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Effect of RANK/RANKL/OPG pathway on bone demineralization and vascular calcification in chronic kidney disease

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Summary

Introduction: In cases of chronic kidney disease (CKD), bone and mineral metabolism changes occur which favor soft tissue calcification. Alterations in the RANK/RANKL/OPG system could also favor vascular calcification, a major cause of morbidity and mortality in CKD.

Objective: In an *in vivo* experimental model of chronic renal failure progression, we assess the effect of CKD on vascular calcification and bone loss correlating these changes in the RANK/RANKL/OPG pathway. An *in vitro* system was used to confirm findings.

Material and Methods: Two models of vascular calcification were used: an *in vivo* rat model with chronic renal failure fed on a diet with different phosphorus content, and an *in vitro* model in vascular smooth muscle cells (VSMC) subjected to different calcifying stimuli.

Results: At 20 weeks, 50% of animals with a diet high in phosphorus presented aortic calcification accompanied by increased aortic expression of RANKL. In contrast, OPG decreased probably as a consequence of an inflammatory component.

At 20 weeks, expression of RANKL and OPG in the tibia increased, while the increase in OPG occurred at earlier stages.

In VSMC, the addition of uremic serum and calcification medium increased calcium content and expression of RANKL and OPG. The addition of OPG and silencing of RANK inhibited this increase.

Conclusions: Our results confirm RANK/RANKL/OPG system involvement in the vascular calcification process.

Key words: RANK, RANKL, OPG, chronic kidney disease, vascular calcification.

Introduction

Vascular calcification is a process in which vascular smooth muscle cells (VSMC) and other populations of blood vessel cells undergo a transformation and begin to resemble osteoblasts¹. This process is regulated in a manner similar to bone mineralization, with several bone proteins being implicated^{2,4}. Osteoblasts are cells responsible for the formation of bone that also regulate the activity of osteoclasts and therefore play an important role in the homeostasis of calcium (Ca) and phosphorus (P)⁵. Osteoblasts secrete the NF- κ B activator receptor ligand (RANKL) that binds to its receptor (RANK) in osteoclast precursors promoting formation, activation and survival^{6,7}. In addition, osteoblasts secrete osteoprotegerin (OPG), which acts as a soluble receptor lure of RANKL and inhibits the binding of this ligand to its transmembrane receptor RANK. There is considerable scientific evidence linking the RANK/RANKL/OPG system to vascular calcifications, which may be an important autocrine/paracrine system involved in the process. The pathway by which RANKL promotes calcification through binding to its RANK receptor with the consequent activation of the NF- κ B alternative pathway and bone morphogenetic protein 4 (BMP4)⁸ has been implicated in the osteogenic transition of VSMCs^{9,10}.

Chronic kidney disease (CKD) is characterized by changes in bone and mineral metabolism that favor the calcification of soft tissues and vessels. Alterations in the gene expression of the RANK/RANKL/OPG system could be favoring vascular calcification, one of the main causes of mortality in CKD. It is interesting to investigate the differences in the regulation of the RANK/RANKL/OPG system in bone and vessel in order to design strategies aimed at protecting the bone without having negative effects on vascular calcification.

Therefore, this study aims: a) to evaluate in a rat model the effect of CKD and diets with different P content on vascular calcification quantified by Ca content analysis and bone mineral density (BMD), quantified by bone densitometry; B) to correlate these changes with alterations in the RANK/RANKL/OPG system gene expression in arteries and bones of these animals; And c) to use an *in vitro* system to confirm the findings found *in vivo*.

Materials and methods

***In vivo* studies:**

Vascular calcification model

The protocol was approved by the University of Oviedo's Ethical Committee of Animal Experimentation.

The study was performed with male Wistar rats (n=55) at 4 months of age (350-400 g). Surgical intervention, following inhalation of isoflurane anesthesia, involved inducing chronic renal failure (CRF) (7/8) in a single surgical procedure. Complete nephrectomy of the right kidney and then subtotal nephrectomy of the left kidney were

carried out by lateral incision. This procedure preserves approximately one fourth of the renal mass. The rats with CRF were divided into two groups: one, CRF C, fed a standard rodent diet with normal P content (0.6% P, 0.6% Ca, and 20% protein content, Panlab, Barcelona, Spain), and the other, CRF P, fed a diet with high P content (0.9% P, 0.6% Ca, and 20% protein content, Panlab). The study lasted 20 weeks (CRF 20C and CRF 20P), time required to induce vascular calcifications. We also included a Sham group (n=10) that was followed up to week 20. Intermediate evaluations were also performed throughout the study, with sacrifices at 8 and 12 weeks (CRF 8C, CRF 12C, CRF 8P and CRF 12P). Twenty-four hours before slaughter, the rats were housed in metabolic cages and received diet and water *ad libitum*. They were sacrificed using CO₂ anesthesia, and serum samples were taken for analysis. From each rat the abdominal aorta was removed down to the bifurcation of the iliac crests and divided into three portions: the first fragment was used for the extraction of RNA, the second fragment to determine the Ca content, and the third fragment was stored in paraffin for future studies.

At the time of sacrifice the two tibia were removed. The left was preserved in alcohol to measure bone mineral density (BMD). The remaining tibia was frozen at -80°C until processed for the study of gene expression.

Biochemical markers

Serum urea, creatinine, Ca and P were measured using a Hitachi 717 multi-channel automatic analyzer (Boehringer Mannheim, Berlin, Germany). Parathyroid hormone (PTH) was measured by ELISA (Immutopics, San Juan Capistrano, USA) following the manufacturer's protocol.

Bone densitometry

BMD was measured in tibia at three levels: proximal octave, seven/eighth distal and total tibia, with a Hologic QDR-1000 dual-energy digital radiological densitometer (Hologic, Bedford, USA) equipped with a specific program for small animals.

Analysis of aortic calcification

Calcification of the rats' abdominal aorta was analyzed by two methods: total Ca content and von Kossa staining.

To determine total Ca content, a fragment of the abdominal aorta (the cm proximal to the iliac bifurcation) was homogenized with an Ultraturrax (OmniHT) in 0.6 N HCl. After shaking at 4°C for 24 hours the samples were centrifuged. The Ca content was determined in the supernatant by the o-cresolphthalein complexone method (Sigma-Aldrich, St. Louis, USA), and the pellet was resuspended in lysis buffer (125 mM Tris and 2% SDS, PH 6.8) for protein extraction and quantification by the method of Lowry (Bio-Rad, Hercules, USA). The Ca content was normalized by expressing as μ g Ca per mg protein.

To carry out von Kossa staining, another fragment of the abdominal aorta was included in methyl methacrylate (Sigma-Aldrich). Five 5 mm thick sections were obtained using a Polycut S Microtome (Reicher-Jung, Heidelberg, Germany) and stained following the von Kossa method.

Gene expression study

RNA extraction was carried out by the guanidinium-phenol-chloroform thiocyanate method. DNA copy (cDNA) was synthesized using the high capacity kit (Applied Biosystems, Foster City, USA). The RANK, RANKL and OPG gene expression was analyzed by real-time PCR (qPCR) on Applied Biosystems ABI Prism 7000 equipment. Assay on-demand assays designed by Applied Biosystems employing specific oligos and fluorescent Taqman probes were used for each of the PCRs. GAPDH was used to quantify and normalize the expression of the constitutive gene.

In vitro studies:

Primary culture of vascular smooth muscle cells (VSCM)

VSCM from primary culture of aorta explants from healthy Wistar rats at 2 months of age was used, sacrificing 12 rats and using CO₂ anesthesia. Abdominal aortas were removed and introduced into cold PBS with 100 units/mL penicillin and 100 mg/mL streptomycin (Biochrom AG, Berlin, Germany). After washing abundantly with cold PBS, the aortas were cut longitudinally; The endothelial layer was carefully removed and subsequently cut into fragments (explants) of 2 to 3 mm². The explants were plated in six-well culture plates (Sigma-Aldrich) pretreated with fibronectin (10 mg/cm²; Sigma-Aldrich). Once the explants were placed, 1 mL of DMEM (Dulbecco's Modified Eagle Medium, Biochrom AG) supplemented with 20% fetal bovine serum (FBS) (Biochrom AG) was added. The medium was renewed every 2 days. When the cells reached subconfluency, the tissue fragments were removed and the cells were enzymatically separated (0.25% trypsin and 1 mM EDTA).

Cells were seeded at a density of 10⁵ cells per culture dish (Sigma-Aldrich) with DMEM supplemented with FBS (10%). Cells obtained by this method were identified as VSCM by the following criteria: (1) cells grow in the characteristic valley and choline pattern; And (2) immunostaining was positive for alpha-actin (mAb from Sigma-Aldrich).

Cells between passages 2 and 8 were used, using three wells per condition and the experiments were performed in triplicate.

Induction of calcification in VSCM

In order to analyze the uremia-induced calcification and to know the implication of the RANK/RANKL/OPG system, two different conditions were used.

For the first condition, the VSCM cultures were treated with DMEM supplemented with 15% uremic rat serum (a set of 8-week CRF rat sera con-

taining 10.8 mg/dL Ca, 6.7 mg/dL P, and 898 pg/mL PTH). As a control condition DMEM was used with 15% serum from healthy rats (a pool of sera containing 10.4 mg/dL Ca, 3.6 mg/dL P and 25 pg/mL PTH).

In a second condition, to confirm the effect of P, the VSCMs were cultured with calcifying medium: DMEM F12+0.1% bovine serum albumin (BSA) with 2 mM Ca and 3 mM P). DMEM control F12+0.1% BSA was used as condition. In both cases, Ca deposition was determined 4 and 8 days after addition of the stimuli.

The effect of OPG (100 pM), silencing of the RANK receptor (increasing concentrations between 100 pM and 100 nM) was tested in VSCM in which calcification was induced with DMEM F12+0.1% BSA with 2 mM Ca and 3 mM of P.

Gene expression study

We proceeded in the same manner as detailed in the section on *in vivo* studies.

Lentiviral production and infection/RANK silencing by shRNA

The RANK gene was silenced in the VSCM by small forks of RNA (shRNA), which were cloned into a lentivirus-based vector (FSVsi). In it were introduced shRNAs whose target was TTAGCTGAGGATGCTGAGGAT and scramble sequences. All of them were co-transfected with the virion packaging elements (VDV-G) in a 293T cell culture using polyethyleneimine. Infectious particles were produced by culturing the cells 3-4 days in medium for VSCM. The medium was then centrifuged at 1,000 g for 5 min and the supernatant was added to a VSCM culture, being replaced by the conventional medium after overnight incubation. Finally, the VSCMs were collected after 4 days and the silencing of RANK with qPCR and Western Blot was checked.

Western Blot

After transfer, the membranes were incubated for 12 hours with anti-RANK antibodies (1:1,000, Cells Signaling Technology, Danvers, USA), and anti-tubulin (01:10,000, Sigma-Aldrich). Binding of the secondary antibody was detected with the Western Blot detection kit ECL Advance (Amersham Bioscience, Buckinghamshire, UK) and the VersaDoc 4000 (Bio-Rad) imaging system.

Statistical analysis

For the statistical analysis of the results, the SPSS 17.0 program was used. In the case of variables with normal distribution, the comparison of the treatment groups was performed using ANOVA with the Bonferroni test. In the case of variables with non-normal distribution, the Kruskal-Wallis test was used.

Results

1. Biochemistry

In the groups that received the diet with a high P

content (CRF 8P, CRF 12P, CRF 20P), a slight deterioration of renal function was observed with respect to their controls (CRF 8C, CRF 12C, CRF 20C). Aggravated at 20 weeks (Table 1). In the high P diet group, serum Ca significantly decreased only at week 20 (CRF 20P), while serum P increased in all groups with a high P diet, particularly at 20 weeks of treatment. Parallel to P, PTH increased as treatment time increased, being statistically significant from week 12 and particularly at week 20, where severe secondary hyperparathyroidism was observed (Table 1).

2. Densitometric study

Although there was a slight decrease in the BMD of the groups of animals with high diet in P regarding their controls in all the studied sectors, this was only significant at 20 weeks (Table 2). Losses were predominant at the distal level, where there is a higher content of cortical bone, on losses at the proximal level (Table 2).

3. *In vivo* effect of uremia and P overload on vascular calcification, bone activity and RANK/RANKL-OPG system

Although the Ca content of the aortas of animals fed a normal P-content diet was slightly affected by uremia, administration of a diet with high P content increased Ca significantly in a time-dependent manner with respect to the Sham group. Animals receiving the high P diet increased the aortic content of Ca with respect to their respective controls from week 12, with this effect being magnified at week 20. Despite the generalized increase in aortic Ca content, von Kossa revealed visible calcifications in the aorta in only 50% of the animals with diet with high content in P (Figure 1).

Parallel to the increase in Ca content there was an elevation of RANKL expression in the aorta (Figure 2A). RANK expression did not show any differences along the course of CRI (Figure 2B),

whereas OPG decreased in all uremic groups, particularly those receiving a high P diet (Figure 2C).

In the tibia, an increase in RANKL and OPG expression was observed at week 20 of the high P diet group (Figures 3A, 3C). OPG expression also increased in all groups receiving high P diet, noting the increase observed at week 20. In contrast, RANK expression remained similar in all groups.

4. *In vitro* effect of uremia and P overload on vascular calcification and the RANK-RANKL-OPG system

Uremic serum induced a significant increase in Ca content at 4 and 8 days (Figure 4A). There was a significant increase in the expression of RANKL (at 4 and 8 days) and OPG (at 8 days of treatment) (Figures 4B, 4C and 4D).

Calcifying medium-treated VMCV (DMEM F12, 2 mM Ca, 3 mM P) showed a significant increase in time-dependent Ca content (Figure 5A). In parallel increased RANKL and OPG expression (Figures 5B, 5C and 5D).

5. *In vitro* effect of the addition of OPG on calcification induced by uremic serum

To confirm the idea that increased RANKL expression is responsible for the Ca content increase in VSCM treated with uremic serum, 100 pM OPG added to the culture medium, which led to a significant decrease of OPG (Figure 6).

6. *In vitro* effect of RANK silencing on calcification induced by uremic serum

Similarly, silencing of the RANK receptor by the shRNA technique significantly reduced the Ca content of the VSCM treated with uremic serum (Figure 7).

Discussion

CKD, a disease characterized by a progressive loss of renal function, leads to the appearance of mul-

Table 1. General biochemistry in the different treatment groups

	SHAM (n=10)	CRF 8C (n=9)	CRF 8P (n=9)	CRF 12C (n=7)	CRF 12P (n=10)	CRF 20C (n=10)	CRF 20P (n=10)
Urea (mg/dL)	34±9	108±27	137±45	119±29	143±51	100±70	200±70
Creatinine (mg/dL)	0.4±0.1	1.0±0.3	1.4±0.5	1.3±0.4	1.3±0.6	1.5±1.3	2.2±0.8*
Calcium (mg/dL)	11.4±0.6	11.8±0.9	11.4±0.8	12.4±0.6	11.4±2.0	12.5±0.6	11.1±0.4*
Phosphorus (mg/dL)	4.8±1.0	5.7±1.1	10.0±3.7*	6.0±1.9	9.4±3.4*	5.6±0.8	12.1±2.8*
PTH (pg/mL)	22 (4-74)	28 (19-55)	139 (59-933)	62 (27-121)	236 (128-1,113)*	80 (54-115)	1,901 (1,117-2,517)*

CRF 8C, CRF 12C, CRF 20 C: groups of rats fed diets with normal phosphorus content sacrificed at 8, 12 and 20 weeks, respectively; CRF 8P, CRF 12P, CRF 20 P: groups of rats fed a high phosphorus diet sacrificed at 8, 12 and 20 weeks, respectively; *P<0.005 relative to its control group with normal diet in P.

Table 2. BMD values in the tibia in the different treatment groups

	SHAM (n=10)	CRF 8C (n=9)	CRF 8P (n=9)	CRF 12C (n=7)	CRF 12P (n=10)	CRF 20C (n=10)	CRF 20P (n=10)
Proximal T. (mg/cm ²)	335±22	307±13	303±24	318±19	302±19	332±18	311±29
Distal T. (mg/cm ²)	276±10	243±12	236±15	247±12	239±9	263±13	236±20*
Total T. (mg/cm ²)	288±12	256±11	250±14	261±13	252±11	277±12	251±21*

CRF 8C, CRF 12C, CRF 20 C: groups of rats fed diets with normal phosphorus content sacrificed at 8, 12 and 20 weeks, respectively; CRF 8P, CRF 12P, CRF 20 P: groups of rats fed a high phosphorus diet sacrificed at 8, 12 and 20 weeks, respectively; *P<0.001 relative to its control group with normal diet in P.

tiple complications and alterations of the cardiovascular system. In order to simulate CKD, we used the animal model with normal CKD from our laboratory.

According to what was observed in the biochemical markers analyzed, the development of CKD was accompanied by alterations in bone and mineral metabolism which were aggravated by hyper-phosphoremia and the development of secondary hyperparathyroidism. It is well recognized that the latter increases bone turnover, negatively affecting the cortical bone (seven/eighths of the tibia or distal area) more severely than the trabecular bone (octave of the tibia or proximal area), which is corroborated in our animals. Although PTH is able to stimulate the expression of OPG, as demonstrated in the tibia of animals with CRF with severe hyperphosphoremia, this hormone is also capable of inducing the expression of RANKL in osteoblasts¹¹, and this gene may be responsible for the BMD decrease recorded in the densitometric study.

The RANK/RANKL/OPG system has also been associated with vascular calcification. Initial evidence of its implication in this process derives from the study with null mice for OPG that, in addition to a severe decrease in BMD and a high incidence of fractures, calcifications of the aorta and renal arteries¹².

OPG has the ability to inhibit osteoclastic activity and thus prevent the onset of vascular calcification. In fact, in our *in vitro* model, vascular calcification induced by uremic serum was attenuated by the addition of OPG.

As some authors have described, a direct relationship between calcification and increase of RANK/RANKL/OPG at the bone level has been observed. The decrease of OPG in aortic tissue induced in our animal model by uremia is in line with what has been reported by other authors. While RANKL was clearly detectable in patients with calcified aortic stenosis, OPG levels were not detectable¹³. These reductions of aortic OPG by uremia could be due to the process of inflammation during the calcification that occurs with decreases in OPG^{14,15}.

Figure 1. A) Ca content in aortas of 7/8 nephrectomy rats fed a diet with normal content in P (0.6%) (gray bars) and high P content (0.9%) (black bars), sacrificed at 8 and 20 weeks. Data represent the mean ± standard deviation. *P<0.05 vs same week with normal P (0.6%). B) von Kossa staining of the aortas in the different treatment groups

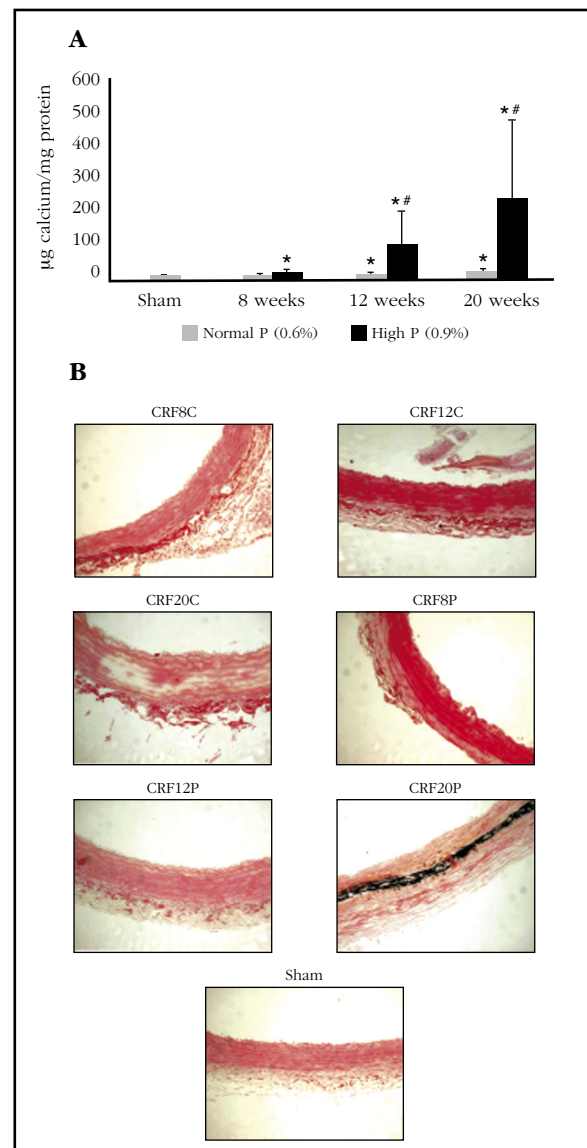


Figure 2. Expression of RANKL (A), RANK (B) and OPG (C) in the aortas of rats with nephrectomy 7/8 fed with diet with normal content in P (0.6%) (gray bars) and high content in P (0.9%) (black bars), sacrificed at 8 and 20 weeks, as determined by q-PCR. Data represent the mean \pm standard deviation. * $P < 0.05$ vs Sham group # $p < 0.05$ vs same weeks with normal P (0.6%)

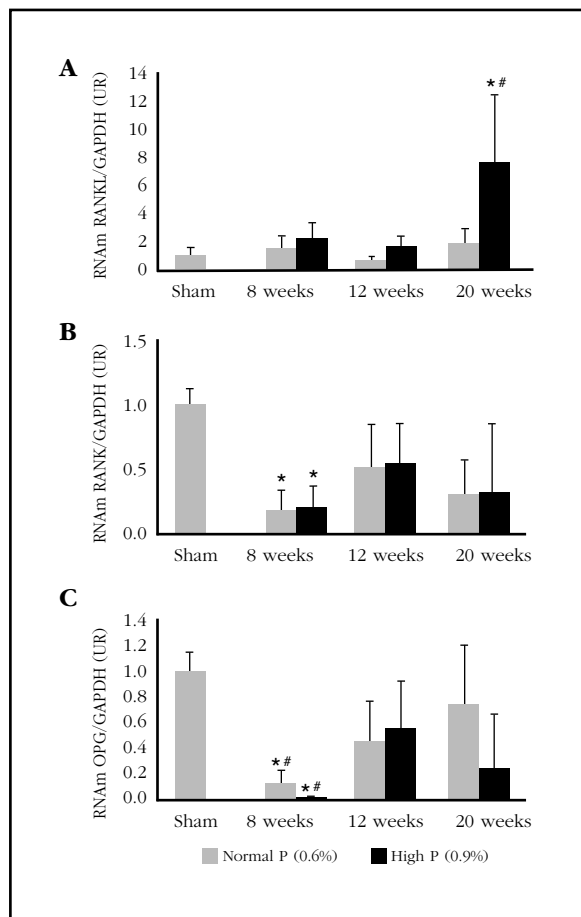
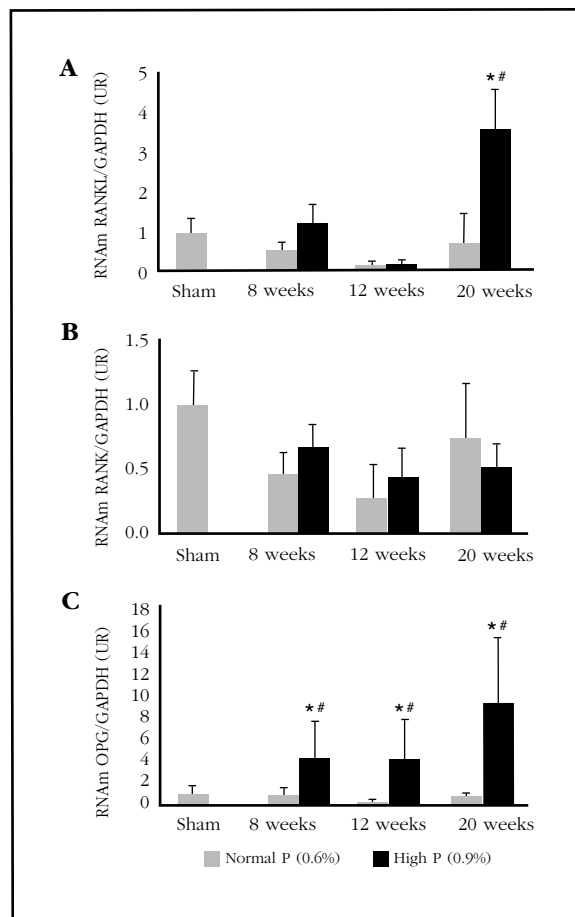


Figure 3. Expression of RANKL (A), RANK (B) and OPG (C) in the tibia of rats with nephrectomy 7/8 fed with diet with normal content in P (0.6%) (gray bars) and high content in P (0.9%) (black bars), sacrificed at 8 and 20 weeks, as determined by q-PCR. Data represent the mean \pm standard deviation. * $P < 0.05$ vs Sham group # $p < 0.05$ vs same weeks with normal P (0.6%)



Some authors have described vascular calcification as an active process and regulated by various factors. The VSMC in the early stages of the calcification process undergo a change in its phenotype and begin to express osteogenic markers, which would allow the mineralization of the extracellular matrix. One of these proteins is RANKL, whose expression is abundant in osteoblasts. Both in the aortas and tibias of the rats and in the VSMC there was an increase in the expression of RANKL, aggravated by the increase of P in the diet. In our paper, we show the direct relationship between the increase in calcification and the increase of RANKL. Osteoblasts secrete RANKL, a process that can be reversed by OPG, a protein that sequesters RANKL thus inhibiting the formation of osteoclasts by preventing RANKL from binding to its RANK receptor. The silencing of RANK in our *in vitro* model of calcification with uremic serum inhibited the calcification process by preventing the binding of RANKL to RANK.

Other studies have also shown that RANKL expression increases in calcified areas¹⁶⁻¹⁸, as occurred in the aortas of the animals studied. While in

bone an increase of RANKL favors demineralization by an increase in osteoclastic activity, in the vessels it stimulates osteogenesis and, therefore, calcification¹⁹. In fact, Kindle L et al. suggest that the endothelial cells of the vessel produce a microenvironment favorable to the formation of calcified tissue, stimulating the migration and adhesion of monocytes through the endothelium that can be differentiated into osteoclasts in the presence of RANKL²⁰. It has recently been demonstrated that VSMC incubated in a calcifying medium to which RANKL is added increases its Ca content and alkaline phosphatase activity, whereas coinubation with OPG is able to inhibit calcification induced by RANKL⁸.

The hypothesis that the RANK/RANKL/OPG system could explain part of the relationship between osteoporosis and vascular calcification is based on multiple epidemiological studies that have revealed the association between bone and vascular metabolism, noting that the decrease in bone mass and increased fractures were associated with a higher prevalence and progression of vascular calcifications in the general population and in populations at risk²¹⁻²⁶, with the latter being those with CKD.

Figure 4. Ca (A) content and expression of RANKL, RANK and OPG in the VCAM of rats treated with DMEM supplemented with 15% of control serum (gray bars) or uremic (black bars) at baseline (0), 4 and 8 days, as determined by q-PCR. Data represent the mean \pm standard deviation. * $P < 0.05$ vs basal; # $p < 0.05$ vs same time with DMEM and serum control

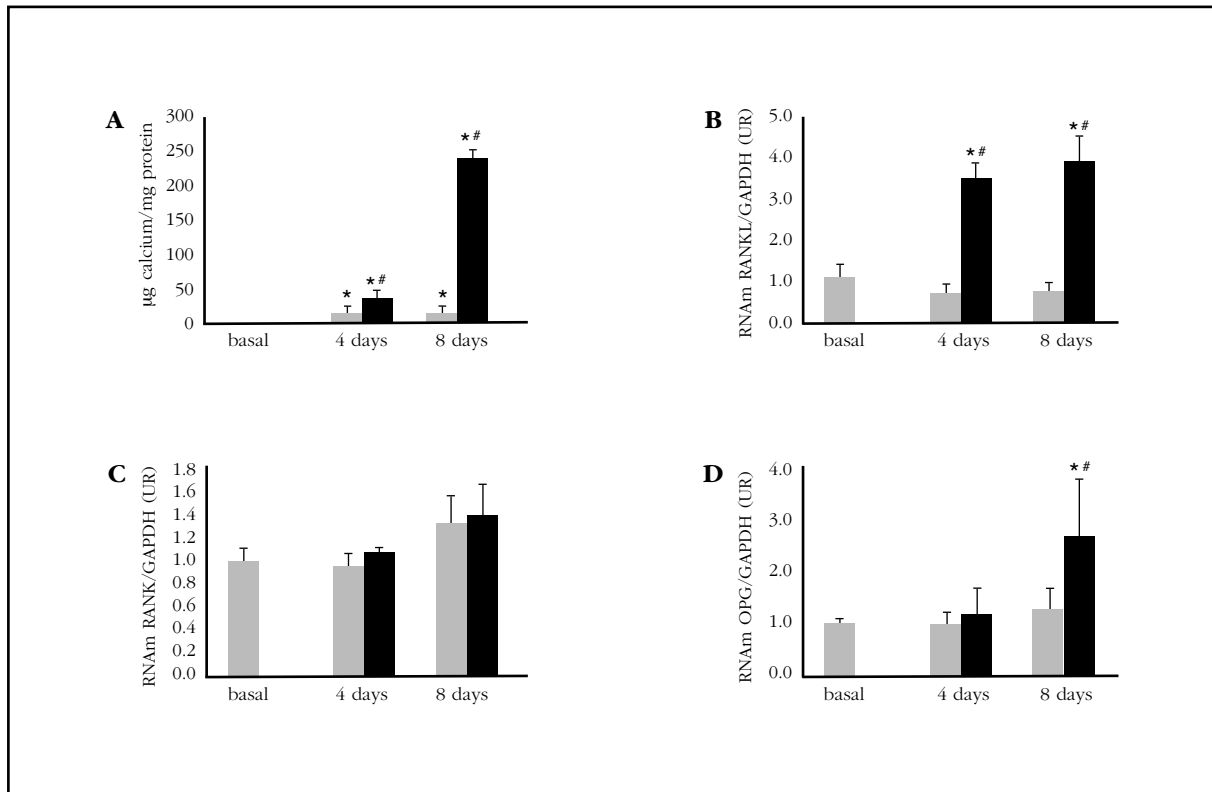


Figure 5. Ca (A) content and expression of RANKL (B), RANK (C) and OPG (D) in the VMCs of rats treated with DMEM control (1mM Ca 1mM P) (gray bars) or calcifying (2mM Ca 3mM P) (black bars). Data represent the mean \pm standard deviation. * $P < 0.05$ vs basal; # $p < 0.05$ vs same time with DMEM control

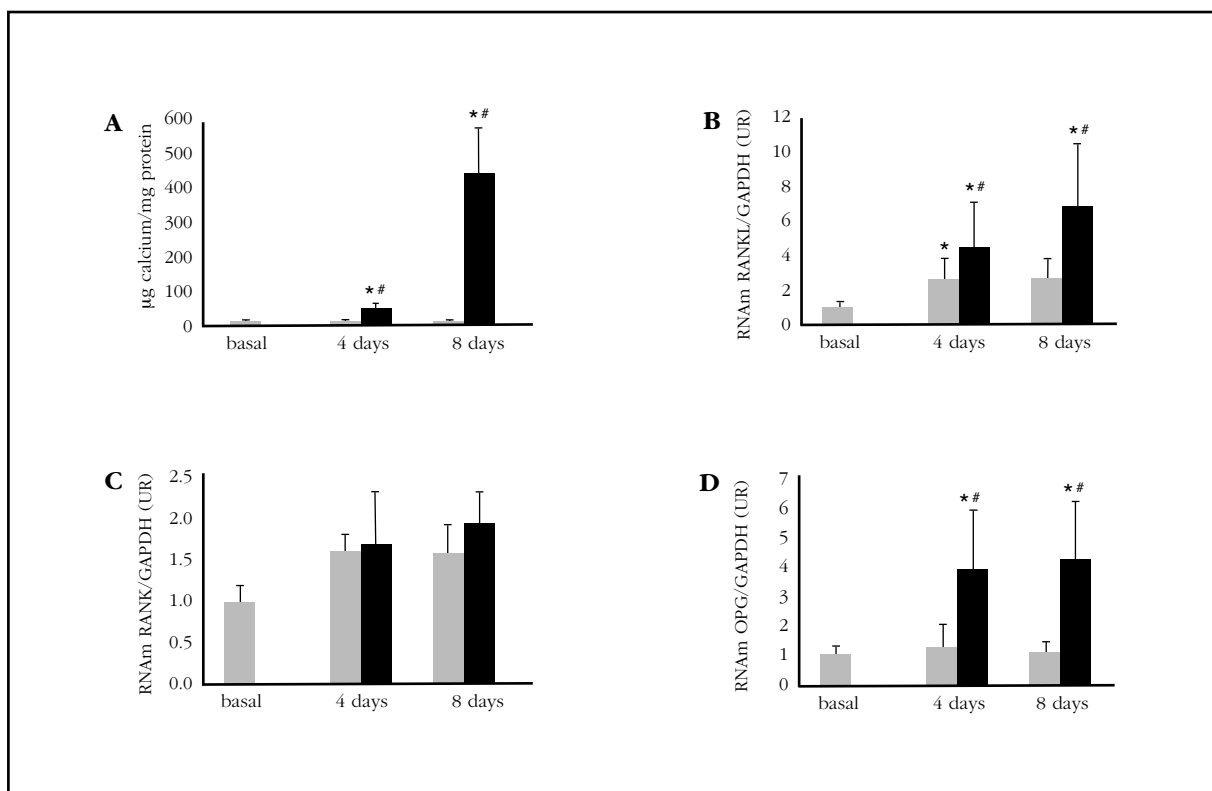
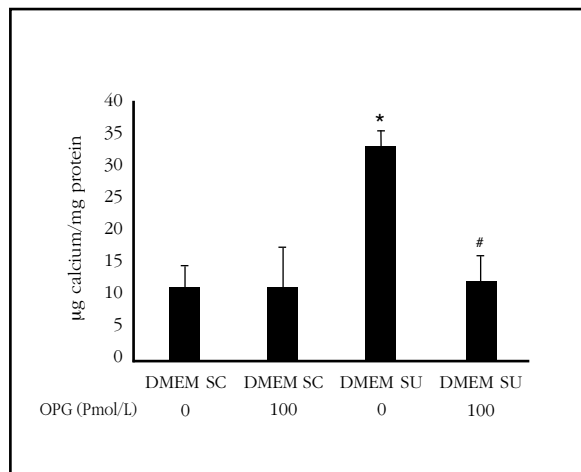


Figure 6. Ca content in VSCM of rats treated with DMEM + 15% control serum (DMEM SC) or DMEM + 15% uremic serum (DMEM SU) with or without 100 pM OPG. Data represent the mean \pm standard deviation. *P<0.05 vs DMEM SC; #p<0.05 vs DMEM SU

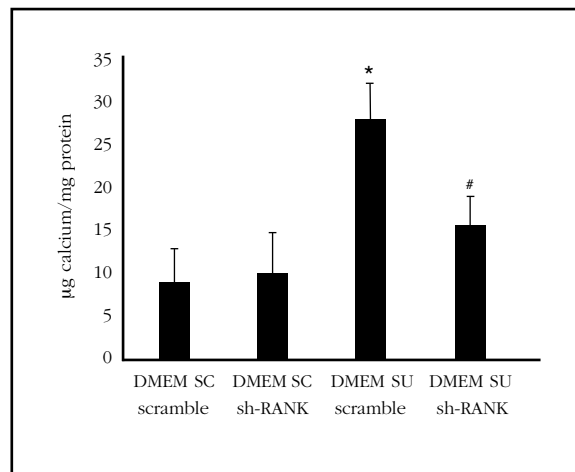


The Wnt pathway is an intracellular signaling pathway involved in bone formation. Due to the similarities between bone formation and calcification, it has been suggested that the inactivation of the Wnt pathway could attenuate the calcification process, as has been described by several authors²⁷⁻²⁹. Data from our group, in the same experimental model, have shown an increase in the gene expression of inhibitors of the Wnt pathway in the group of animals with vascular calcification, suggesting a protective mechanism of the progression of calcification³⁰. On the other hand, we should not forget that a negative balance of inhibitors of calcification, such as fetuin A, could also favor the calcification process^{31,32}.

Our *in vivo* results indicate the involvement of the RANK/RANKL/OPG axis in vascular calcification and changes in BMD as a consequence of CKD and of stimuli favoring the former. Moreover, in our *in vitro* model, the addition of OPG as well as the silencing of RANK reduced calcification, indicating that the RANK/RANKL/OPG system acts in this process, opening the doors to new investigations in this area. Because of their importance in the regulation of bone turnover, RANK/RANKL/OPG axis members could be used in the future as useful biomarkers in assessing bone function in patients with CKD.

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Figure 7. VSCM Ca content of rats transfected with scramble (control) or with sh-RANK with DMEM + 15% serum control (DMEM SC) or DMEM + 15% uremic serum (DMEM SU). Data represent the mean \pm standard deviation. *P<0.05 scramble with DMEM SC; #p<0.05 vs scramble with your DMEM SU



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