

Phytate reduces age-related cardiovascular calcification

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1. ABSTRACT

The aim of this research was to evaluate the effect of dietary phytate on cardiovascular calcification in rats during aging. Male Wistar rats (10 weeks old) were randomly assigned to four diet groups. The control group was fed with a balanced diet (UAR-A04) containing phytate. The AIN group was fed a purified diet (AIN-76A) with an undetectable level of phytate. The PHY group was fed with a purified diet (AIN-76A) enriched with phytate (phytin, as the calcium magnesium salt). The MOD group was fed with the AIN-76A diet (phytate undetectable) enriched with MgO, inositol and CaHPO₄. At 76 weeks of age all rats were sacrificed, and the aortas, hearts, kidneys, livers and femurs were removed for chemical analysis. The most significant differences were found in the aorta calcium content. Phytate-treated rats (the control and PHY groups) had significantly lower levels of calcium in the aorta compared to nonphytate-treated rats (AIN and MOD groups). The present study demonstrated that dietary phytate treatment significantly reduced age-related aorta calcification.

2. INTRODUCTION

Human arterial and valvular calcification increases progressively during aging and is amplified by vascular pathologies such as hypertension or arteriosclerosis (1-3). Moreover, age-linked ectopic calcification has poor prognosis and there are no drugs available to prevent its occurrence.

Development of pathological tissue calcification requires a pre-existing injury to act as an inducer (heterogeneous nucleus) of calcification: dead cells and/or their membranes act as important heterogeneous nuclei for calcium phosphate (hydroxyapatite) crystallization (4). Another major factor in arterial wall calcification is a deficiency in calcification repressor factors (cellular defense mechanisms and/or crystallization inhibitors). A number of studies have documented expression of several mineralization-regulating proteins in soft tissue calcification leading to the suggestion that these proteins play a role in vascular calcification acting as cellular or molecular regulators. The common characteristic of these

proteins is calcium ion affinity. These proteins include osteopontin (5-9), osteoprotegerin (10-12), matrix Gla protein (13-15) and osteocalcin (also known as bone Gla protein) (16,17). These cell activity-modulating proteins have also been shown to act as crystallization inhibitors (18-20) *in vitro*, but these data were obtained using protein, free calcium and phosphate concentrations outside typical physiological ranges. Some of these proteins have also been reported to be calcification promoters due to heterogeneous nucleation activity (21-23). Despite the reports that these proteins have crystallization inhibitor/promoter activity, it is likely that their major role in calcification is as modulators of osteoclast/osteoblast-like cell activity and cell differentiation.

Crystallization inhibiting substances are normally non-signaling molecules that bind to the crystal nucleus or faces and, as a consequence, prevent or disrupt crystal development. Crystallization inhibitors are effective at significantly lower concentrations than chelating agents, which act by decreasing target ion supersaturation. Some molecules have been reported to inhibit crystallization associated with cardiovascular calcification, including pyrophosphate (24-26), bisphosphonates (e.g. etidronate, alendronate and ibandronate) (27-30) and phytate (myo-inositol hexakisphosphate) (31,32). We recently showed that phytate significantly inhibited calcification in the aorta and heart of male Wistar rats treated with vitamin D and nicotine (31). Phytate has also been shown to act as a vascular calcification inhibitor in male Sprague-Dawley rats treated with high doses of vitamin D, and the effect was clearly superior to that of etidronate (32).

The aim of the present study was to evaluate the role of dietary phytate in cardiovascular calcification in rats during aging.

3. MATERIALS AND METHODS

3.1. Animals and diets

Sixty male Wistar rats of approximately 250 g (10 weeks of age; Harlan Iberica S. L., Barcelona, Spain) were acclimated over 7 days in our animal house. Animals were kept on a 12 h light:dark cycle in Plexiglas cages (three animals per cage) at 21 \pm 1°C and relative humidity of 60 \pm 5%. After acclimation the animals were randomly assigned to 4 groups (control, AIN, PHY and MOD) of 15 rats each. The control group was fed a balanced diet that contains phytate (UAR-A04; Panlab S.L., Barcelona, Spain), the AIN group was fed a purified diet in which phytate is undetectable (AIN-76A; Ssniff Spezialdiäten GmbH, Soest, Germany), the PHY group was fed a modified AIN-76A diet enriched by the addition of phytate at a concentration equivalent to that in UAR-A04 (AIN-76A + 12990 mg/kg phytin; Ssniff Spezialdiäten GmbH, Soest, Germany), and the MOD group was fed with a modified AIN-76A diet in which phytate is undetectable (no added phytin) but with the same magnesium, calcium and phosphorus concentrations as the PHY diet (AIN-76A + 1220 mg/kg MgO + 1720 mg/kg inositol + 9050 mg/kg CaHPO₄; Ssniff Spezialdiäten GmbH, Soest, Germany). The mineral compositions of the diets are listed in Table 1.

Experimental procedures were performed according to Directive 86/609/EEC concerning the treatment of animals used for experimental and other scientific purposes. Permission to perform these animal experiments was obtained from the Bioethical Committee of our University. The present study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.2. Echocardiographic study

At 76 weeks of age, 7 and 8 rats randomly chosen from the AIN and PHY groups respectively (termed AIN and PHY subgroups, respectively) were subjected to an echocardiographic examination by an experienced echocardiographer using a Vivid-i echo platform (General Electric and Vigmed Ultrasound Systems, Horten, Norway) with a 10 MHz sector phased array probe. The rats were sedated with 0.2 mg intraperitoneal ketamine (Merial, Lyon, France) plus 0.02 mg medetomidine (OrionPharma, Espoo, Finland). The aortic root wall, mitral annulus and aortic valve were analyzed, and digital file records of the studies were reviewed to score the degree of calcification according to previously described echocardiographic criteria (33): 1 – no calcification, 2 – mild calcification, 3 – moderate calcification, 4 – severe calcification. The frame rate used was at least 250 fps. The echocardiographer had no knowledge of which animal group was being analyzed.

3.3. Monitoring and sampling

Animal weights and diet intake were monitored throughout the study. At 76 weeks of age, all rats were sacrificed and aortas, hearts, kidneys, livers and femurs were removed.

3.4. Histological analysis

Aortas from 3 rats of each group were placed in 4% buffered (pH = 7) formaldehyde (Panreac, Barcelona, Spain) and fixed for 24 h at room temperature. After fixation, two sections of each aorta, one from aortic arch and one from abdominal aorta, were embedded in paraffin blocks. Histological 4 μ m sections were obtained and stained with Hematoxylin Eosin and Von Kossa stain. All sections were examined by an experienced pathologist.

3.5. Chemical analysis

Organs, tissues and diets were lyophilized and weighed, and then digested using a 1:1 HNO₃:HClO₄ mixture in a sand bath until the solution was clear. Digested samples were diluted with distilled water to a volume of 20 ml. The concentrations of calcium, magnesium, manganese, iron and zinc were determined using inductively-coupled plasma atomic emission spectrometry (Optima 5300DV spectrometer; Perkin-Elmer S. L.) and a corresponding calibration curve.

3.6. Statistics

Chemical analysis values are expressed as mean \pm SE. A one-way ANOVA was used to determine the significance of differences among groups. Student's *t* tests were used to assess differences between means. In the echocardiographic study, Fisher's Exact test was used to

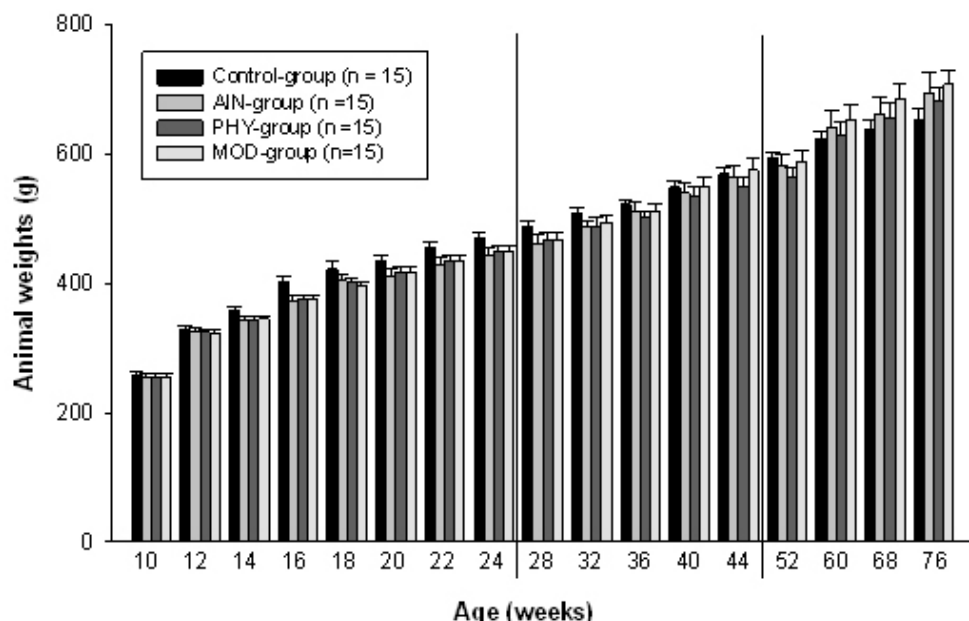


Figure 1. Animal weight trajectories for rats in the control, AIN, PHY and MOD groups.

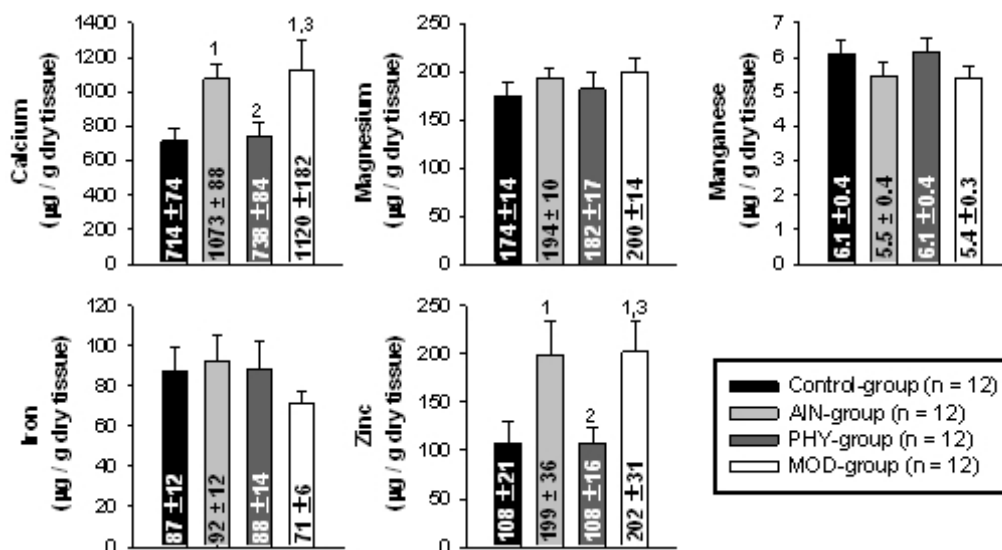


Figure 2. Chemical analysis (Ca, Mg, Mn, Fe, Zn) of the aorta for rats in the control, AIN, PHY and MOD groups.

Statistics. ¹ $p < 0.05$ vs. the corresponding value for the control group; ² $p < 0.05$ vs. the corresponding value for the AIN group; ³ $p < 0.05$ vs. the corresponding value for the PHY group.

determine significant differences among groups. Conventional Windows software was used for statistical computations. A p value < 0.05 was considered to indicate a significant difference.

4. RESULTS

No significant differences in body weight were found among the groups throughout the study. Figure 1 shows the body weight trajectory for each group.

Figure 2 and Table 2 show the results of chemical analysis (Ca, Mg, Mn, Fe, Zn) of the aorta, heart, kidney, liver and femur for the four groups. The most significant differences were found in the aorta chemical content (Figure 2). Phytate-treated rats (the control and PHY groups) had significantly lower levels of calcium and zinc in the aorta compared to nonphytate-treated rats (AIN and MOD groups). These results clearly suggest that dietary phytate treatment results in a significant reduction of zinc accumulation and aortic calcification due to aging.

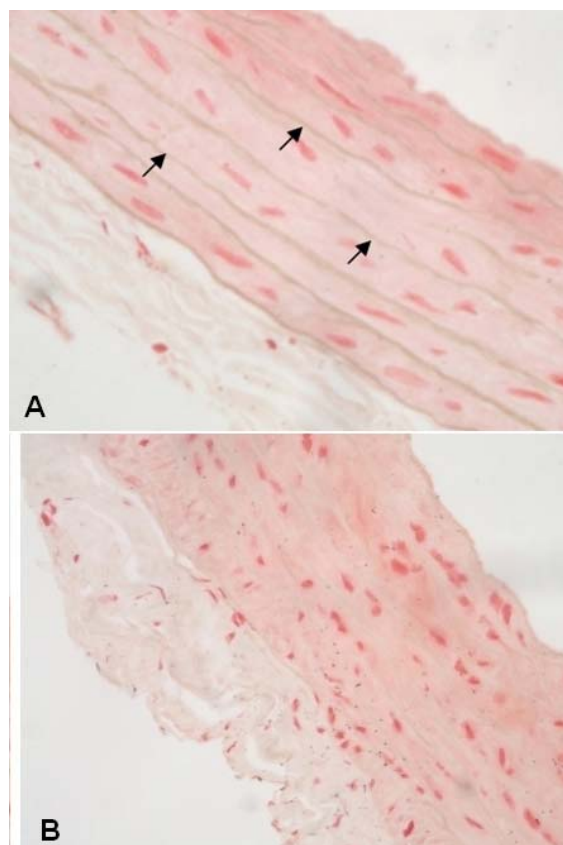


Figure 3. Aorta sections (original magnification $\times 100$) after Von Kossa stain: (a) AIN group (b) PHY group. Aortic calcification (dark purple areas, arrows) was greater for the AIN and MOD groups than for the control and PHY groups. Calcium deposits were associated with medial elastic fibers.

Histological analysis revealed that aortic calcified deposits were mainly associated with the medial elastic lamellae (Figure 3).

No significant differences were found in the calcium, magnesium, manganese or zinc content among groups with respect to the mineral content of the heart, kidney and liver. However, significant differences in iron content were found between the control group and the other three groups, which may be a consequence of the higher iron content of the UAR diet relative to the other diets (Table 1). Significant differences were also found in the femur chemical analysis between the control group and other three groups, with respect to calcium, magnesium and manganese levels. These results indicate that phytate (as calcium magnesium salt) did not affect element bioavailability (Table 2).

Table 3 and Figure 4 show the results of the echocardiographic study. Most rats in the PHY subgroup had significantly less calcification in the aortic root wall and mitral annulus compared to rats in the AIN subgroup. No differences in aortic valve calcification were detected between these subgroups.

5. DISCUSSION

As has previously been described, the calcium content in arteries of rats increases with age (2). The present study found that dietary phytate treatment significantly reduced aortic calcification during aging.

It has been known since the 1930s that the presence of trace amounts of molecules including polyphosphates can act as water softeners by inhibiting crystallization of calcium salts such as calcium carbonate. However, the use of such compounds as regulators of calcification under physiological conditions was not explored until the 1960s. During that decade, Fleisch *et al.* showed that pyrophosphate, a naturally occurring polyphosphate, was present in serum and urine, and could prevent calcification by binding to hydroxyapatite (34,35). However, studies using animal models found that pyrophosphate could inhibit ectopic calcification in blood vessels and kidneys only when injected rather than ingested. Oral administration was found to cause hydrolysis and hence inactivation of pyrophosphate, and this led to a search for more stable analogues. Bisphosphonates, a group of synthetic polyphosphates, were found to have a high affinity for hydroxyapatite and to prevent calcification both *in vitro* and *in vivo*, even when administered orally (36).

Phytate is abundant in plant seeds, and has been shown to inhibit vascular calcification and calcium salt crystallization in urine and soft tissues (31,32,37,38), as do other polyphosphates such as pyrophosphate and bisphosphonates (39). Phytate is found in all mammalian organs, tissues and fluids (40,41) which are dependent upon an exogenous supply, either oral (40,41) or topical (42,43). Administration of phytate as a food salt (phytin, as calcium magnesium phytate) in amounts corresponding to the so-called 'Mediterranean diet' (1–2 g phytate/day) has not been found to have any negative effect (44), and phytate is only toxic at very high doses (45), with an oral LD50 (as the sodium salt) of 1.3 mmol/kg for male rats.

The present study demonstrated that phytate decreased calcification in the aorta during aging. Histological analysis showed that calcified deposits were mainly associated with the medial elastic lamellae (Figure 3). A striking feature of arterial aging is the progressive thinning, splitting, fraying and fragmentation of elastic lamellae (46,47), and the intensity of calcification during aging follows the elastin gradient, with larger arteries having proportionately higher levels of elastin and calcification (47). Accordingly, gradual disruption of elastic fibers could act as an inducer of calcification through heterogeneous nucleation of calcium phosphate crystals on such fibers, and so facilitate the age-linked calcification process. The action of crystallization inhibitors which avoid hydroxyapatite development appears to involve re-absorption of injured tissue by the immune system. With worse injuries, re-absorption is more problematic and there is greater likelihood of high-level calcification. Indeed, phagocytosis of hydroxyapatite has been observed in implants (48), and basic calcium phosphate crystals can stimulate the endocytotic activity of

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Table 1. Mineral composition of the diets

	UAR-A04 (n = 3)	AIN-76A (n = 3)	AIN-76A + phytin (n = 3)	AIN-76A modified (n = 3)
Calcium (mg/g dry weight)	9.3 +/- 0.2	6.2 +/- 0.11 ¹	8.1 +/- 0.4 ²	8.9 +/- 0.1 ²
Magnesium (mg/g dry weight)	1.75 +/- 0.05	0.69 +/- 0.01 ¹	1.62 +/- 0.01 ²	1.60 +/- 0.01 ²
Manganese (mg/g dry weight)	0.069 +/- 0.006	0.056 +/- 0.001	0.055 +/- 0.001	0.057 +/- 0.01
Iron (mg/g dry weight)	0.25 +/- 0.03	0.056 +/- 0.001 ¹	0.057 +/- 0.003 ¹	0.059 +/- 0.002 ¹
Zinc (mg/g dry weight)	0.051 +/- 0.002	0.045 +/- 0.001	0.040 +/- 0.004	0.041 +/- 0.003
Phytate (mg/g dry weight)	7.3 +/- 0.2	< 0.01 ¹	10 +/- 0.3 ^{1,2}	< 0.01 ^{1,3}

Mineral composition of the four diets used in the study: UAR-A04, AIN-76A, AIN-76A enriched with phytate (AIN-76A + 12990 mg/kg phytin), and AIN-76A modified diet (AIN-76A + 1220 mg/kg MgO + 1720 mg/kg inositol + 9050 mg/kg CaHPO₄). Statistics. ¹ p < 0.05 vs. the corresponding value for UAR-A04; ² p < 0.05 vs. the corresponding value for AIN-76A; ³ p < 0.05 vs. the corresponding value for AIN-76A + phytin.

Table 2. Chemical analysis of metals

	Control group (n = 15)	AIN group (n = 15)	PHY group (n = 15)	MOD group (n = 15)
Heart				
Calcium (µg/g dry weight)	196 +/- 10	196 +/- 8	204 +/- 16	186 +/- 9
Magnesium (µg/g dry weight)	514 +/- 76	495 +/- 68	440 +/- 65	498 +/- 66
Manganese (µg/g dry weight)	1.2 +/- 0.1	1.1 +/- 0.1	1.0 +/- 0.1	1.1 +/- 0.1
Iron (µg/g dry weight)	301 +/- 11	266 +/- 13 ¹	242 +/- 11 ¹	263 +/- 7 ¹
Zinc (µg/g dry weight)	64 +/- 7	60 +/- 7	62 +/- 7	58 +/- 6
Kidney				
Calcium (µg/g dry weight)	372 +/- 11	351 +/- 16	360 +/- 14	344 +/- 21
Magnesium (µg/g dry weight)	706 +/- 22	677 +/- 25	660 +/- 19	649 +/- 23
Manganese (µg/g dry weight)	3.0 +/- 0.2	3.0 +/- 0.1	2.9 +/- 0.1	2.6 +/- 0.1
Iron (µg/g dry weight)	673 +/- 32	587 +/- 22 ¹	564 +/- 22 ¹	542 +/- 19 ¹
Zinc (µg/g dry weight)	78 +/- 6	81 +/- 9	74 +/- 5	67 +/- 3
Liver				
Calcium (µg/g dry weight)	158 +/- 7	173 +/- 26	168 +/- 12	189 +/- 10
Magnesium (µg/g dry weight)	454 +/- 20	462 +/- 38	451 +/- 20	499 +/- 18
Manganese (µg/g dry weight)	4.8 +/- 0.2	4.4 +/- 0.3	4.7 +/- 0.2	4.7 +/- 0.3
Iron (µg/g dry weight)	835 +/- 75	641 +/- 50 ¹	650 +/- 43 ¹	647 +/- 27 ¹
Zinc (µg/g dry weight)	65 +/- 2	63 +/- 3	67 +/- 5	68 +/- 2
Femur				
Calcium (mg/g dry weight)	263 +/- 3	248 +/- 6 ¹	246 +/- 5 ¹	247 +/- 3 ¹
Magnesium (mg/g dry weight)	4.4 +/- 0.1	3.9 +/- 0.1 ¹	3.9 +/- 0.2 ¹	4.1 +/- 0.1 ¹
Manganese (µg/g dry weight)	0.61 +/- 0.03	0.77 +/- 0.03 ¹	0.73 +/- 0.03 ¹	0.75 +/- 0.02 ¹
Iron (µg/g dry weight)	41 +/- 3	44 +/- 4	37 +/- 3	36 +/- 3
Zinc (µg/g dry weight)	214 +/- 4	210 +/- 3	207 +/- 3	216 +/- 8

Chemical analysis (Ca, Mg, Mn, Fe, Zn) of the heart, kidney, liver and femur for rats in the control, AIN, PHY and MOD groups. Statistics. ¹ p < 0.05 vs. the corresponding value for the control group.

Table 3. Echocardiographic results

	Aortic root wall		Aortic valve		Mitral annulus	
	AIN subgroup	PHY subgroup ¹	AIN subgroup	PHY subgroup	AIN subgroup	PHY subgroup ¹
No or mild calcification (scores 1 and 2)	0	5	5	6	2	7
Moderate or severe calcification (scores 3 and 4)	7	3	2	2	5	1

Echocardiographic results (expressed as number of rats) for the AIN (n = 7) and PHY (n = 8) subgroups according to the following criteria: 1 – no calcification, 2 – mild calcification, 3 – moderate calcification, 4 – severe calcification. Statistics. ¹ p < 0.05 vs. the corresponding value for the AIN subgroup.

cells (49,50). Hence, crystallization inhibitors such as phytate can prevent excessive calcium phosphate precipitation, facilitating phagocytosis and post-injury

re-absorption. Phytate has also been demonstrated to have antioxidant properties as a consequence of its capacity to avoid free radical formation (51). The results

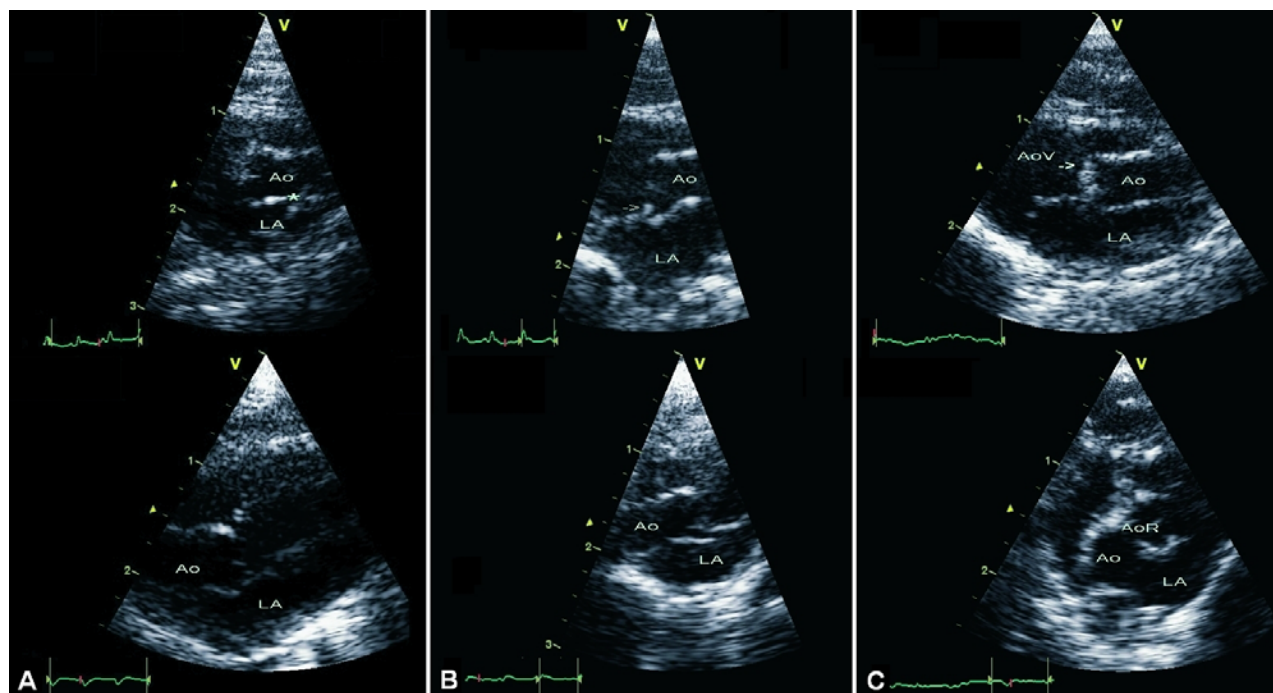


Figure 4. Images of the echocardiographic study (upper: long axis view, bottom: subcostal view). The degree of calcification was scored according to previously described echocardiographic criteria (33): (a) example of score 2 – mild calcification. Echo intensity increased in small isolated spots (star) in aortic wall; (b) example of score 3 – moderate calcification. More uniform thickening of the aortic wall extended to the posterior cusp of the aortic valve; (c) Example of score 4 – severe calcification. Severe thickening affecting the aortic root and the aortic valve. Abbreviations. Ao: aorta; AoV: aortic valve; AoR: aortic root; LA: left atrium.

obtained support the current view that whole-grain is an important dietary component associated with reduced risk of cardiovascular events (52-54), with phytate probably being beneficially involved due to its activity as a crystallization inhibitor and also as an antioxidant.

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