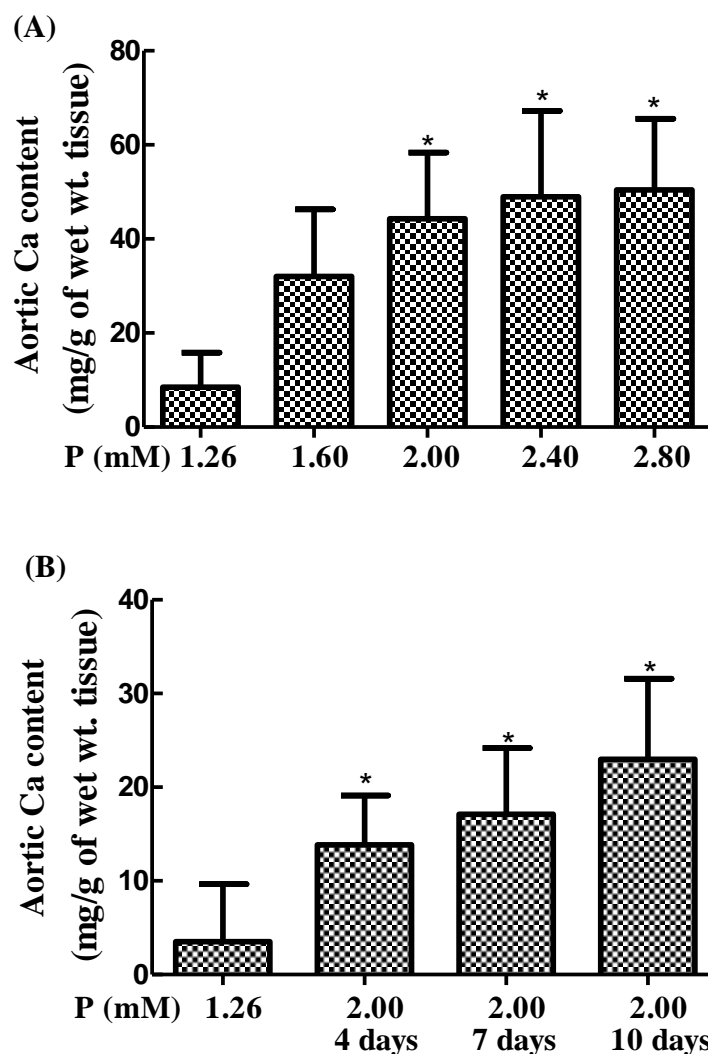


Figure 1: Elevated phosphate concentration induced calcification in rat aortas dose- and time-dependently. (A) Rat aortas were treated with the indicated concentrations of phosphate for 10 days. Aortic calcium content was stepwise increased along with elevated phosphate concentration. (* $p < 0.05$, N=5) (B) Rat aortas were treated with 2.00 mM phosphate for different days. Control group was cultured in normal medium contained 1.26 mM phosphate for 10 days. The amount of calcification was gradually increased comparable to treated times. (* $p < 0.05$, N=5) P: phosphate, Ca: calcium.

gradually increased from 1.00 to 2.00 mM. After 10-day culture, magnesium showed a dose-dependent reduction of aortic calcification induced by high phosphate content (Figure 2A). Meanwhile, this high-phosphate induced calcification was completely inhibited by PFA. When magnesium concentration was greater than 1.60 mM ($p < 0.00$), which is equal to a serum level of 3.84 mg/dl (1 mM = 2.4 mg/dl) in human, its inhibitory ability on aortic calcification was similar to that of PFA. Therefore, the minimal concentration of magnesium in culture medium to inhibit a high-phosphate induced calcification was

determined as 1.60 mM. The inhibitory effect of aortic calcification by magnesium was partially, but not completely, antagonized by 2-APB, an inhibitor of TRPM7 (Figure 2B). This finding speculated that there were different effects of magnesium on inhibiting calcification other than involvement of cellular control by TRPM7.

To investigate the preventive effects of magnesium, aortic segments were cultured for 14 days in a high-phosphate condition. Magnesium concentration of 1.60 mM was introduced since day -2 in the pre-treatment group. In other groups, magnesium was given since day 0 and this

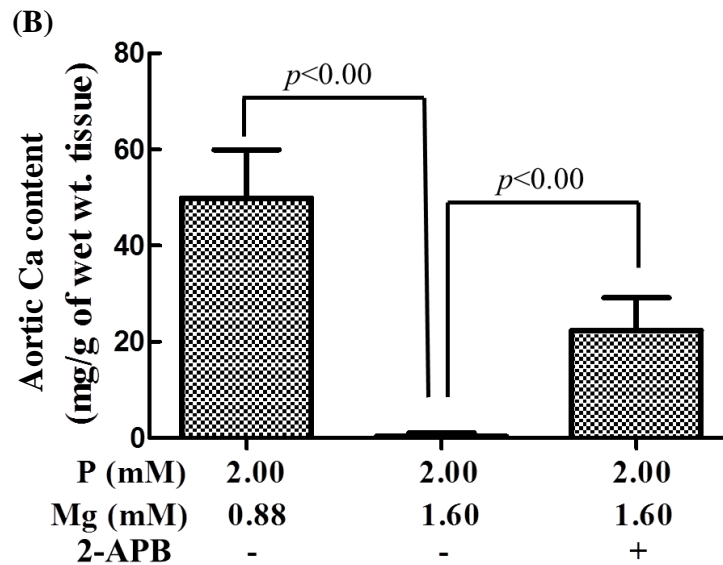
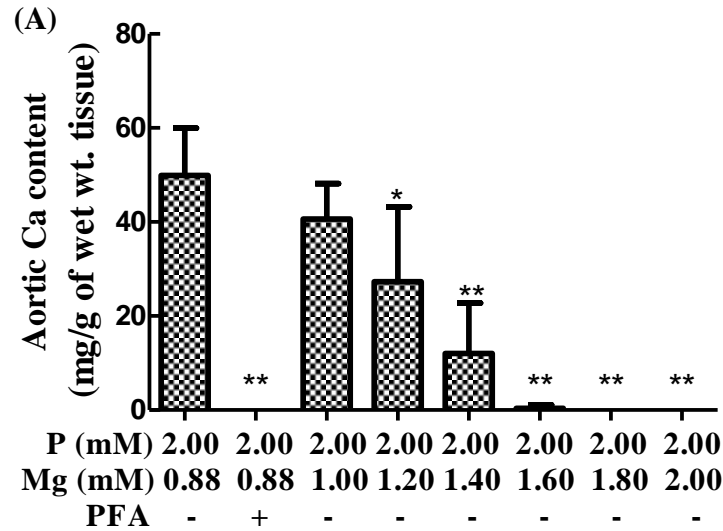


Figure 2: Magnesium reduced high phosphate-induced calcification dose-dependently. (A) Rat aortas were treated with high phosphate (2.00 mM) and elevated magnesium concentration (from 1.00 to 2.00 mM) for 10 days. The control medium contained 0.88 mM magnesium. PFA was used as an inhibitor of Pit-1 for blocking calcification process. Magnesium reduced calcification dose-dependently. (* $p < 0.05$, ** $p < 0.00$ vs control, $N = 5$). (B) Magnesium (1.60 mM) reduced high phosphate-induced calcification significantly ($p < 0.00$, $N = 5$), and the inhibition ability was partially blocked by 2-APB ($p < 0.00$). PFA: phosphonoformic acid. 2-APB: 2-aminoethoxy- diphenylborate . P: phosphate, Ca: calcium, Mg: magnesium.

concentration was maintained for 3, 6 or 10 days, respectively. As shown in Figure 3, the calcification of aortas was dramatically inhibited in the pre-treatment group ($p < 0.00$). The prevention of calcification by magnesium was also present in other groups time-dependently, even only after 3 days of exposure.

In another experiment on the rescuing effects of

magnesium, aortic segments were cultured with high phosphate concentration for 14 days. Magnesium concentration of 1.60 mM was given since day 0, 3 or 6. It showed that calcification was significantly lessened in all groups ($p < 0.00$) (Figure 4). Even after 5 days of exposure to a high phosphate concentration, calcification was still partially resolved by magnesium.

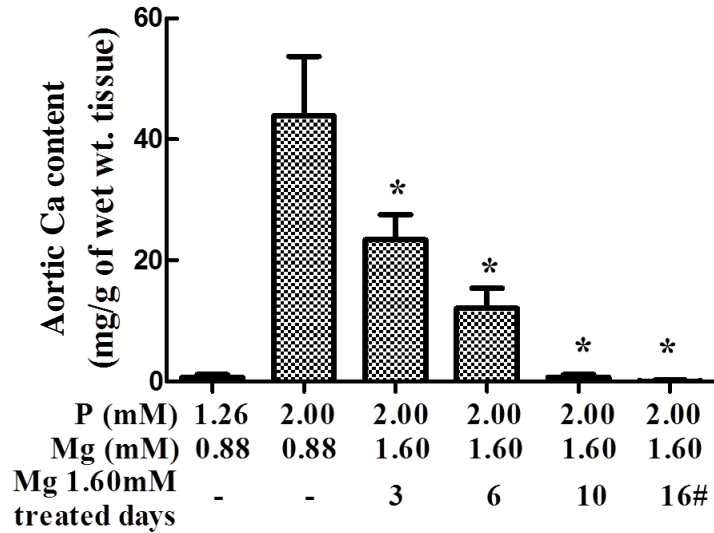


Figure 3: Magnesium prevented high phosphate-induced calcification time-dependently. Rat aortas were treated with 2.00 mM phosphate for 14 days. Magnesium 1.60 mM was introduced for 3, 6 or 10 days. In the pre-treatment group (#), magnesium was given 2 days before high phosphate intervention and was maintained for the later 14 days (total 16 days). Magnesium significantly prevented high phosphate induced calcification in all groups (* $p < 0.00$, $N = 5$). The inhibition ability was more prominent in pre-treatment group (#) and was comparable to treated days. P: phosphate, Ca: calcium, Mg: magnesium.

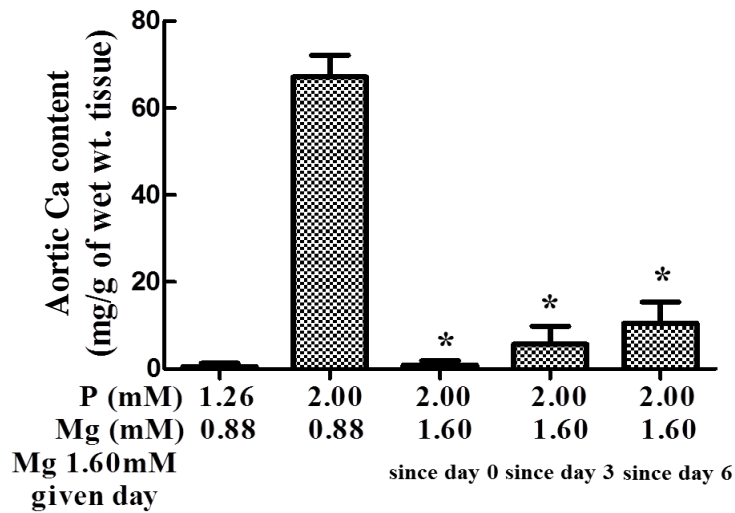


Figure 4: Magnesium rescued calcification even after 2 or 5 days exposure to high phosphate. Rat aortas were cultured with 2.00 mM phosphate for 14 days. Magnesium 1.60 mM was given since day 0, 3 and 6. Even after 5 days exposure to high phosphate concentration, calcification still could be reduced by magnesium significantly (* $p < 0.00$, $N = 5$). P: phosphate, Ca: calcium, Mg: magnesium.

DISCUSSION

Vascular calcification is an emerging problem beyond phosphate control in patients with CKD. From previous *in*

vitro studies using VSMCs (Kircelli et al., 2012; Montezano et al., 2010; Louvet et al., 2013) or using a uremic rat model (De Schutter et al., 2013), magnesium was observed to reduce calcification induced by high phosphate. We fill the

gap using a rat aortic culture model to demonstrate the preventive effects of magnesium, and also point out the possibility of rescuing the progression of vascular calcification by magnesium even after the aortas are already exposed to a calcified condition.

Firstly, we confirmed the effects of high phosphate concentration on inducing aortic calcification. The severity of calcification was closely related to exposure dose and treated times, and this calcification was almost completely inhibited by PFA.

A concentration of magnesium in culture medium greater than 3.84 mg/dl was found to reduce high-phosphate induced vascular calcification dose- and time-dependently in our model. Furthermore, magnesium could prevent calcification and had a legacy effects even after it had been withdrawn. The experiment of the present study also showed that rat aortic calcification could be significantly reduced by magnesium even after calcification had been established following 5 days exposure to high phosphate.

TRPM7, an important regulator of magnesium homeostasis, had been implicated as a signaling kinase involved in VSMCs growth, apoptosis, adhesion, contraction, cytoskeletal organization and migration. Montezano et al. (2010) studied the role of TRPM7 in the regulation of magnesium effects in rat VSMCs cultures. TRPM7 activation was decreased in calcification medium, while magnesium restored TRPM7 activity and inhibited osteogenic differentiation of VSMCs. Interestingly, the inhibition of calcification was not totally blocked by 2-APB in the present study. Our findings showed that the mechanisms responsible for the inhibitory effect of magnesium on vascular calcification are via TRPM7 and beyond.

Magnesium compounds have been evaluated as phosphate binders for CKD patients since the 1980s. The CALMAG study, a large randomized multicenter study, showed a similar phosphate control with good tolerability by calcium acetate/magnesium carbonate as compared with sevelamer hydrochloride in HD patients (de Francisco et al., 2010). In a small open-label pilot study (Spiegel et al., 2007), magnesium carbonate combined with calcium carbonate controlled serum phosphate well and reduced elemental calcium ingestion, with no progression of coronary artery calcification or bone mass changes over an 18-month period in HD patients. In another study, magnesium citrate did not change phosphate control but improved carotid intima media thickness as compared with calcium acetate over a 2-month period (Turgut et al., 2008). Put our experimental results together, magnesium might be a candidate for a new phosphate binder and not only to treat hyperphosphatemia but also to inhibit development or progression of vascular calcification.

Conclusion

In conclusion, the data of the present study support the preventive effects of magnesium on vascular calcification in

rat aortas. Since magnesium-containing phosphate binders are relatively inexpensive, it can be an attractive treatment option for CKD patients not only for the control of hyperphosphatemia, but also for a better cardiovascular protection. However, further studies are needed to establish the beneficial effects and the mechanisms of magnesium on inhibition of vascular calcification.

REFERENCES

- Abedin M, Tintut Y, Demer LL (2004). Vascular calcification: mechanisms and clinical ramifications. *Atheroscler. Thromb. Vasc. Biol.* 24(7): 1161-70.
- Adamopoulos C, Pitt B, Sui X, Love TE, Zannad F, Ahmed A (2009). Low serum magnesium and cardiovascular mortality in chronic heart failure: a propensity matched study. *Int. J. Cardiol.* 136(3): 270-277.
- de Francisco AL, Leidig M, Covic AC, Ketteler M, Benedyk-Lorens E, Mircescu GM, Scholz C, Ponce P, Passlick-Deetjen J (2010). 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.* 25(11): 3707-3717.
- Gorgels TGMF, Waarsing JH, Wolf A, ten Brink JB, Loves WJ, Bergen AA (2010). Dietary magnesium, not calcium, prevents vascular calcification in a mouse model for pseudoxanthoma elasticum. *J. Mol. Med.* 88(5): 467-75.
- Hruska KA (2009). Vascular smooth muscle cells in the pathogenesis of vascular calcification. *Circ. Res.* 104(6): 710-711.
- Ishimura E, Okuno S, Yamakawa T, Inaba M, Nishizawa Y (2007). Serum magnesium concentration is a significant predictor of mortality in maintenance hemodialysis patients. *Magnes. Res.* 20(4): 237-244.
- Johnson RC, Leopold JA, Loscalzo J (2006). Vascular calcification: pathobiological mechanisms and clinical implications. *Circ. Res.* 99(10): 1044-1059.
- Jono S, McKee MD, Murry CE, Shioi A, Nishizawa Y, Mori K, Morii H, Giachelli CM (2008). Phosphate regulation of vascular smooth muscle cell calcification. *Circ. Res.* 87(7): e10-17.
- Jono S, Shioi A, Ikari Y, Nishizawa Y (2008). Vascular calcification in chronic kidney disease. *J Bone Miner Metab.* 24(2): 176-181.
- Kircelli F, Peter ME, Sevinc Ok E, Celenk FG, Yilmaz M, Steppan S, Asci G, Ok E, Passlick-Deetjen J (2012). Magnesium reduces calcification in bovine vascular smooth muscle cells in a dose-dependent manner. *Nephrol. Dial. Transplant.* 27(2):514-521.
- London GM, Guérin AP, Marchais SJ, Métivier F, Pannier B, Adda H (2003). Arterial media calcification in end-stage renal disease: impact on all-cause and cardiovascular mortality. *Nephrol. Dial. Transplant.* 18(9):1731-1740.
- Louvet L, Büchel J, Steppan S, Passlick-Deetjen J, Massy ZA (2013). Magnesium prevents phosphate-induced calcification in human aortic vascular smooth muscle cells. *Nephrol. Dial. Transplant.* 28(4): 869-878.
- Meema HE, Oreopoulos DG, Rapoport A (1987). Serum magnesium level and arterial calcification in end-stage renal disease. *Kidney Int.* 32(3): 388-394.
- Montezano AC, Zimmerman D, Yusuf H, Burger D, Chignalia AZ, Wadhwa V, van Leeuwen FN, Touyz RM (2010). Vascular smooth muscle cell differentiation to an osteogenic phenotype involves TRPM7 modulation by magnesium. *Hypertension.* 56(3): 453-62.
- Spiegel DM, Farmer B, Smits G, Chonchol M (2007). Magnesium carbonate is an effective phosphate binder for chronic hemodialysis patients: A pilot study. *J. Ren. Nutr.* 17(6): 416-422.
- Turgut F, Kanbay M, Metin MR, Uz E, Akcay A, Covic A (2008). Magnesium supplementation helps to improve carotid intima media thickness in patients on hemodialysis. *Int. Urol. Nephrol.* 40(4): 1075-1082.
- Tzanakis I, Virvidakis K, Tsomi A, Mantakas E, Grousis N, Karefillakis N, Papadaki A, Kallivretakis N, Mountokalakis T (2004). Intra- and extracellular magnesium levels and atheromatosis in haemodialysis patients. *Magnes. Res.* 17(2): 102-108.