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Effect of Crystallization Inhibitors on Vascular Calcifications Induced by Vitamin D

— A Pilot Study in Sprague-Dawley Rats —

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Background Pathological calcification in soft tissues (ie, ectopic calcification) can have severe consequences. Hydroxyapatite is the common mineral phase present in all tissue calcifications. In general, the development of tissue calcifications requires a pre-existing injury as an inducer (heterogeneous nucleant), whereas further progression requires the presence of other promoter factors (such as hypercalcemia and/or hyperphosphatemia) and/or a deficiency in calcification repressor factors (crystallization inhibitors and cellular defense mechanisms). The present study investigated the capacity of etidronate (a bisphosphonate used in osteoporosis treatment) and phytate (a natural product) to inhibit vascular calcification in rats.

Methods and Results Six male Sprague-Dawley rats in each of the 3 treatment groups were subcutaneously injected with either a placebo (physiological serum solution), etidronate ($0.825\,\mu mol \cdot kg^{-1} \cdot day^{-1}$) or phytate ($0.825\,\mu mol \cdot kg^{-1} \cdot day^{-1}$) for 8 days. Four days into this regimen, calcinosis was induced by subcutaneous injections of 500,000 IU/kg vitamin D at 0 h, 24 h and 48 h. Ninety-six hours after the final vitamin D injection, the rats were killed and aortas and their hearts were removed for histological and calcium analyses. The data showed that phytate-treated rats had lower levels of aortic calcium than placebo-treated rats. All groups had similar heart calcium levels.

Conclusions The present study found that phytate acted as a vascular calcification inhibitor. Thus, the action of polyphosphates could be important in protecting against vascular calcification. (*Circ J* 2007; **71:** 1152 – 1156)

Key Words: Crystallization inhibitors; Etidronate; Phytate; Vascular calcification; Vitamin D

Pathological calcification in soft tissues (ie, ectopic calcification) can have severe consequences when it occurs in vital organs such as the vascular or renal systems. Ectopic calcification in arteries can cause thrombosis, arterial rupture and myocardial infarction[1–3] In the heart, the most common valvular lesion is aortic stenosis because of valvular calcification, and this can lead to heart failure and death[4] In the kidneys, tissue calcification can be associated with the formation of the calcium oxalate monohydrate papillary calculi-type kidney stone[5] and in extreme cases can cause renal failure[6]

Hydroxyapatite (basic calcium phosphate crystals) is the common mineral phase present in all tissue calcifications. In general, the development of tissue calcification requires a preexisting injury as an inducer (heterogeneous nucleant), whereas further progression requires the presence of other promoter factors (such as hypercalcemia and/or hyperphosphatemia) and/or a deficiency in calcification repressor factors (crystallization inhibitors and cellular defense mechanisms).

Several proteins modulate calcification in mammalian tissues and their activity can either enhance or inhibit the ability of macrophages to destroy hydroxyapatite deposits (ie, osteoclastic activity)[7–9] A common characteristic of the proteins involved in calcification is calcium ion affinity, and there are 2 major groups of such proteins: phosphoproteins and carboxyproteins. Major phosphoproteins include osteopontin[10–13] and osteoprotegerin[14–16] and a major carboxyprotein is matrix Gla protein[17–19] or bone-Gla-protein, also known as osteocalcin[9,20] Although these proteins have been suggested to have some crystallization inhibitor activity, the in vitro experiments that demonstrated such effects involved the use of nonphysiological concentrations of these proteins and of calcium and phosphate[21–23] Moreover, these proteins have been reported to also have some calcification promoter activity because of their heterogeneous nucleant capacity[24–26] It appears that the major calcification modulator role of these proteins is as regulators of osteoclast/osteoblast cell activity[7,8,27]

Crystallization inhibitors obstruct or prevent crystal development. In general, crystallization inhibitors bind to the crystal nucleus or to the crystal face and hence prevent or disturb crystal development without any cellular signaling capacity. Pyrophosphate[28] bisphosphonates (such as etidronate, alendronate and ibandronate)[29–31] and phytate (myo-inositol hexakisphosphate)[32] have been shown to inhibit crystallization in the form of vascular calcification. Etidronate is used to treat osteoporosis[33] and phytate is a naturally occurring compound that can either be ingested[34,35] or absorbed topically[36]

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Table 1 Composition of the UAR-A04 Diet

Moisture (g/kg)	*119*
Crude protein (g/kg)	*161*
Crude oil (g/kg)	*31*
Nitrogen free extract (g/kg)	*600*
Crude fibre (g/kg)	*3.9*
Calcium (mg/kg)	*8,400*
Phosphorus (mg/kg)	*5,700*
Sodium (mg/kg)	*2,500*
Potassium (mg/kg)	*6,400*
Manganese (mg/kg)	*70*
Copper (mg/kg)	*17*
Phytate (mg/kg dry matter)	*9,000*

The present study investigated the ability of etidronate and phytate to act as inhibitors of vascular calcification.

Methods

Animals and Diets

Male Sprague-Dawley rats (approximately 450 g) were purchased from Harlan Iberica S.L. (Barcelona, Spain). Rats were given 7 days to acclimatize to the animal house conditions before the experimentation. Rats were fed a UAR-A04 diet (Panlab S.L., Barcelona, Spain; Table 1), and were kept in Plexiglas cages (3 per cage) at a temperature of 21±1°C and relative humidity of 60±5%, under a 12 h light/dark cycle.

Experimental procedures were performed according to the Directive 86/609/EEC regarding treatment of animals used for experimental and other scientific purposes. Permission to perform these animal experiments was obtained from the Bioethical Committee of the University.

Treatments

Placebo, Etidronate and Phytate Treatments Rats (6 per group) were administered a placebo, etidronate or phytate subcutaneously once daily for 8 days (ie, for the duration of the experiment). Placebo was administered at 200 µl/day as sodium chloride 0.9% solution, etidronate was administered at 0.825 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ in the acid form (Fluka, Buchs, Schweiz), and phytate at 0.825 $\mu mol \cdot kg^{-1} \cdot day^{-1}$ as a sodium salt (Sigma, St Louis, MO, USA).

Calcinosis Induction The rats were treated for 4 days, then underwent calcinosis induction, as described previously[31] Briefly, the rats were given 3 subcutaneous injections of 500,000 IU/kg vitamin D (Fort Dodge Veterinaria S.A., Fort Dodge, USA) at 0, 24 and 48 h. They were monitored every 12 h and at 96 h after the 3rd injection they were killed and their aortas and hearts removed for calcium determination and histological analysis.

Histological Analysis

Aortas and hearts were placed in 4% buffered formaldehyde at pH 7 (Panreac, Barcelona, Spain) and fixed for 24 h at room temperature. Tissues were then embedded, sectioned (4 µm) and stained with hematoxylin-eosin. Tissue analysis was performed by an experienced pathologist.

Calcium Determination

Aortas and hearts were lyophilized and weighed, and then digested using a 1:1 HNO_3:$HClO_4$ mixture in a sand bath until the solution was clear. For calcium determination, digested samples were diluted with distilled water to a volume of 20 ml, and the concentration of calcium was determined using inductively coupled plasma atomic emission spectrometry (Perkin-Elmer SL, Optima 5300DV spectrometer) and a corresponding calibration curve.

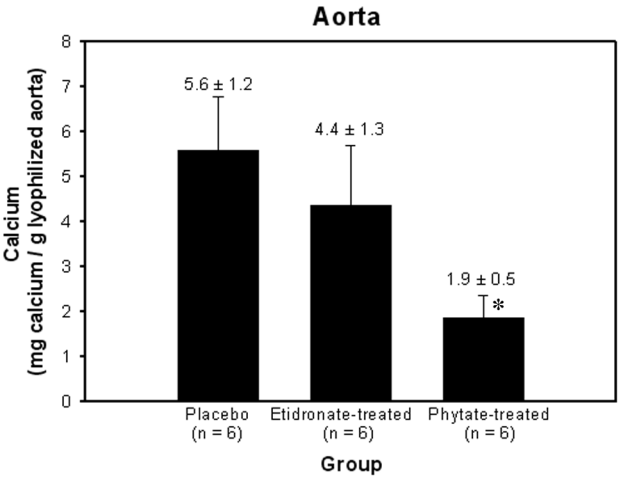

Fig 1. Calcium content of the aortas of placebo-treated, etidronate-treated and phytate-treated rats. *p<0.05 vs the placebo group.

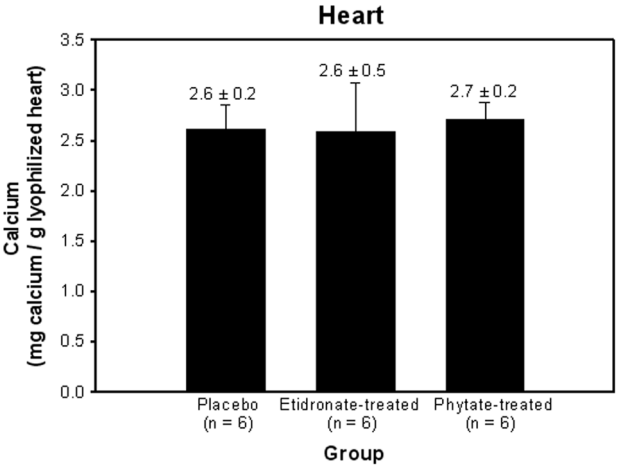

Fig 2. Calcium content of the hearts of placebo-treated, etidronate-treated and phytate-treated rats.

Statistical Analysis

Values are expressed as mean ± SE. One-way ANOVA was used to determine the significance of differences between groups. Student's t-tests were used to assess differences between means. Conventional Windows software was used for statistical computations. A p-value <0.05 was considered to indicate a significant difference.

Results

Analysis of the aortas showed that phytate-treated rats had lower levels of calcium in the aorta (1.9±0.5 mg calcium/g lyophilized aorta) than did placebo-treated rats (5.6±1.2 mg calcium/g lyophilized aorta) (Fig 1). Although etidronate-treated rats (4.4±1.3 mg calcium/g lyophilized aorta) also appeared to have lower aortic calcium levels than placebo-treated rats, this difference was not statisti-

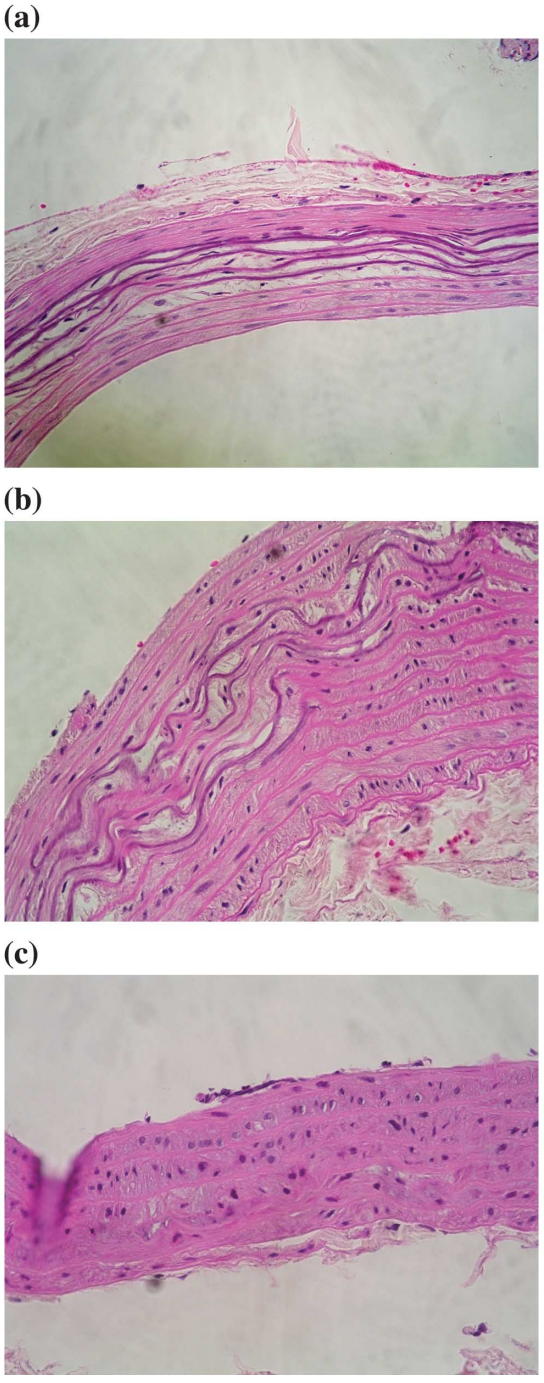

Fig 3. Sections of aortas from placebo-treated (original magnification ×100) (a), etidronate-treated (original magnification ×200) (b) and phytate-treated (original magnification ×100) (c) rats. Each section is stained with hematoxylin-eosin solution, which stains calcification dark purple.

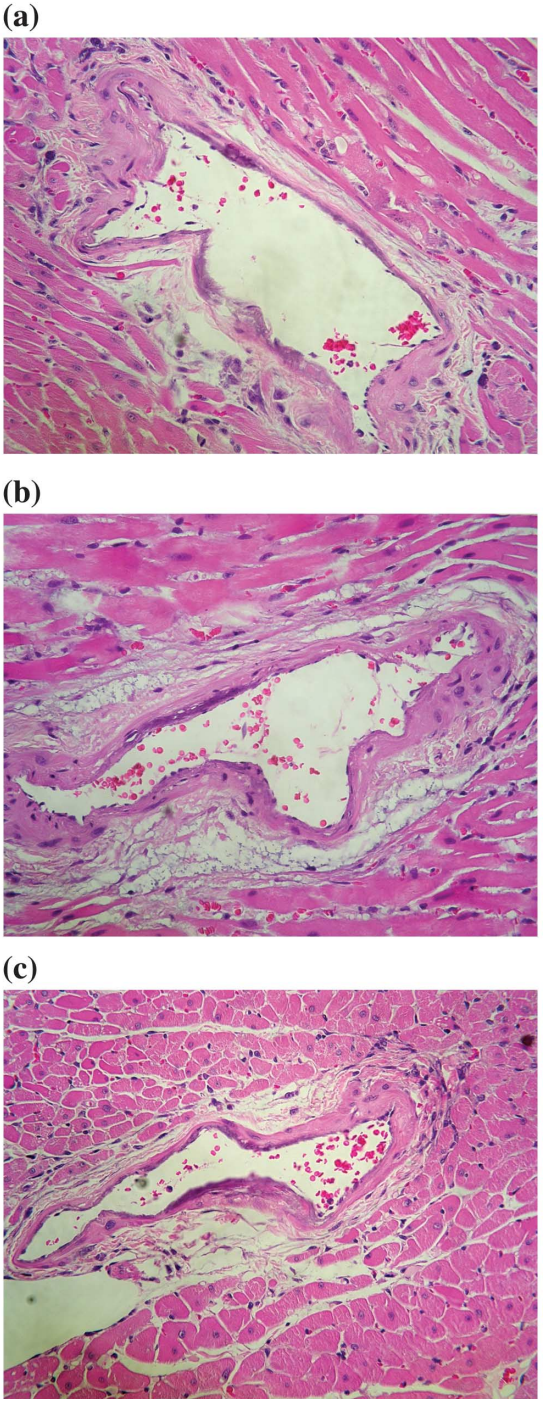

Fig 4. Sections of hearts from placebo-treated (original magnification ×200) (a), etidronate-treated (original magnification ×200) (b) and phytate-treated (original magnification ×200) (c) rats. Each section is stained with hematoxylin-eosin solution, which stains calcification dark purple.

cally significant (Fig 1).

No significant differences were observed in the heart calcium content between the phytate-treated (2.7±0.2 mg calcium/g lyophilized heart), etidronate-treated (2.6±0.5 mg calcium/g lyophilized heart) and placebo-treated (2.6±0.2 mg calcium/g lyophilized heart) rats (Fig 2).

The calcium deposits in the aorta were found to have developed predominantly in the internal tunica vessel layers (Fig 3). Heart calcium deposits developed in the walls of the coronary vessels and in the adjacent myocardium (Fig 4). In addition, hearts showed evidence of multiple myocardium necrosis and inflammation (ie, heart attack).

Discussion

The present study examined the effect of etidronate and

phytate on the calcification of vascular tissue in vitamin D-treated rats. The results indicate that phytate treatment reduced aortic calcification, whereas neither etidronate nor phytate reduced calcification in the heart tissue.

The development of ectopic soft tissue calcification, such as vascular calcification and nephrocalcinosis, can be linked to high doses of vitamin D;[31,37–39] a process that is associated with the consequent hypercalcemia that dramatically increases the supersaturation of calcium phosphate salts in the blood. A previous study using both vitamin D (300,000 IU/kg im) and nicotine (25 mg/kg po) reported higher levels of calcification in the rats' arteries and hearts compared with the current study[32] That study used lower levels of vitamin D, and thus involved lower levels of calcium phosphate supersaturation than the present study. These observations demonstrate the importance of a pre-existing lesion (caused by nicotine in the other study) in the development of tissue calcification.

The present study found that phytate decreased calcification in the aorta, but had no effect in the heart. A previous study found that phytate could inhibit vitamin D and nicotine-induced calcification in both the aorta and the heart of Wistar rats.[32] The difference between the findings of the 2 studies may be related to less lesions in the absence of nicotine, and that as a consequence of the higher hydroxyapatite supersaturation (because of increased blood calcium and phosphorus levels), the calcium deposits may have developed directly through homogeneous nucleation and not as a consequence of hydroxyapatite heterogeneous nucleation induced by the injured tissue. The tissue calcium content data for the 2 studies shows that more calcium was deposited when using vitamin D and nicotine than using only high vitamin D doses, despite the former experiments being half the duration of the latter. Aortic lesions were more severe than heart lesions in the present experiments, making the activity of crystallization inhibitors more obvious.

Although etidronate appeared to reduce the aortic calcium content, this effect was not statistically significant. A previous study[31] reported that the bisphosphonate ibandronate inhibited vitamin D-induced calcification in arteries. It is pertinent to note that other studies have reported that the activity of crystallization inhibitors decreases as supersaturation increases.[40] In the present study, the protective effect of phytate on aortic calcification was clearly superior to that of etidronate, most likely because phytate has a greater capacity to inhibit hydroxyapatite crystallization than etidronate.[41] The effect of phytate depends on the level of calcium phosphate supersaturation (free calcium and phosphate plasma concentrations) and the severity of the injury. Under normal dietary circumstances the phytate plasma concentration in rats of 5×10^{-7} mol/L is enough to manifest important inhibitory capacity.[42] However, phytate levels must be increased in order to inhibit crystallization under conditions of supersaturation and severe injury, such as in the present experiments. Administration of phytate as the natural food salt (phytin: calcium magnesium phytate) at amounts that provide maximum absorption, equivalent to doses that correspond to the consumption of the so-called "Mediterranean diet" (1–2 g phytate/day) were not found to have any chronic effect.[43] The chronic effects of phytate can be attributed to the decreased availability of oligo-elements such as zinc and iron. Obviously, administration via subcutaneous injection would avoid this problem. Previous work found that phytate only manifested toxic effects at very high doses (the LD50 value for male rats was 1.3 mmol/kg administered as sodium phytate).[44]

The inhibiting of crystallization appears to facilitate the reabsorption of injured tissue by the immune system. The greater the injury, the more difficult is reabsorption, and the more likely is high-level calcification. Indeed, phagocytosis of hydroxyapatite has been observed in implants;[45] and basic calcium phosphate crystals can stimulate the endocytotic activity of cells.[46,47] Hence, crystallization inhibitors can prevent excessive calcium phosphate precipitation facilitating phagocytosis and calcified injury reabsorption. It has been demonstrated that osteoclast adhesion to hydroxyapatite depends on the presence of specific extracellular adhesive proteins.[7,8,27] Thus, the role of some proteins in the calcification processes is to signal the presence of calcification and hence modulate cell activity, rather than act as crystallization inhibitors.

Phytate has shown a powerful capacity as an inhibitor of hydroxyapatite crystal formation in both in vitro and in vivo experiments.[48,49] Phytate levels found in tissues reflect dietary intake;[34,35] as well as topical absorption.[36] Bisphosphonates are commonly used for treating osteoporosis, and have also been shown to act as crystallization inhibitors both in vitro and in vivo.[29–31] Pyrophosphate, another well-known inhibitor of calcium salt crystallization, is also reported to prevent vascular calcification.[28] Notably, these molecules have structural similarities (polyphosphates), which explains their common activity. It appears that the action of polyphosphates (of natural origin or not) could be important in protecting against vascular calcification. These compounds, plus the action of cell modulator proteins, can combine to result in both the minimization of the size of the calcified lesions and the reabsorption of such lesions.

Conclusion

The present study found that phytate acted as a vascular calcification inhibitor. Thus, the action of polyphosphates could be important in protecting against vascular calcifications.

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