Magnesium Modifies the impact of Calcitriol Treatment on Vascular Calcification in Experimental Chronic Kidney Disease

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C) Text pages: 17

Tables: 0

Figures: 9

References: 55

Abstract: 236

Introduction: 698

Discussion: 1160

D) List of Abbreviations

CKD Chronic Kidney Disease
CVD Cardiovascular disease
DBP Diastolic blood pressure
ESRD End stage renal disease
FGF-23 Fibroblast growth factor 23
VEGF Vascular endothelial growth factor
MAP Mean arterial pressure
Mg Magnesium
PP Pulse pressure
PTH Parathyroid hormone
PWV Pulse wave velocity
RUNX2 runt-related transcription factor 2
SBP Systolic blood pressure
TRPM7 transient receptor potential cation channel, subfamily M, member 7
VC Vascular Calcification
VSMC Vascular smooth muscle cells

E) Cardiovascular
Abstract:

Chronic kidney disease (CKD) patients are commonly treated with vitamin D analogs, such as calcitriol. Recent epidemiological evidence revealed a significant interaction between vitamin D and magnesium, since an inverse relationship between vitamin D levels and mortality mainly occurs in patients with a high magnesium intake. The aim of the study was to assess the mechanisms involved by determining whether magnesium alone or combined with calcitriol treatments differentially impact vascular calcification (VC) in male Sprague-Dawley rats with adenine-induced CKD. Treatment with moderate doses of calcitriol (80ug/kg) suppressed PTH to near or slightly below control levels. Given alone, this dose of calcitriol increased the prevalence of VC; but when magnesium was given in combination, the severity of calcification was attenuated in the abdominal aorta (51% reduction), iliac (44%) and carotid arteries (46%) compared to CKD controls. The decreases in vascular calcium content were associated with 20-50% increase in vascular Mg. Calcitriol treatment alone significantly decreased TRPM7 protein (↓ to ~11%) whereas the combination treatment increased both the mRNA (1.7X) and protein (6.8X) expression compared to calcitriol alone. In summary, calcitriol increased VC in certain conditions; but magnesium prevented the reduction in TRPM7 and reduced the severity of VC, thereby increasing the bioavailable magnesium in the vascular microenvironment. These findings suggest that modifying the adverse effect profile of calcitriol with Mg may be a plausible approach to benefiting the increasing number of CKD patients being prescribed calcitriol.
Introduction

Vascular calcification (VC) is a common manifestation of cardiovascular disease (CVD) in chronic kidney disease (CKD) (Foley and Parfrey, 1998; Jono et al., 2006). Aberrant mineral metabolism, involving hyperphosphatemia, hyperparathyroidism and vitamin D insufficiency, are hallmarks of progressing CKD. These indicators have been consistently linked to the severity of VC and mortality in these patients (Kestenbaum et al., 2005; Cannata-Andia et al., 2006; Holden et al., 2010). VC is now recognized as an active mineralization process, involving transformative changes in medial vascular smooth muscle cells (VSMC) and the surrounding microenvironment that negatively impacts the circulation (Giachelli, 2004). Recent evidence also demonstrates significant cross-talk between endothelial cells and VSMCs through humoral factors, such as angiopoietin 2 and vascular endothelial growth factor (VEGF). These factors have been implicated in the regulation of vascular function in health and various disease states, for example vascular calcification (Bourque et al., 2011; David et al., 2012; Yao et al., 2013; Chang et al., 2014).

The mineral magnesium also plays an important role in various aspects of cellular physiology, including the regulation of vascular tone, cardiac electrophysiology, and bone metabolism. Magnesium entry into these tissues is regulated, at least in part, by the activity of the transient receptor potential melastatin 7 cation channel (TRPM7), a divalent ion transporter with a high affinity for magnesium. In population-based studies, magnesium deficiency has been implicated in several disorders such as diabetes, (Garg et al., 2014) hypertension (Cunha et al., 2012) and CVD (Reffelmann et al., 2011.) Although not currently considered to be a hallmark of CKD, abnormal serum magnesium has also
been associated with increased cardiovascular events and mortality in end stage renal disease patients (ESRD) (Navarro-Gonzalez et al., 2009; Kanbay et al., 2012). Despite these associations, the mechanism by which magnesium attenuates the progression and development of VC in CKD has not been well studied. Mechanistically linking abnormalities in magnesium regulation with CVD could be important, as several in vitro studies have demonstrated that magnesium has the potential to attenuate hydroxyapatite growth, the basic building block of calcification (Cheng et al., 1988; Kircelli et al., 2012; Louvet et al., 2013). These studies also showed that magnesium up regulated anti-calcification proteins, and prevented VSMC trans-differentiation into an osteoblast-like-phenotype. Using a 5/6-nephrectomy model to induce experimental CKD, Inagaki et al. (1996) investigated whether in vivo magnesium supplementation attenuated VC. Their results were not definitive as the CKD phenotype in their model lacked hyperphosphatemia, hyperparathyroidism, and frank VC. Despite this limitation they found that aortic calcium content, but not phosphate, was slightly higher in animals on a magnesium deficient diet (Inagaki et al., 1996); a finding that may or may not be generalizable to other models (Shobeiri et al., 2010). Further studies are needed to investigate whether magnesium treatment beneficially impacts CKD in a model in which all the key aspects of the pathogenic phenotype are evident.

Magnesium has also been implicated in vitamin D synthesis and metabolism. Magnesium is as a cofactor for vitamin D binding protein in the serum, as well as a modulator of the expression and activities of 1-α-hydroxylase and 25-hydroxylase (Risco and Traba, 1992). Furthermore, Verberckmoes et al. (2007), discovered that uremic rats supplemented with calcitriol (1,25(OH)2D3) had a significantly elevated level of
magnesium relative to calcium (↓ Ca : Mg ratio) in aorta tissue compared to non-calcitriol
supplemented controls (Verberckmoes et al., 2007). This relative increase in vascular
magnesium suggested there was a potential interaction between magnesium and vitamin
D receptor activation. Vitamin D insufficiency is a well-established occurrence in the
progression of CKD, and has been linked to hyperparathyroidism and adynamic bone
disease in these patients (Helvig et al., 2010; Holden et al., 2010). Furthermore, clinical
studies have found that treatments with vitamin D receptor activators, such as calcitriol,
reduce the risk of mortality in early-stage CKD patients and ESRD patients on dialysis
treatments (Duranton et al., 2013). However, more recent evidence from the National
Health and Nutrition Examination Survey (NHANES) and confirmed by Mursu et al.
(2015) indicate that the association between serum 25(OH)D and mortality is modified by
magnesium intake (Deng et al., 2013; Mursu et al., 2015). The inverse relationship
between mortality and vitamin D levels was only identified in patients with high
magnesium intake. Together, these data suggest a mechanistic interaction between
magnesium and vitamin D is likely but the scope of this interplay requires further
elucidation.

Despite the reported beneficial effects of calcitriol in clinical studies, most studies
in experimental CKD have shown that calcitriol exacerbates hypercalcemia,
hyperphosphatemia and increases the severity of VC (Jono et al., 1998; Cardus et al.,
2007; Koleganova et al., 2009). This calcitriol paradox likely relates to the dose, as
amounts below 30ng/kg have been shown to reduce VC (Mathew et al., 2008; Lau et al.,
2012) whereas higher doses induce VC (Cardus et al., 2007; Koleganova et al., 2009;
Duranton et al., 2013). These data suggest that calcitriol has a very narrow therapeutic
window, thereby limiting its utility and benefit in therapy. Expanding and defining the beneficial therapeutic window for vitamin D type treatments would clearly be clinically useful. The present study hypothesizes that magnesium has a potential role as an adjunct treatment in CKD by attenuating the adverse actions of calcitriol. To test this concept, the present study was designed to determine whether changes in dietary magnesium status (to reflect likely translational approaches), alone or in combination with calcitriol treatment impacts the susceptibility to and/or severity of VC in a rodent model of adenine-induced CKD.

Methods

Animal Preparation:

All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US national Institutes of Health, the guiding principles of the Canadian Council on Animal Care (CCAC) and with the approval of the Queen’s University Animal Care Committee (UACC).

Male Sprague-Dawley rats (n=32, 14 weeks of age) were individually housed and maintained on a 12-h light/dark cycle. All animals were acclimatized for a one week period prior to the study during which they were provided with standard rat chow (LabDiet 5001, Ren’s Pets Depot, Oakville, ON, Canada) and water ad libitum. At 15 weeks of age, CKD was generated using an adenine model as previously described (McCabe et al., 2013; Shobeiri et al., 2013). Briefly, the standard rat chow was
exchanged with a specially formulated adenine diet (Harlan, Teklad, Madison, WI). This diet contained 0.25% adenine with 1% phosphate, 1% calcium, 0.05% magnesium, 0.2 mg/kg vitamin K, 1 IU/g vitamin D, and 6% protein. With this approach all animals placed on the adenine diet developed at least moderate to severe renal dysfunction (creatinine greater than 100 uM) by the end of the 7 week experimental period. After 3 weeks of giving adenine to induce CKD, rats were stratified according to serum creatinine levels and then allocated by rank into one of four groups ensuring that there were equivalent average level and range of kidney dysfunction at the onset of the treatments.

Animals were stratified into one of 4 treatment groups; ensuring that each group had. For the next 4 weeks, each group was maintained on: 1) regular adenine diet (CKD; 0.25% adenine, 0.05% Mg food, n=8), 2) high magnesium diet (CKD-M; 0.25% adenine + 0.2% Mg food, n=8), 3) calcitriol (CKD-C; 80ng/kg/day, 0.25% adenine, n=8), or 4) high dietary magnesium (CKD-CM; 0.25% adenine + 0.2% Mg food) + calcitriol (80ng/kg/day, n=8). Weights and food intake were monitored on a daily basis, and animals were supplemented with normal chow and/or Nutri-Cal if a decline in body weight reached 10%. These supplements do not contain increased amounts of calcitriol, any form of vitamin D, magnesium or vitamin K. At 7 weeks (3-7 week treatment interval) rats were sacrificed under a high level of general anesthetic (5% isoflurane). Blood was drawn (8-10 ml) using a 22g hypodermic needle inserted into the left ventricle of the heart while the animal was under anesthesia. The heart was excised and right ventricle was separated from the left ventricle at the septum. The following tissues were rapidly collected, cleaned, and weighed: left and right ventricle, thoracic aorta, abdominal
aorta, renal artery, carotid artery, superior mesenteric artery and iliac artery. Tissues were collected and snap-frozen in liquid nitrogen and stored at -80°C for further analysis.

Calcitriol and Magnesium Dosages:

The clinical dose of calcitriol, converted to rat equivalent doses based on body surface area, is approximately 15-50 ng/kg/day. Based on preliminary dose-ranging studies, the selected moderately high dose of 80 ng/kg/day was found to meet the target criteria for promoting a mild adverse event profile including vascular calcification, mild hypercalcemia and suppression of PTH to near normal levels. The overarching goal of the present studies was to determine whether adjunct treatment with high dietary magnesium could attenuate the development of these calcitriol-induced characteristics in CKD.

In healthy humans, the normal range of total serum magnesium is ~0.65-1.05 mmol/L. In the present study, the CKD rats on the adenine diet alone (0.05% mg diet) had serum magnesium levels of 1.38±0.7 mmol/L. The animals on the high magnesium diet (0.2% mg diet) had serum magnesium of 1.72 ± 0.3. This amount of magnesium was well tolerated by the animals and did not cause any obvious abnormalities such as diarrhea or seizures. Preliminary in vitro assessments using aortic ring vessel segments in DMEM showed that magnesium concentration-response (0.8 to 2.5 mM) had a protective effect against vascular calcification starting between 1.38 mM and 1.72 mM.

Hemodynamic measurements:
Prior to sacrifice, the animals were anesthetized to a surgical plane using isoflurane (2.5%). Rats body temperature was maintained at 37°C using a thermistor temperature controller, and heating pad (Yellow Springs Instruments, Yellow Springs, OH). Pulse wave velocity (PWV) was assessed using the foot-to-foot method as previously described (Shobeiri et al., 2013). Briefly, two catheters were inserted into the superior (via carotid) and inferior (via iliac) portion of the aorta and arterial pressure was recorded as a pulsatile waveform (1000Hz) using Chart version 5 software. Arterial pressure was measured using PE-50 heparinized (50IU/mL) saline-filled cannula (ID 0.58mm, OD 0.965mm; Belton Dickson, Sparks, MD), and the distance between the tips of superior and inferior catheters was measured using 1-0 silk stretched between the two points. PWV was measured as (propagation distance)/(propagation time) between the superior and inferior catheter placements. At least 10 consecutive waveforms at each time point were individually analyzed then averaged. Systolic blood pressure, diastolic blood pressure and pulse pressure were measured from the carotid catheter. PWV was normalized to diastolic pressure to account for the known dependency of this variable on changes in arterial pressure (Spronck et al., 2015).

Vessel Phosphate, Calcium and Magnesium Content:

Vessels were weighed, and then fully dissolved in 1.0 N hydrochloric acid for 24h at 4°C. The samples were then spun and the calcium content was determined colorimetrically using the O-cresolphthalein complexone method (Sigma-Aldrich Canada Co., Oakville, ON, Canada). This reagent generates a purple color when in a complex with calcium; the linear range of detection is at least 40 fold. The absorbance for this complex was measured for both standards and the tissue homogenates at 540nm (SynergyHT
Microplate Reader, Bio-Tek Instruments, Winooski, VT). Tissue phosphate levels from the same samples as calcium were quantified using the malachite green method as previously described (Heresztyn and Nicholson, 2001). In brief, when ammonium molybdate is added, a green complex containing malachite green, molybdate, and free phosphate forms. The absorbance for this complex was measured for standards, tissue homogenates and plasma at 650 nm. Magnesium content was quantified colorimetrically using a modified Magnesium Reagent Kit (Pointe Scientific Inc., Canton, MI). Briefly, in this assay, magnesium ions react with xylidyl blue in an alkaline solution to produce a red complex that is measure spectrophotometrically at an absorbance of 530nm.

**Blood Biochemistry:**

Blood from the saphenous vein was obtained from all animals to measure serum creatinine and phosphate at baseline, 3, 5, and 7 weeks of adenine treatment, to track the progression of CKD. At the end of the experiment, serum calcium and magnesium were also determined. Creatinine levels were measured using the QuantiChrom Creatinine Assay Kit (DICT-500) (BioAssay Systems, Hayward, CA). Serum phosphate, calcium and magnesium assays were the same as for tissue samples.

At sacrifice (7 weeks), serum and plasma were procured from cardiac puncture for subsequent analysis. Parathyroid hormone (PTH) and FGF-23 (C-Term) concentration were measured using a commercially available Rat Intact Elisa (Immutopics, Clemente, California). Angiopoietin 2 was measured in serum using a commercially available Rat Quantikine Elisa kit (R&D Systems, Minneapolis, Minnesota)

**Western Blot Analysis:**
Protein extracts were prepared from vascular tissue samples by homogenization in RIPA buffer (0.150M NaCl, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.05M tris base). Protein concentrations were measured using Bio-Rad DC™ Protein Assay. Protein extracts (7.5µg) were separated by 10% SDS-PAGE followed by transfer to PVDF membrane. For the analysis of transient receptor potential cation channel, subfamily M, member 7 (TRPM7), Western blots were probed with rabbit anti-human TRPM7 antibody (1:1000 dilution, sc-98250; Santa Cruz Biotechnology, Santa Cruz, CA) at a overnight and incubated in the secondary (1:20,000 dilution, goat anti-rabbit immunoglobulins HRP, Dako) for 1 hr at room temperature. To assess the level of de-differentiation of the tissue, analysis for runt-related transcription factor 2 (RUNX2) by Western blot was performed. The gels were probed with rabbit anti-mouse RUNX2 antibody (1:5000 dilution, sc-10758; Santa Cruz Biotechnology, Santa, Cruz, CA) overnight and incubated in secondary (1:20000 dilution, goat anti-rabbit immunoglobulins HRP (Dako) antibody for 1 hr at room temperature. As a loading control, the blots were probed with anti-β-actin antibody HRP (1:10000 dilution, ab20272; Abcam Inc., Cambridge, MA) for 1 hr. TRPM7 and RUNX2 protein expression was expressed relative to the loading control and an internal control. Densitometry using ImageJ was used to quantify relative protein levels.

Real-time Polymerase Chain Reaction:

RNA was extracted using QIAGEN RNeasy Minikit (Canada – QIAGEN Inc., Mississauga, ON, CAN) following homogenization by Brinkmann Polytron (PT 10-20 sec, 3500 rpm; Bohemia, NY, USA). Aliquots of RNA were reverse transcribed using Applied Biosystems (ABI) high-capacity cDNA reverse transcription kit (ABI, Foster
City, CA, USA) and then amplified with SYBR Green PCR Master Mix (ABI). Forward and reverse primers, respectively, were as follows: β-actin, 5’ ACAACCTTCTTGAGTCCTCCTC-3’, 5’ CATACCCACCACCATCACACCCCTGG 3’; Runx2, 5’ CCAGATGGGACTGTGGTTACC 3’, 5’ ACTTGGTGCAGAGTTCAGGG 3’; TRPM7, 5’ GTCTCCCCACCAACCAAAAT 3’, 5’ CAGCAGCACCCACATGTTTCC 3’; SM22, 5’ CCAAGCCTTTTCTGCCTCAA 3’, 5’ ACAATCCACTCCACTAGCCG 3’ Plates were run in a Bio-Rad CFX96 Touch Thermocycler (Bio-Rad Laboratories Inc., Hercules, NY, USA) and expression data was analyzed using CFX Manager Software (Bio-Rad). Expression levels were calculated using the 2(-Delta Delta C(T)) method and normalized to β-actin expression levels (Schmittgen and Livak, 2008). Data are expressed relative to non-CKD age match controls.

Von Kossa method of assessing vascular calcification

Sections of abdominal aorta were fixed in neutral phosphate-buffered saline (10x) with 4% paraformaldehyde prior to embedding in paraffin. Embedded tissue was sectioned into five micrometer segments and placed on a glass slide. Vascular calcification was visualized using the Von Kossa method (McCabe et al., 2013).

Statistical analysis

Statistical analysis and graphical representation was performed using GraphPad Prism 6.0. Figure 3 and Figure 8 were presented as mean ± standard error of the mean (SEM). All other data were presented as mean ± standard deviation (SD). Statistical differences between treatment groups were determined with a two-way analysis of variance.
(ANOVA). As required, a Tukey correction was used for post hoc comparison of means for significant interaction. Data were log transformed to normalize distribution prior to statistical analysis (one way ANOVA) when required. P <0.05 was considered to be significantly different.

Results

Results of the biochemical analyses of serum samples appear in Figure 1 and Figure 2. To monitor the progression of kidney disease, serum creatinine (Figure 1A) and phosphate (Figure 1B) were measured at baseline, 3, 5, and 7 weeks on an adenine diet (0.25%). There was a significant main effect of duration of CKD on serum creatinine (Figure 1A, F(3, 106)=55.11, p<0.0001) and phosphate (Figure 1B, F(3, 100)=44.05, p<0.0001). Serum creatinine was slightly elevated in the CKD-M group compared to the CKD-C treatment group at 5 weeks (p<0.05). The decline in renal function after 7 weeks of adenine treatment was comparable between all groups (Figure 1A/B). Serum phosphate was similarly elevated in all of the treatment groups at all time points. After 7 weeks of CKD, total serum calcium levels were significantly elevated with calcitriol treatments. There was significant interaction between magnesium and calcitriol treatments on serum calcium, indicating that magnesium differentially modified calcium homeostasis (↓) in calcitriol treated rats (p<0.05, Figure 2A). There was a significant main effect of calcitriol (↑ 32%) and magnesium (↑ 24%) treatments on serum magnesium (p<0.05, Figure 2B). As expected, calcitriol treatment significantly suppressed PTH towards control levels and FGF-23 was significantly elevated (8.8X, Figure 2C/D) compared to the non-calcitriol groups (p<0.05). There was an overall main
Effect (^) of magnesium treatment on FGF-23 (p<0.05) and a specific within group FGF-23 increase in the calcitriol treated group (p<0.05). Calcitriol treatment also significantly lowered angiopoietin 2 levels (10.76 ± 2.85 pg/mL non-calcitriol vs. 8.18 ± 2.3 pg/mL calcitriol; p<0.05). There was also a significant main effect (↓ by 20%) of magnesium treatment on angiopoietin 2 levels (p<0.05).

Haemodynamic Consequences of Treatment:

Although there was no difference in mean arterial pressure (MAP) between treatment groups (Figure 3A), calcitriol treated rats had significantly elevated systolic blood pressure (SBP, Figure 3C; p<0.05), decreased diastolic blood pressure (DBP, Figure 3D, p<0.05) resulting in significantly increased pulse pressures (Figure 3B). The effects were most evident in changes to pulse wave velocity (PWV, Figure 3F); i.e. calcitriol alone induced a 4.2 fold elevation in PWV versus controls whereas magnesium-calcitriol treatment only induced a 2.0 fold increase versus the magnesium treated control (p<0.05).

Vascular Calcification:

To test the hypothesis that calcitriol and magnesium treatments modify vascular calcification severity in CKD, calcium and phosphate content was measured in the thoracic and abdominal aorta and iliac, carotid, renal, and superior mesenteric arteries (Figure 4). A main effect of calcitriol treatment was observed, such that treatments increased VC severity in four of the six vessel segments studied: thoracic aorta and carotid, renal and iliac arteries (p<0.05). To test whether magnesium status can further
modify VC severity, calcium content was measured in CKD rats supplemented with a high magnesium diet and CKD rats supplemented with calcitriol and magnesium. A main effect of magnesium treatment was observed in the abdominal aorta and the carotid, iliac and superior mesenteric arteries as magnesium treatments significantly attenuated VC severity (p<0.05). Post hoc analysis revealed within group differences in the calcitriol treated rats, such that magnesium treatment significantly attenuated VC severity in the abdominal aorta (52% reduction), iliac artery (45% reduction), and carotid artery (43%; p<0.05). The post hoc analysis did not identify any within group differences in the thoracic aorta and renal and superior mesenteric arteries. Calcitriol and magnesium treatments similarly impacted vessel phosphate content (data not shown). In all treatment groups, vessel phosphate content (nmol PO$_4^{3-}$/mg tissue) increased stoichiometrically with calcium content in a ratio predicted by hydroxyapatite crystal formation (Figure 5).

Staining of the vessels with von Kossa confirmed the presence of severe VC that was localized to the vascular media. Figure 6 shows representative images of the abdominal aorta from the different treatment groups.

*Vascular Magnesium Status:*

To determine whether calcitriol or magnesium treatments modify vascular magnesium status, magnesium content was measured across the vasculature (Figure 7). Calcitriol treatments significantly increased magnesium content in the thoracic aorta and the iliac, carotid and renal arteries (p<0.05). Post hoc analysis did not identify any effect of magnesium treatment on total vascular magnesium content. However, magnesium is incorporated stoichiometrically into the vasculature throughout the VC process (data not
shown). Therefore, relative magnesium status was assessed using the calcium to magnesium ratio (Ca/Mg; Figure 8). Calcitriol treatment alone decreased the relative vascular magnesium content (↑Ca/Mg ratio) in the thoracic aorta and the carotid and renal arteries (Figure 8; p<0.05). However, a main effect of magnesium treatment was identified in the abdominal aorta and carotid, iliac and superior mesenteric arteries such that magnesium treatments significantly improved the relative vascular magnesium status (↓Ca/Mg; p<0.05). Post hoc analysis revealed within group effects of magnesium treatment. Calcitriol + magnesium co-supplementation significantly improved relative vascular magnesium content compared to calcitriol treatment alone in abdominal aorta (42% ↓Ca/Mg) and carotid arteries (51% ↓Ca/Mg; p<0.05). These changes demonstrate a relative deficiency in vascular magnesium content occurs in the progression of CKD and with calcitriol treatment alone this is corrected with magnesium supplementation.

**Gene and Protein analysis:**

Analysis of the mRNA (Figure 9A) and protein (Figure 9 D) expression of TRPM7 (transient receptor potential cation channel, subfamily M, member 7) in segments of abdominal aorta revealed contrasting effects across the different treatment groups. Calcitriol treatments significantly decreased TRPM7 protein by 89%. Combination treatment with magnesium and calcitriol increased both the mRNA (1.7X) and protein (6.8X) expression compared to the calcitriol treatment alone (p<0.05). There were no differences in mRNA or protein expression of RUNX2 (runt-related transcription factor 2), and mRNA expression of SM22 (Smooth Muscle 22), in the abdominal aorta.
Discussion:

This study revealed that (i) overall, increasing dietary magnesium significantly attenuated the severity of VC across the vasculature in experimental CKD, (ii) high dose calcitriol treatment increased the susceptibility of blood vessels to calcification and (iii) increasing dietary magnesium attenuated the adverse effect of high dose calcitriol on calcification. The mechanism of this therapeutic benefit appears to only partially involve changes in the expression of the magnesium transporter, TRPM7; i.e. the calcium-magnesium ratio is significantly suppressed in 4 out of the 6 tissues assessed.

The prevalence of vitamin D insufficiency is well documented in CKD and is the acknowledged cause of secondary hyperparathyroidism and worsening calcium and phosphate homeostasis in these patients (Bergwitz and Juppner, 2010; Holden et al., 2010). In fact, up to 80% of CKD patients at the initiation of chronic hemodialysis have levels of 1,25(OH)2D3 and 25(OH)D3 below normal (Wolf et al., 2007). It is also well established that there are substantial alterations in the vitamin D metabolome in adenine-induced CKD in rats (Ikeda et al., 2010). Epidemiological evidence indicates that vitamin D deficiency is associated with adverse cardiovascular outcomes. According to the DOPPS Annual Report in Canada, treatment with vitamin D and analogs in CKD patients on dialysis is common and on the rise (50.6% in 2002 to 65.9% in 2011). This practice is largely based on the salutary effect on serum PTH and bone demineralization. Accordingly, careful monitoring of PTH and mineral levels, in particular, is important to avoid the iatrogenic adverse effects of these treatments and to emphasize the beneficial effects. In the current study, a key objective was to assess whether the negative impact of a moderate dose of calcitriol (80 ng/kg/day), previously shown to cause symptoms of
calcitriol toxicity, namely hypercalcemia, PTH over-suppression and VC, could be modified by concomitant magnesium (Krejs et al., 1983; Koleganova et al., 2009; Helvig et al., 2010; Lau et al., 2012). The results confirmed the anticipated dose-dependent, calcitriol-induced pathogenesis in this adenine model of CKD: i.e. suppression of PTH back to control levels, increased serum calcium by ~35% (but not serum phosphate), elevated FGF-23 and increased VC, with consequent increases in systolic blood pressure, pulse pressure and PWV. Angiopoietin-2 is a growth factor specific for the vascular endothelium, and is an important regulator of inflammation (Chang et al., 2014). Angiopoietin-2 is elevated in CKD patients and has been associated with mortality and vascular stiffness in these patients (David et al., 2012; Chang et al., 2014). Work is currently underway to elucidate the direct effect of angiopoietin-2 on vascular calcification. The present study demonstrates the calcitriol decreases angiopoietin-2 levels, and is further modified by magnesium status. Although speculative, it is possible that magnesium, which has anti-inflammatory properties (Moslehi et al., 2012), also inhibits VC through angiopoietin-2 dependent pathways.

The selected dose of calcitriol (Inagaki et al., 1995; Henley et al., 2005) clearly increased the susceptibility of vessels to VC, and to the associated pathogenic haemodynamic alterations (increased PWV, PP). Although this study did not elucidate the specific mechanism by which calcitriol increases the susceptibility of vessels to calcification, the findings did reveal that hallmark increases in FGF-23 and serum calcium could have played a significant role, consistent with previous work (Koleganova et al., 2009). Despite pre-clinical evidence of the adverse impact of vitamin D analogs on VC, many human epidemiological studies have demonstrated a survival benefit in
patients with higher serum vitamin D levels (Mursu et al., 2015). However, recent evidence suggests that this beneficial effect might only be in patients that have a high magnesium intake (Deng et al., 2013). Importantly, the present findings are consistent with this concept, demonstrating for the first time, that combining magnesium treatment with calcitriol, reduces the severity of both hypercalcemia and vascular calcification in conductance and peripheral vascular beds. Interpreting the mechanism underlying the increased severity of VC with calcitriol alone is complicated by the finding that serum magnesium is increased with this treatment. However, evidence is emerging that it is the concentration of magnesium at the tissue level that is critical and this level does not necessarily directly reflect the circulating milieu (Lim et al., 1969; Montezano et al., 2010).

Several in vitro vascular studies have identified magnesium as a potential anti-calcification moiety (Ennever and Vogel, 1981; Cheng et al., 1988; Montezano et al., 2010). The present study extends and confirms the previous in vitro findings to in vivo using an approach that uses a moderate level of magnesium supplementation to attenuate VC severity in various arteries. The mechanism of inhibition is likely dependent on the entry of magnesium into the vascular microenvironment, a process thought to be regulated, at least in part, by the transient receptor potential melastatin 7 cation channel (TRPM7). TRPM7 is a divalent ion transporter with a high affinity for magnesium. The overall decrease in the Ca:Mg ratio in magnesium-treated rats provides evidence of a differential effect on magnesium levels in the vascular microenvironment. Levels of intracellular magnesium are known to inversely affect channel activity through a feedback mechanism involving the phosphotransferase activity of TRPM7’s kinase.
domain (Schmitz et al., 2003; Macianskiene et al., 2012). The present study shows that the CKD rats given calcitriol have markedly lower TRPM7 protein expression in the vasculature compared to untreated controls. Decreased TRPM7 expression could be the key basis for the increased prevalence of VC in calcitriol treated animals, a suggestion supported by previous *in vitro* studies demonstrating that vessels readily calcify when TRPM7 channels are pharmacologically blocked (Montezano et al., 2010). Although the decreased expression of this protein herein may be due to the elevated magnesium levels in animals given calcitriol, a direct effect of calcitriol on TRPM7 expression remains to be elucidated. Furthermore, combining calcitriol with magnesium partially rescued the calcitriol-induced suppression of TRPM7 indicating there is significant cross-talk between the pathways. While our results indicate that the improved vascular magnesium status may be dependent, in part, on TRPM7 mediated processes, further research is also needed to confirm this effect.

It is well known that calcium and phosphate accrual occurs in the vascular microenvironment in a stoichiometric manner with increasing VC severity, in both uremic and aged patients (Contiguglia et al., 1973; Ongkana et al., 2007; Matsumoto et al., 2012). Our findings in rats with adenine-induced CKD confirm that magnesium is also incorporated within crystals of calcifying vascular tissues. In this study, one hypothesis was that for a given vascular calcium level, increasing magnesium content or availability would protect against further progression. Therefore, given that calcium sequestration is actively occurring, vascular magnesium content is more appropriately expressed relative to the calcification severity (Ca/Mg). In a relative magnesium deficient state, vessels exposed to the uremic milieu would readily form the calcium-phosphate
complexes, resulting in rapid crystal proliferation (Boskey and Posner, 1980). *In vitro* evidence suggests that magnesium does not directly affect hydroxyapatite growth; rather, it acts to stabilize the precursor amorphous calcium-phosphate phase (Nielsen, 1973; Tomazic *et al.*, 1975). The current study clearly demonstrates that magnesium supplementation increases the magnesium content relative to calcium. The study further demonstrates that magnesium treatment generates a significant reduction in VC severity that is generalizable to both proximal and distal vasculature. The robust reduction in VC severity parallels the increased availability of magnesium relative to calcium in the magnesium treatment groups; an effect which was even more pronounced in the combined calcitriol-magnesium group. Although currently a putative concept, the findings suggest that magnesium co-treatment might be able to extend the therapeutic window for calcitriol therapy in CKD, an outcome which could have important therapeutic implications. Further studies, investigating the full dose-response effects of calcitriol in combination with magnesium, will be required to fully validate this concept.

In addition, the study revealed that combining magnesium with calcitriol can both reduce hypercalcemia and yet similarly suppress PTH, while protecting, at least in part, the vasculature from calcium and phosphate accrual. The results confirm, as predicted, that calcitriol can increase VC under certain circumstances; an effect that is attenuated in the presence of increased magnesium. Importantly, the calcitriol-induced reduction in vascular TRPM7 protein expression was abrogated, at least in part, by magnesium co-treatment. Taken together, these data suggest that the benefit of the combined treatment likely involves (i) preventing reductions in TRPM7 expression and (ii) increasing the relative entry and availability of magnesium (↓Ca:Mg ratio) into the VC susceptible...
microenvironment. Modifying the adverse effect profile of calcitriol is important as the number of CKD patients’ prescribed calcitriol or vitamin D analogs is on the rise (Tentori et al., 2015). Given the increasing prevalence of vitamin D and analog treatments in CKD, the development of adjunctive treatment strategies that ameliorate the iatrogenic adverse effects continues to be an important area of research.

**Authorship Contributions:**

Participated in research design: Zelt, McCabe, Holden and Adams
Conducted experiments: Zelt, McCabe, Svajger, Barron, Laverty
Contributed new reagents or analytic tools: Laverty, Svajger, Barron
Performed data analysis: Zelt, McCabe, Holden, Adams
Wrote or contributed to the writing of the manuscript: Zelt, Holden, Adams

**Conflict of Interest Disclosures:** All authors have no conflicts of interest to disclose
References


Footnotes:

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Figure Legends:

Figure 1 Time course of serum creatinine (A), and phosphate (B) in rats treated with 0.25% adenine (Chronic Kidney Disease, CKD) at 0, 3, 5 and 7 weeks. CKD rats were treated with either the standard adenine diet (0.25% adenine, CKD, n=8), high magnesium diet (0.2%, CKD-M, n=8), high magnesium + calcitriol (0.2% Mg, 80ng/kg/day calcitriol, CKD-CM, n=8), or calcitriol (80ng/kg/day, CKD-C, n=8). Data are presented as mean ± SD. # p<0.05, CKD-C vs. CKD-M. Two-way ANOVA, as required, a Tukey correction was used for post hoc comparison of means for significant interaction.

Figure 2 Serum calcium (A), magnesium (B), parathyroid hormone (C) and fibroblast growth factor 23 (D) after 7 weeks of CKD (0.25% adenine, CKD). Rats were treated with either the standard adenine diet (0.25% adenine, CKD) or calcitriol (80ng/kg/day, 0.25% adenine). * Significant within group differences, p<0.05. # Main effect of magnesium treatment, p<0.05. Φ Main effect of calcitriol treatment, ω significant interaction, p<0.05. Two-way ANOVA, as required, a Tukey correction was used for post hoc comparison of means for significant interaction.

Figure 3 Mean arterial pressure (A), pulse pressure (B), Systolic blood pressure (C), diastolic blood pressure (D) heart rate (E), and pulse wave velocity (F) (Insert: Fold increase in PWV with calcitriol treatments from control), in anaesthetized rats (carotid catheter) treated with 7 weeks CKD (0.25% adenine n=32, 8 animals per group). Data are presented as mean ± SD. * Significant within group differences, p<0.05. Φ Main effect of
calcitriol treatment, p<0.05. Two-way ANOVA, as required, a Tukey correction was used for post hoc comparison of means for significant interaction.

Figure 4. Vascular calcium content (nmol/mg tissue) in the thoracic aorta (A), abdominal aorta (B), iliac artery (C), carotid artery (D), renal artery (E) and superior mesenteric artery (F) of rats treated with 7 weeks CKD (0.25% adenine; CKD) or CKD + calcitriol (80 ng/kg/day; 0.25% adenine). * Significant within group differences, p<0.05. # Main effect of magnesium treatment, p<0.05. Φ Main effect of calcitriol treatment, p<0.05. Two-way ANOVA, as required, a Tukey correction was used for post hoc comparison of means for significant interaction.

Figure 5 Calcium content as a function of tissue phosphate (thoracic and abdominal aorta and carotid, iliac, renal, superior mesenteric arteries) in CKD (A), CKD-M (B), CKD-C (C), and CKD-CM (D). This figure displays the average of all 5 calcium and phosphate values measured from individual vasculature segments. The dotted line reflects the stoichiometry (10:6) of hydroxyapatite.

Figure 6 Calcification of the aorta. Ex vivo photograph depicts the thoracic aorta from a calcitriol treated animal (80ng/kg/day) with adenine induced CKD (A). Von Kossa-stained abdominal aorta from health control rat (B) and animals treated with CKD generating diet (C-F). CKD-M (C), CKD-C (D), CKD-CM (E), and CKD (F).
Figure 7. Vascular magnesium content (nmol/mg tissue) in the thoracic aorta (A), abdominal aorta (B), iliac artery (C), carotid artery (D), renal artery (E) and superior mesenteric artery (F) of rats treated with 7 weeks CKD (0.25% adenine; CKD) or CKD + calcitriol (80 ng/kg/day; 0.25% adenine). * Significant within group differences, p<0.05. Φ Main effect of calcitriol treatment, p<0.05. Two-way ANOVA, as required, a Tukey correction was used for post hoc comparison of means for significant interaction.

Figure 8. Vascular calcium to magnesium ratio (Ca/Mg) in the thoracic aorta (A), abdominal aorta (B), iliac artery (C), carotid artery (D), renal artery (E) and superior mesenteric artery (F) of rats treated with 7 weeks CKD (0.25% adenine; CKD) or CKD + calcitriol (80 ng/kg/day; 0.25% adenine). * Significant within group differences, p<0.05. # Main effect of magnesium treatment, p<0.05. Φ Main effect of calcitriol treatment, p<0.05. Two-way ANOVA, as required, a Tukey correction was used for post hoc comparison of means for significant interaction.

Figure 9 Effects of treatments on mRNA expression (A-C) and protein expression (D-F) in the abdominal aorta. Levels of mRNA TRPM7 (A), RUNX2 (B), and SM22 (C) were determined by quantitative real-time PCR in triplicate and normalized to B-Actin mRNA levels. mRNA Data are expressed as fold increase above non-CKD control, mean ± SEM (n=8). Comparison of magnesium transporter TRPM7 (D) and Runx2 (E) protein expression in the abdominal aorta. Representative blots are provided (F). * Significant within group differences, p<0.05. Φ Main effect of calcitriol treatment, p<0.05. CKD rats were treated with either the standard adenine diet (0.25% adenine, CKD, n=8), high
magnesium diet (0.2%, CKD-M, n=8), high magnesium + calcitriol (0.2% Mg, 80ng/kg/day calcitriol, CKD-CM, n=8), or calcitriol (80ng/kg/day, CKD-C, n=8). Two-way ANOVA, as required, a Tukey correction was used for post hoc comparison of means for significant interaction.
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