Tissue calcification: Phytate versus immune system

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Introduction

Crystallization inhibitors (such as phytate) and several proteins (such as osteopontin or osteoporotegerin) have demonstrated to have a role in soft tissue calcification. Soft tissue calcification is an undesirable disorder that implies the pre-existence of an injury which induce hydroxyapatite formation through heterogeneous nucleation. The present study examined the role of food phytate during the development of soft tissue calcification during ten days and the paper of osteopontin in an animal model of calcinosis induction.

Material and Methods

Male Wistar rats were assigned in two groups (n=16). Non-phytate-treated rats were fed with AIN-76A diet (a purified diet in which phytate is undetectable) and phytate-treated rats were fed with AIN-76A enriched with phytin diet. After a period of 21 days consuming corresponding diets, all rats were subjected to calcinosis induction by subcutaneous injection of $KMnO_4$ in two positions on either side of the interscapular region. At 2, 5, 8 and 10 days after calcinosis induction, four rats of each group were sacrified and the injured tissues (hydroxyapatite and surrounding tissue) were removed for hystological analysis (calcium deposits, macrophages and osteopontin detection) and calcium determination.

Histological analysis

For histological analysis, blocks of excised tissue from each sacrificed animal were fixed in 4% buffered formaldehyde at pH 7 (Panreac S.A., Barcelona, Spain) for 24 hours at room temperature, embedded in paraffin, and sectioned at 4 to 6 µm. The tissue sections were stained with hematoxylin and eosin using standard techniques. Calcium in sections was enhanced by von Kossa stain. Macrophages were detected using a cd68 monoclonal antibody (Dako Denmark A/S), lymphocytes were detected using a panel of cd3, cd20 and cd45 antibodies (Dako Denmark A/S), and osteopontin was detected using MPIIIB10 antibody from DHSB. (University of Iowa, Iowa City, USA) using the Envision (R) system (Dako Denmark A/S) in an automated stainer (Autostainer Plus, Dako Denmark A/S). All tissues was examined and evaluated semi-quantitatively by an experienced pathologist.

Analysis of calcium and magnesium

Excised injured tissues were lyophilized, placed in a mixture of HNO_3 and $HClO_4$ (1:1) in a sand bath, and digested until the solution was clear. Digested samples were diluted with distilled water to a volume of 10 ml. The concentrations of calcium and magnesium were determined using inductively coupled plasma atomic emission spectrometry (Optima 5300DV spectrometer, Perkin-Elmer SL) and an appropriate calibration curve.

Statistical analysis

Values are expressed as mean ± SE. Student's t tests were used to assess differences between means. Windows software was used for statistical computations. A p value < 0.05 was considered to indicate a significant difference.

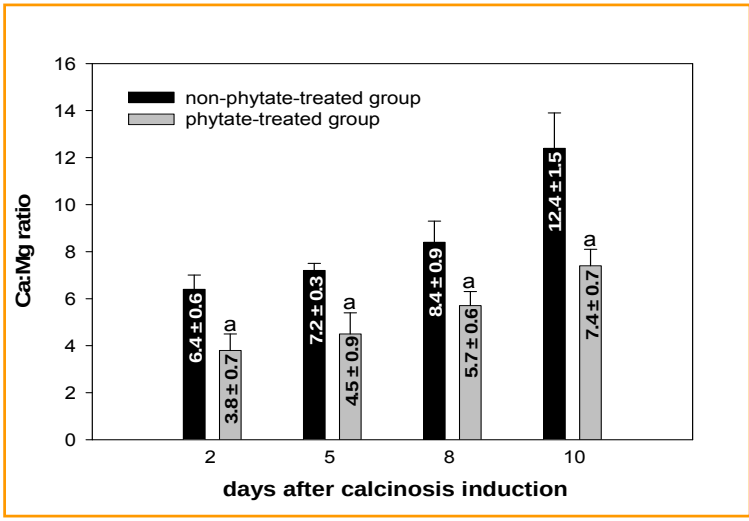
Results

The development of calcification in the injured tissues for the phytate-treated and non-phytate-treated rats, evident in changes in the calcium:magnesium ratio (Figure 1). Rats treated with phytate had notably and significantly reduced development of calcification in comparison with non-phytate-treated rats.

Development of tissue calcification of phytate-treated rats was notably and significantly reduced in comparison with non-phytate-treated rats Calcified deposits appeared as soon as 2 days after calcinosis induction, macrophages weren't present until the 5th-day and osteopontin wasn't detected until the 8th-day and from this day, it was clearly detected and associated with calcified areas (Figure 2).

Figure 1

Calcium:magnesium ratio in the injured tissues of non-phytate-treated and phytate-treated rats at 2, 5, 8 and 10 days after calcinosis induction.
Values are expressed as mean ± SE (n = 4).
a: $p < 0.05$ vs. the corresponding nonphytate-treated value.



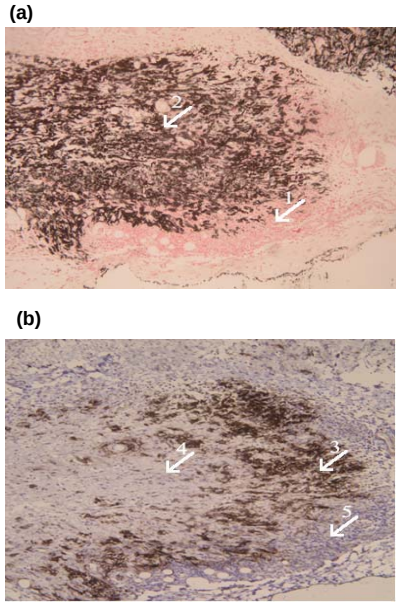


Figure 2

Injured tissue 8 days after calcinosis induction, visualized with von Kossa stain (a) and by immunocytochemistry for osteopontin detection (b). The same pattern was observed in both non-phytate-treated and phytate-treated rats:

(a) granulomatous inflammation reaction is located around dermic calcified regions (arrow 1). Calcium deposits associated with collagen fibers (arrow 2) were detected by von Kossa staining;

(b) osteopontin was only observed in the peripheral layers (arrow 3) of the calcified region (arrow 4) and not in central calcified layers (arrow 4), suggesting osteopontin diffusion from the granulomatous inflammation area (arrow 5) to the calcified region.

Original magnification x 100.

Conclusions

The results suggest an important role of phytate as crystallization inhibitor in the first steps of calcification formation, avoiding hydroxyapatite crystals development. Hence the inhibition of crystal development would facilitate the reabsorption of injured tissue by the immune system. Histological analysis indicated that osteopontin appears to be related with the control of calcification, regulating the activity of macrophage and macrophage-derived cells (i.e., osteoclasts), thus facilitating phagocytosis and enhancing hydroxyapatite deposits destruction.