

Phytate acts as an inhibitor in formation of renal calculi

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1. ABSTRACT

The aim of this study was to assess the inhibitory action of phytate in formation of renal calculi. Hypertension (induced by nicotine) combined with hypercalcemia (induced by D vitamin) was used to induce calcification in renal tissue in male Wistar rats that were fed a purified phytate free diet. Phytate non-treated rats developed significant calcium deposits in kidneys and papillae, as well as in kidney tubules and vessels, whereas calcium deposits were absent in control and phytate treated rats. Fragments of hydroxyapatite (HAP) calculi exhibited the capacity to induce the growth of calcium salts on their surfaces. Presence of 1.5 mg/L of phytate in the synthetic urine inhibited the formation of calcium oxalate monohydrate on HAP renal calculi in normocalciuric conditions. The findings show that the action of phytate as a crystallization inhibitor takes place both in the intrapapillary tissue and urine.

2. INTRODUCTION

Pathological calcification is an undesirable process that frequently occurs in soft tissues. Development of calcification involves complex physicochemical and molecular biological events. Injury acts as an inducer of calcification (hydroxyapatite, HAP) but continuation of this process depends on presence of other promoter conditions (hypercalcemia, hyperphosphatemia) and/or the deficit of inhibitory factors (altered cellular calcification modulators, deficit of crystallization inhibitors).

Papillary renal calculi are small size uroliths, mainly composed of calcium oxalate monohydrate (COM). They exhibit a typical morphology consisting of a concave face (zone of union with the papillary tissue) and an opposite smooth convex face. According to recent studies, around 13% of all renal calculi are of renal papillary type (1). A COM papillary stone can only develop from a nidus

comprised of several crystals and/or organic matter that attach to the kidney papilla. This nidus can remain on site, in close contact with urine, and with an altered epithelium that resists nidus permanence. Urine remains supersaturated with calcium oxalate (2,3). HAP has been identified as the major component of the nidus (core) in 39.2% of papillary calculi (1). In the 1930's, Randall described a pre-calculus lesion in the renal papilla and proposed that a subepithelial calcification of renal papilla becomes the nidus of COM papillary calculi, as a consequence of the disruption of the papillary epithelial layer by the HAP plaque (4-10). Recently, it was found that in patients susceptible to the development of calcium renal calculi, plaque is initiated in thin-loop basement membranes, whereas in patients who have undergone bypass surgery, these plaques are formed in intratubular HAP crystals in renal collecting ducts (7-10).

Myo-inositol hexaphosphate (InsP₆, phytate) is abundant in plant seeds (~ 1.5-6.4%) and is present in all mammalian tissues and fluids, at a significantly low concentration (11,12). The levels found in blood and mammalian tissues are dependent on the amount in the dietary intake (11-13). Phytate inhibits crystallization of calcium salts in urine and soft tissues, and prevents the formation of renal calculi (13-16), acts as an antioxidant (17,18) and protects against development of colon cancer (19,20). The aim of this paper was to study the factors implied in formation of intrapapillary calcifications, to evaluate the capacity of HAP calcifications to promote crystallization of calcium salts in urine and to assess the inhibitory action caused by phytate in both cases.

3. MATERIAL AND METHODS

3.1. *In vivo* studies

3.1.1. Animals, diets and treatments

Eighteen male Wistar rats of approximately 250 g from Harlan Iberica S.L. (Barcelona, Spain) were acclimated in the course of 7 days. Animals were kept in Plexiglas cages (three animals per cage) at a temperature of 21 ± 1°C and relative humidity of 60 ± 5% with a 12-h on-off light cycle. After this period, animals were randomly assigned into three groups of six rats respectively: control group (subjected to placebo calcinosis induction), phytate non-treated group (subjected to calcinosis induction) and phytate treated group (subjected to calcinosis induction and pre-treated with a phytate containing cream). No control treated group with the moisturizing cream without phytate was included because it was previously found that such moisturizing cream without phytate had no effects on preventing dystrophic calcifications in rats (21). All rats were fed AIN 76-A diet (Ssniff Especialdiät GmbH, Soest, Germany), a purified diet in which phytate is undetectable. The procedures used in this experiment were carried out according to the Directive 86/609/EEC for protection of animals and after obtaining permission from the Bioethical Committee of the University.

3.1.2. Phytate containing cream pre-treatment

After a period of 16 days on a AIN 76-A diet, urinary phytate became undetectable. Then, phytate treated

group received once a day for the duration of the experiment (20 days), topical skin application of 4 g of a cream containing 2.0% potassium salt of phytate (22). The application skin surface (~50 cm²) on the back was shaved every four days.

3.1.3. Cream preparation

The moisturizing phytate cream (O/W) was prepared using two different phases, oil and water phases according to Table 1. The oil phase (O) and water phase (W) were previously heated until 65 ± 5 °C and then were mixed to obtain the O/W emulsion. For this purpose the W phase was slowly added to the O phase, with intensive stirring and homogenizing during all the process. Then the obtained system was intensively stirred for 10 min. at 65 ± 5 °C and finally cooled at room temperature maintaining the intensive stirring during all time.

3.1.4. Induction of calcinosis, monitorization and sample acquisition

After a period of 16 days of receiving cream, phytate non-treated group and phytate treated group were subjected to calcinosis induction. Another control group was subjected to placebo calcinosis induction. Calcifications were induced according to P. Kieffer *et al.* (23) by intramuscular injection of 300.000 IU/kg of vitamin D₃ (supplied by Fort Dodge Veterinaria, S.A., Girona, Spain) and 25 mg/kg oral administration of (-)-nicotine hydrogen tartrate salt (5 g/L solution, Sigma Aldrich, Steinheim, Germany). Nicotine administration was repeated ten hours later. Control group received intramuscular injection of NaCl (0.15 M) and distilled water orally. Animals were monitored every 12 hours. After 60 hours of calcinosis induction, all rats of the phytate non-treated group died, and the rest of rats were sacrificed, and kidneys were removed.

3.1.5. Histological analysis

Tissues were fixed in 10% buffered formalin, embedded in paraffin and examined after hematoxylin and eosin staining. The extent of calcium deposits was scored semiquantitatively as absent, low, moderate or high.

3.1.6. Quantitation of phytate

Levels of phytate were quantified in the 24 hr urine samples collected on the day before calcinosis was induced by inductively coupled plasma atomic emission spectrometry (ICP-AES, Perkin-Elmer, USA.) following total phosphorus determination, which has a detection limit of 60 µg/L (24). To do this, 5.0 ml of urine (acidified with HCl 1:1 until pH = 3-4) was transferred to a column containing 0.2 g of anion exchange resin (AG 1-X8, Bio-Rad Laboratories, U.S.A.). The first eluate was discarded, the column was washed first with 50 ml of HCl 50 mM. The second eluate was discarded. Finally, the column was washed with 3.0 ml of HNO₃ 2 M. The ICP-AES conditions were set as follows: outer argon flow 15 L/min, auxiliary argon flow 1 L/min, inner argon flow 1 L/min, nebulizer uptake rate 1 mL/min and wavelength 213.618 nm. The determination of phytate was then carried out through direct phosphorus analysis of the last eluate by ICP-AES using a calibration curve.

Table 1. Composition of the moisturizing cream used

Component	Percentage composition
Oil phase (O)	
• Almond oil	4
• Glyceril stearate	4
• Cetearyl alcohol	4
• Isopropil miristate	3.8
• Vitamin F	2.5
• Lactic acid	1.6
• Stearic acid	1
• Propil paraben	0.1
• Controx VP ®	0.03
Water phase (W)	
• Water	70.05
• Glycerine	4.87
• Potassium phytate	2.85
• Laurylate S-90	0.3
• Imidazolidinil urea	0.3
• Essence	0.3
• Methyl paraben	0.2
• Triethanolamine	0.1

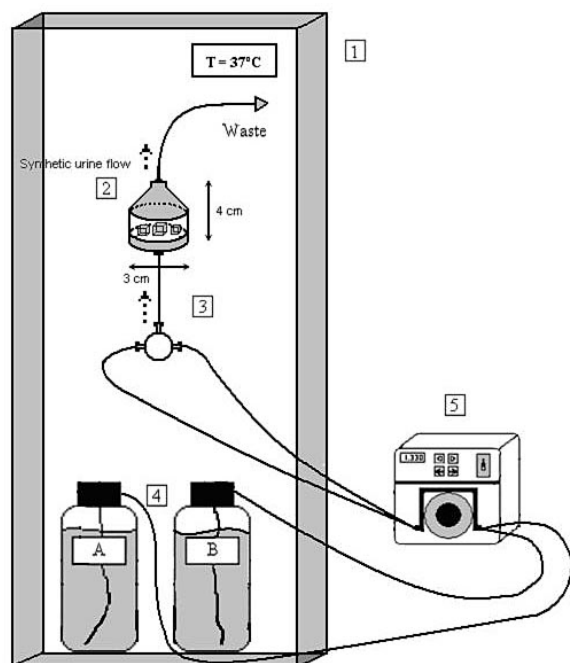


Figure 1. Diagram of the experimental flow system device used for crystallization studies with hydroxyapatite calculi. 1. Temperature-controlled chamber; 2. Flask containing the post-ESWL calculi fragments; 3. Three-way T mixing chamber of A and B solutions; 4. A and B solutions for artificial urine; 5. Peristaltic pump.

3.1.7. Calcium determination

Samples of kidneys were lyophilized and weighed. Dried tissues were digested in a mixture of 1:1 HNO_3 : HClO_4 in a sand bath until the solution was clear. For calcium determination, digested samples were diluted with distilled water to a final volume of 10 mL. The concentration of calcium was determined by ICP-AES using the corresponding calibration curve.

3.1.8. Statistics

Results are expressed as means \pm standard error (SE.) One-way ANOVA was used to calculate significance

of difference between groups. The Student *t*-test was used to assess differences of means. Conventional Windows software was used for statistical computations. A value of $p < 0.05$ was considered as being statistically significant.

3.2. In vitro studies

A collection of 24 spontaneously passed post-ESWL fragments of HAP calculi was collected the same day after ESWL application. The fragments were selected by combination of optical stereomicroscopy, infrared spectrometry and scanning electron microscopy (SEM) equipped with an energy dispersive X-ray analyzer (EDS) (25). All the selected fragments had similar morphology being this representative of that observed in the majority of spontaneously passed post-ESWL HAP pure renal calculi fragments.

The size of the selected fragments oscillated between 2-4 mm. Each of the four temperature-controlled (37°C) hermetic flow chambers (3 cm diameter and 4 cm high) contained three fragments of a HAP calculus with a total of 12 fragments used in each set of the experiments comprised of pH = 6.5 and normocalciuria ($[\text{Ca}^{2+}] = 3.75$ mM) and pH = 6.5 and hypercalciuria ($[\text{Ca}^{2+}] = 6.25$ mM). The fragments were placed in the experimental chamber without any previous pre-treatment process. Synthetic urine (26) was introduced into the flow chambers, freshly prepared, by a multichannel peristaltic pump, with a rate of 750 mL/day through the bottom of the flasks (Figure 1). The system was operating for different duration that allowed the growth of new crystals on the fragments. Growth of the fragments was assessed by weight increase and normalized by using the relative mass increase. In one set of experiments, the system ran for 48 hours under conditions of hypercalciuria/normooxaluria ($[\text{Ca}^{2+}] = 6.25$ mM, $[\text{Oxalate}] = 0.28$ mM). In a second set of experiments, the system was allowed to run for 192 hours under conditions of normocalciuria/ normooxaluria ($[\text{Ca}^{2+}] = 3.75$ mM, $[\text{Oxalate}] = 0.28$ mM).

3.2.1. Synthetic urine

Synthetic urine supersaturated with calcium oxalate was prepared by mixing equal volumes of solutions A and B (Table 2). The pH of both solutions was adjusted to 6.5. Solutions were stored for a maximum period of one week at 4 °C. Chemicals of reagent-grade purity were dissolved in deionized and redistilled water. All solutions were filtered through a 0.45 μm pore filter before use.

3.2.2. Effects of phytate

The inhibitory effects of phytate as sodium salt (Sigma-Aldrich, MO) on crystallization in synthetic urine were assessed in the concentration of 0.76-9.09 μM which is within the normal physiologic concentration of the urine (27).

4. RESULTS

4.1. In vivo studies

The calcium content of kidneys of the three studied groups (control, phytate non-treated and phytate treated) at the end of the experiment is shown in Figure 2

Table 2. Composition of synthetic urine (25)

Solution A (mM)		Solution B (mM)	
Na ₂ SO ₄ · 10H ₂ O	19.34	NaH ₂ PO ₄ · 2H ₂ O	15.45
MgSO ₄ · 7H ₂ O	5.93	Na ₂ HPO ₄ · 12H ₂ O	15.64
NH ₄ Cl	86.73	NaCl	223.08
KCl	162.60	Na ₂ C ₂ O ₄	0.57

Different volumes of a 1 M calcium solution, prepared by dissolving calcium carbonate with hydrochloric acid were added to solution A to obtain a final calcium concentration of 3.75-6.25 mM.

Table 3. Phytate concentration in urine for the subject groups, after 31 days of AIN 76-A diet

	Groups		
	Control	Phytate non-treated	Phytate treated
Phytate concentration in urine (mg/L)	0.08 +/- 0.03 (n = 6)	0.09 +/- 0.03 (n = 6)	36.15 +/- 7.26 ^{1,2} (n = 6)

¹: $p < 0.001$ phytate treated versus control group, ²: $p < 0.001$ phytate treated versus phytate non-treated group

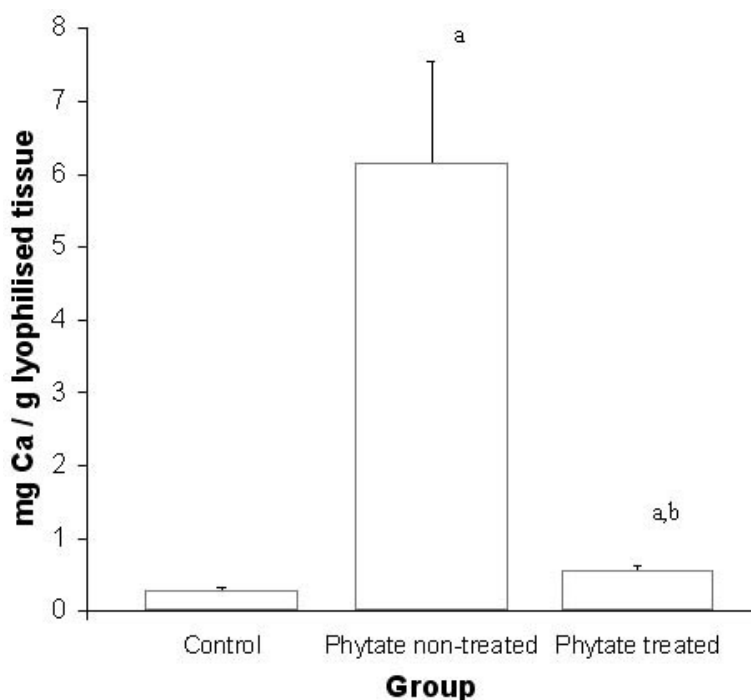


Figure 2. Calcium content in kidney of the subject groups (control, phytate non-treated, phytate treated). : $p < 0.005$ vs control group. : $p < 0.01$ vs phytate non-treated group.

and the concentration of phytate in urine after 31 days of AIN 76-A diet consumption is shown in Table 3. A significantly higher amount of calcium content was observed in the kidney of the phytate non-treated group (6.14 +/- 1.41 mg calcium / g dry kidney tissue) as compared with control (0.29 +/- 0.03 mg calcium/g dry kidney tissue) and phytate treated animals (0.56 +/- 0.06 mg calcium/g dry kidney tissue) (Figure 2). A significantly higher urinary excretion of phytate was detected in the phytate treated group as compared with control and phytate non-treated groups (Table 3).

As is shown in Figure 3, only phytate non-treated rats displayed significant level of calcium deposits in kidneys. The calcium deposits were assessed from histological analysis to be absent in control and phytate treated rats (Figure 3A) and to be present at a high level in phytate non treated rats. In this group, intratubular calcium

deposits (Figure 3B) and calcium deposits in blood vessels (Figure 3C) and calcified areas on papillae (Figure 3D) were also observed.

4.2. In vitro studies

In the *in vitro* conditions (normophosphaturic, normoaxaluric and pH = 6.5 synthetic urine), fragments of HAP calculi exhibited an important capacity to induce the growth of calcium salts on their surface. Thus, in normocalciuric conditions ($[Ca^{2+}] = 3.75$ mM), COM crystals developed, at a rate of 0.36 +/- 0.10 µg/h per mg of HAP calculus fragment (Figure 4A, t = 192 h). Using hypercalciuric urine ($[Ca^{2+}] = 6.25$ mM) brushite crystals were mainly developed, but also calcium oxalate dihydrate (COD) and HAP crystals were observed (Figure 4B, 4C, t = 192h). The overall growth rate in these conditions was 1.87 +/- 0.22 µg/h per mg of HAP calculus fragment. Presence of 1.5 mg/L phytate inhibited the development of COM

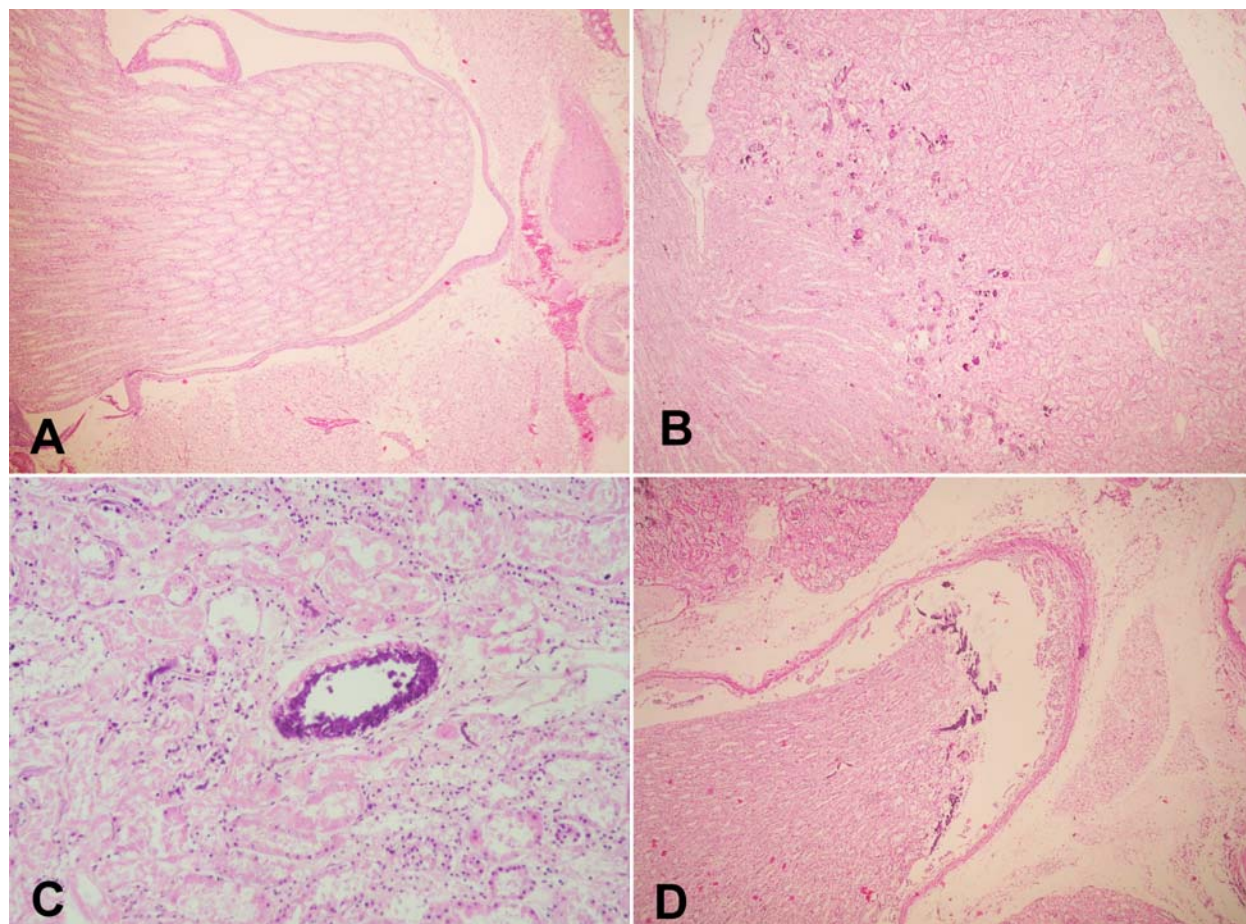


Figure 3. Sections of kidneys of the subject groups: A. Lack of calcium deposits in phytate treated rats (original magnification x 4) and control rats (not shown). B. Intratubular calcium deposits in phytate non-treated kidneys (original magnification x 4). C. Calcium deposits in blood vessels in phytate non-treated kidneys (original magnification x 20) D. Calcified areas in papillary region in phytate non-treated kidneys (original magnification x 4).

crystals on HAP calculi fragments in normocalciuric conditions (Figure 5A). In hypercalciuric conditions, presence of 6.0 mg/L phytate was necessary to inhibit crystal development (Figure 5B).

5. DISCUSSION

The findings reported here show that animals treated with D vitamin and nicotine develop calcified kidney deposit in renal tubules, blood vessels and on papillary epithelium. A significant number of papillary renal calculi (39.2%) initially develop on a subepithelial calcification deposit (Randall's plaque) that erodes the epithelium and gains direct contact with the urine. However, not all papillary calculi develop on a hydroxyapatite plaque and in 60.7% of these calculi no hydroxyapatite can be detected in the origin zone (1). In humans, there are different regions of kidney tissue calcification such as coarse focal deposits in the papillary region, and calcifications around the loops of Henle which are seen at all ages. The calcifications present at the boundary of the inner and outer medullary region are associated with degenerative changes, aging and arteriolar

disease (7, 10, 28). Development of these calcified areas appears to depend on injury or a pre-existing lesion such as papillary necrosis which leads to intratubular calcium phosphate deposits (29). Papillary calcification can be induced in rats with a combination of aspirin and sodium saccharin (30,31) and calcification of the vasa recta can be induced by long term phenacetin (32). Other studies demonstrated that high doses of phenylbutazone, oxyphenbutazone and indomethacin in rats leads to tubular necrosis in the lower nephron and causes calcification (33). Renal papillary necrosis and calcification also develop in diabetes mellitus (34). Hyperoxaluria which leads to hydroxyapatite tubular deposits in the lumen of collecting ducts and calcium oxalate monohydrate crystal deposits on the papillary tips is a precursor to papillary stones (9,35-40). The in vitro studies presented here, demonstrate that HAP fragments in contact with normocalciuric/normoxaluric urine induce COM crystal on their surfaces. Although this mechanism might be important to formation of some renal calculi, they are not the cause of all calculi since HAP is only found in 39.2 % of all COM papillary calculi (1). In the other cases, the nidus might be formed on sites with altered (damaged or just slightly injured) epithelium (2,3).

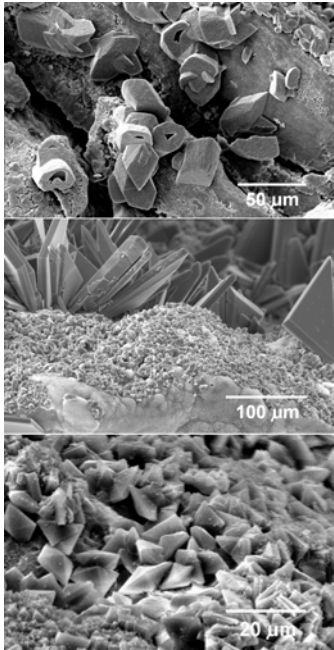


Figure 4. A. Formation of COM crystals after 192 hr on a post-ESWL fragment of a HAP renal calculus in normocalciuric (3.75 mM) and normooxaluric (0.28 mM of oxalate) synthetic urine (pH = 6.5) *in vitro*. B. Brushite, HAP and C. Formation of COD crystals after 192 h on post-ESWL fragments of HAP renal calculi in hypercalciuric (6.25 mM) and normooxaluric (0.28 mM of oxalate) synthetic urine (pH = 6.5) *in vitro*.

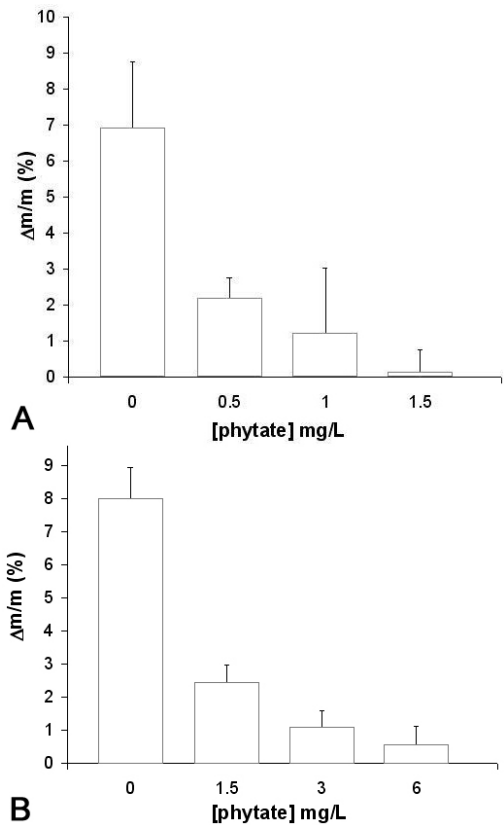


Figure 5. Effect of phytate on the increase of relative weight of post-ESWL fragments of HAP renal calculi maintained in normooxaluric ($[oxalate] = 0.28$ mM) synthetic urine at pH = 6.5. Values are means of 12 fragments \pm SE. Normocalciuric urine ($[Ca^{2+}] = 3.75$ mM). System kept working for 192 hours, Hypercalciuric urine ($[Ca^{2+}] = 6.25$ mM). System kept working for 48 hours.

Although, the development of tissue calcification depends on a preexisting injury which acts as an inducer, continuation of this process is subject to modulators and/or the deficit of crystallization inhibitors. For example, some carboxyproteins act as osteopontin, bind HAP, signal and recruit macrophages that remove these calcifications or prevent their progression (41-46). The crystallization inhibitory action avoiding HAP development (nucleation and growth) has been attributed to low molecular weight compounds such as pyrophosphate, magnesium and phytate (47-49). Here, we show that phytate can act as crystallization inhibitor in both, the intrapapillary tissue and in urine. The *in vitro* results show that phytate prevents the development of calcifications on HAP at concentrations similar to those found in real urine (27).

The phytate levels found in tissues and blood in mammals clearly depend on dietary intake and these levels correlate with that found in the urine (12, 27, 33). Here, we show that phytate can be also readily absorbed from skin and might be clinically useful route of administration in subjects who are prone to the development of renal calculi.

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