Biometals (2008) 21:133–141
DOI 10.1007/s10534-007-9101-z

Bone and faecal minerals and scanning electron microscopic assessments of femur in rats fed phytic acid extract from sweet potato (*Ipomoea batatas*)

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Received: 10 January 2007 / Accepted: 15 May 2007 / Published online: 12 June 2007
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Abstract Phytic acid was extracted from sweet potato (*Ipomoea batatas*) and fed to Wistar rats with or without zinc for 3 weeks. Animals were then sacrificed and bone and faecal minerals were assessed. The ultra-structure of the bones was examined via scanning electron microscopy. Phytic acid extract or commercial phytic acid supplemented diets (D + Zn + PE or D + PE) displayed reduced bone calcium levels (101.27 ± 59.11 and 119.27 ± 45.36 g/kg) compared to the other test groups. Similarly, reduced calcium were observed in the control groups (D + Zn and D) fed formulated diets with or without zinc supplementation (213.14 ± 15.31 and 210 ± 6.88 g/kg) compared to the other test groups. The group fed supplemented commercial phytic acid diet (D + CP) demonstrated the lowest femur magnesium (3.72 ± 0.13 g/kg) while the group fed phytic acid extract supplementation (D + PE) recorded the highest level (4.84 ± 0.26 g/kg) amongst the groups. Femur iron was highest in the group fed commercial phytic acid supplemented diet (D + CP −115.74 ± 2.41 g/kg) compared to the other groups. Faecal magnesium levels were significantly higher in the two test groups fed phytic acid extract with or without zinc (D + Zn + PE or D + PE) compared to all other groups. All the groups which had phytic acid supplemented diets had significantly thinner bone in the trabecular region, compared to the groups fed formulated diet or zinc supplemented formulated diet (D or D + Zn). These observations suggest that the consumption of foods high in phytic acid may contribute to a reduction in the minerals available for essential metabolic processes in rats.

Keywords Phytic acid · Minerals · Bone · Sweet potato

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Introduction

Phytic acid is a hexaphosphorylated sugar and a ubiquitous plant component which may constitute 1–5% by weight of most cereals, nuts legumes and oil seeds (Mega 1982). It is believed to be the primary reserve of phosphate in seeds (Lopez et al. 1998).

The beneficial effects of phytic acid include the prevention of conditions such as kidney stones, high cholesterol, heart and liver diseases (Shamsuddin et al. 1997). A reduction in breast, colon and prostate cancer is also associated with increased consumption of phytic acid-rich legumes and cereal (Singh et al. 2004; Fox and Eberl 2002). Phytic acid is also thought to regulate the process of digestion by binding to some digestion products thereby delaying their absorption. This is a factor which has been attributed to the delay of the onset of diabetes and hyperlipidemia (Potter et al. 1981). Studies carried out by Yoon et al. (1983), also show that phytic acid may affect the starch digestibility through interaction with amylase and/or binding with salivary minerals such as calcium. The presence of phytic acid in seeds is thought to be responsible for their remarkable longevity, up to 400 years in some instances (Owen et al. 1996). The antioxidant properties of phytic acid are thought to be responsible for this. Phytic acid is also a potent factor in lowering blood glucose levels in the presence of zinc (Dilworth et al. 2005).

However, some negative effects of phytic acid have been reported as it binds to essential minerals thus rendering them unavailable for intestinal uptake (Phillipy 2003). These minerals are therefore not able to participate in essential metabolic processes in the body since the first step in mineral absorption requires that minerals remain in the ionic state (Schlussel 2003). This may result in mineral deficiencies in humans and animals (Lott et al. 2000). Studies have shown that zinc and iron bound to phytic acid can pass through the gastrointestinal tract and be egested in the faeces (Dilworth et al. 2004). Overall, phytic acid which is the main storage form of phosphorus in cereals, legumes and oleaginous seeds, has been recognized as the main inhibitor of mineral bioavailability in foods of plant origin (Yonekuru and Suzuki 2003).

Bones are plastic-like tissues containing organic and inorganic components. They consist essentially of intercellular material impregnated with mineral substances, mainly hydrated calcium phosphate (Moore 1985). Bone has two compartments, the cortical bone and trabecular bone. Cortical bone provides the sturdy outer wall while trabecular bone provides support along the lines of stress (www.familydoctor.co.uk 2005). Trabecular bone is supplied with blood vessels and is metabolically active and sensitive to hormones that govern day-to-day deposits and withdrawals of calcium. It therefore readily gives up minerals whenever blood calcium needs replenishing.

It is expected that mineral deficiencies which may arise as a result of unchecked phytic acid consumption may be manifested in the levels of some bone minerals or in a change in bone ultrastructure. Sweet potato which is a staple food in the Caribbean has been reported to contain high level of phytic acid. The effect of Sweet potato consumption on mineral homeostasis is not known especially with reported cases of iron deficiency in the region. Against this background, the levels of some minerals essential to the development and maintenance of bones as well as the maintenance of other key metabolic processes in the body, were assessed in rats fed phytic acid extract from sweet potato. Commercial phytic acid was used for comparison purpose. The femur, being the longest bone in the body was chosen for our assessments.

Methods

Fresh matured sweet potato (*Ipomoea batatas)* tubers were harvested from a local farm in the Parish of Manchester, Jamaica. Tuber samples were washed with distilled water, oven dried at 65°C to constant weight and ground into fine powder.

Extraction and determination of phytic acid

Phytic acid was extracted by a modification of the method of Samotus and Schwimmer et al. (1962). A known amount of sweet potato was blended for 5 min with 10% tri-chloro acetic acid in a Warring Blender. The slurry was filtered by suction in a sintered glass funnel and the residue washed successively with known volumes of 5% tri-chloro acetic acid. Filtrates were combined, neutralized with 5 M NaOH and freeze-dried for use as phytic acid extract. Phytic acid level in the extract was determined by the method of Holt (1955) as described by Davis and Reid (1979). Commercial phytic acid was purchased from Sigma-Aldrich, St. Louis, MO, USA.

Feeding experiments

The experimental animals were 36 adult Wistar rats which were assigned by weight into six groups of six rats each; average body weights 236.4 g. Diets were prepared according to standard methods of diet preparations, AIN-93G Purified Rodent diet. AIN-93G Vitamin mix and AIN-93G Mineral mix (Reeves 1997).

Table 1 Test groups and their respective diets

Groups	Diet composition	Group names
Group I	Formulated diet	D
Group II	Formulated diet + Zn	D + Zn
Group III	Formulated diet + Zn + phytic acid extract	D + Zn + PE
Group IV	Formulated diet + Zn + commercial phytic acid	D + Zn + CP
Group V	Formulated diet + phytic acid extract	D + PE
Group VI	Formulated diet + commercial phytic acid	D + CP

Control diets fed to groups D and D + Zn were formulated without or with zinc supplementation, respectively. Diets D + Zn + PE and D + Zn + CP were formulated to simulate the level of phytic acid to zinc ratio (18:1) observed in sweet potato commonly consumed in the Caribbean. Diets prepared for Groups D + PE and D + CP had phytic extract from sweet potato and commercial phytic acid supplementation, respectively without zinc, in order to assess the effects of phytic acid on low dietary zinc intake. The levels of phytic acid and zinc in the extract were 9.46 and 0.02 mg/g, respectively. Zinc was added as part of the mineral mix to the diets of groups D + Zn, D + Zn + PE and D + Zn + CP animals, in the form of zinc carbonate at a concentration of 1.65 mg/kg (Reeves 1997). Zinc levels in groups D, D + PE and D + CP were negligible as determined by Atomic Absorption Spectrophotometry. The respective diets fed to different groups are listed below (Tables 1, 2).

Rats were housed in stainless steel cages in a room kept on a 12 h light-dark cycle, and were allowed access to their respective diets and water freely. The cages were cleaned daily and the faeces collected and pooled for further analyses. Prior to the start of the experiment, all the animals were fed the control diet (formulated diet with zinc) for 1 week to allow for acclimatization to the new diet. The rats were then fed their respective test diets for 3 weeks. Body weight change and total food intakes were recorded weekly. At the end of the experimental period, the rats were sacrificed by a blow to the head after an overnight fast. Approval for the study was obtained after presentation of the protocol to the Board of the Department of Basic Medical Sciences, University of the West Indies, Mona, Jamaica.

Table 2 Formulated diet composition

Ingredients	(%)
Casein	20.000
Corn starch	39.748
Dextrin	13.200
Sucrose	10.000
Soya bean oil	7.00
Powdered cellulose	5.00
AIN 93G mineral mix	3.500
AIN 93G vitamin mix	1.000
L-Cysteine	0.300
Choline bitartate	0.25
t-Butylhydroquinone	0.0015

(Reeves 1997)

Mineral analyses

For bone mineral analyses, the right-sided femur was excised from all the animals, then cleaned of soft tissue using stainless steel scissors and forceps. They were dried in an oven at 100°C for 3 h or until constant weight was attained. The femurs were then ashed in silica glass crucibles (Momcilovic et al. 1975). Ca, Mg, Zn and Fe were determined according to standard AOAC methods (AOAC 2000) as described below. The samples were initially placed in a furnace and the temperature gradually raised from room temperature to 450°C over a 30 min period, where it was kept for 24 h for complete ashing. One millimeter of concentrated high purity nitric acid was then added. After the bones had dissolved, the solutions were taken to dryness on a hotplate, then heated for 30–60 s over a Bunsen burner. The resulting residues were dissolved in 5 ml of 20% HCl, after which the crucibles were covered and the solution digested at low heat for about

15 min. Each digested solution was allowed to cool and made up to 25 ml in a volumetric flask, using deionized water.

Samples were read using a Unicam 939 atomic absorption spectrophotometer equipped with background correction and cathode lamps. The determination of mineral components in the samples was carried out using a nitrous oxide/acetylene mixture, at the following wavelengths: 422.7 nm for calcium and 248.3 nm for iron and Zinc. A slit width of 0.2 mm was used for Iron and Magnesium while 0.7 mm was used for Zinc and Calcium. In determining the calcium content, a solution of lanthanum chloride was added to all the analysed solutions and reference samples to attain a 0.5% concentration of La^{3+}.

The accuracy of the analytical method was confirmed through a series of certified analyses on reference materials. Specific samples were spiked with known amounts of minerals to determine percentage recovery. Faecal minerals were determined using the same method described above.

Sample preparation for scanning electron microscopic analyses

Femur samples dried as previously indicated and the proximal end removed, were each cut longitudinally from the distal end to the shaft, with a stainless steel scalpel. Each bone was then pried open and one-half mounted (fractured surface facing upward) with Leit-C carbon cement to a 12.5 diameter aluminium pin stub. A streak of silver paint was extended from an edge of the fractured face to the aluminium stub, thus providing a conducting path to remove charges that would result from the incident electron beam of the Scanning Electron Microscope striking the specimen. The mounting stubs were placed in an oven at 70°C for 15 min to allow for evaporation of volatile solvents from the carbon element and silver paint. The mounted stubs were then transferred to an Edwards S15OB sputter coater and the femurs coated with a thin film of 60:40 gold/palladium alloy at a coating current of 20 mA for 2 min. The sputter coated specimens were then imaged in a Philips SEM 505 scanning electron microscope at an accelerating voltage of 7 kV. Thickness of the trabecular bone plates was determined by comparison with the standard 1 mm scale bar.

Statistical analyses

Results were expressed as mean ± Standard Error or the Mean (SEM). Analysis of Variance (ANOVA) was used to test for differences between the groups. Duncan's Multiple Range Test at significance level $P < 0.05$, was used to test for significant difference among the means (Sokal and Rohlf 1969).

Results and discussion

The feeding programme in this study was aimed at investigating the effects of consumption of phytic acid extract from sweet potato on minerals in the presence and absence of supplemented zinc. Rats which were fed diets supplemented with commercial phytic acid were also assessed for comparative purposes.

Assessment of bone minerals

There were no significant changes in calcium levels in the femur of rats fed commercially obtained phytic acid compared to the control groups. However, rats in the two test groups which consumed diets supplemented with phytic acid extract (D + Zn + PE and D + PE) displayed the lowest levels of bone calcium (Table 3). This suggests that consumption of phytic acid played a vital role in the determination of calcium levels in the bones of Wistar rats (Weaver 1992). Bone calcium levels observed in the two test groups fed commercial phytic acid were also lower than the levels observed in the control groups. This reduction in bone calcium depicted in the groups fed phytic acid supplemented diets, is a direct reflection of the increased faecal output observed in those groups. This indicates that dietary calcium which is bound by phytic acid is made unavailable to the body as it passes through the gastrointestinal tract and eventually egested in the faeces. In such scenarios, the blood calcium levels would be expected to remain normal as any shortfall would be replaced by the bone reserves (Guyton and Hall 1996). The crude phytic acid extract was found to contain small amounts of tannins which are shown to inhibit calcium channels (Zhu et al. 1997) and otherwise reduce calcium levels in the body (Chiesi and

Table 3 Calcium and Magnesium levels in the femur of rats fed diets supplemented with or without phytic acid extract

Diets administered	Ca (g/kg)	Mg (g/kg)
Formulated diet (D)	210.5 ± 6.88[a]	3.89 ± 0.131[a]
Formulated diet + Zinc (D + Zn)	213.14 ± 15.31[a]	4.17 ± 0.33[a]
Formulated diet + Zinc + phytic acid extract (D + Zn + PE)	101.27 ± 19.11[a]	4.20 ± 0.15[a]
Formulated diet + Zinc + commercial phytic acid (D + Zn + CP)	203.58 ± 40.46[a]	4.16 ± 0.17[a]
Formulated diet + phytic acid extract (D + PE)	119.27 ± 8.36[a]	4.84 ± 0.26[a,c]
Formulated diet + commercial Phytic acid (D + CP)	188.85 ± 30.55[a]	3.72 ± 0.13[a,b]

Figures that share different letter superscripts are significantly different ($P < 0.05$). Mean ± SEM, $n = 3$

Schwaller 1994). This may translate to a reduction in bone calcium levels. Our data also confirm the role of phytic acid in sequestering calcium as reported by Anshu and Neelam (2002).

The lowest level of magnesium in the bone was observed in the test group fed formulated diet + commercial phytic acid (D + CP). The level of bone magnesium in this group was significantly lower than the level observed in the group fed formulated di et al ong with phytic acid extract (D + PE). This suggests that in the absence of zinc supplementation, commercial phytic acid played a greater role in reducing femur magnesium compared to phytic acid extract. This may be because the tannins and other factors responsible for reduced availability of calcium were not as effective against magnesium. The additional of zinc to the diets resulted in bone magnesium levels which did not vary significantly at the $P < 0.05$ level. This we theorize may be due to competition for the phytic acid active binding sites by zinc, resulting in increased levels of magnesium availability for absorption. Other differences in bone magnesium levels were not significant. As observed with bone calcium, there was no correlation between dietary zinc supplementation and bone magnesium levels.

The lowest femoral zinc levels were observed in the test groups with the lowest dietary zinc. Similarly, test groups which had zinc supplement diets had higher bone zinc levels compared to the groups which had diets void of zinc (Fig. 1). Zinc deficiency was however not observed at the level of the bones, as the bone zinc levels did not vary significantly among groups.

Animals fed diets supplemented with phytic acid extract plus zinc and commercial phytic acid, respectively (D + Zn + PE and D + CP) had significantly higher femur iron levels compared to the other test groups. The levels of iron observed in the other test groups (D + Zn + CP and D + PE) were lower than the levels observed in the control group, but this difference was not significant (Fig. 1). This suggests that a critical shortage of iron did not exist in the bones of these test groups. This is important as iron stored in the bones, liver and spleen is critical to the production of ferritin, an iron storage protein (www.webmd.com 2006). The extra-phytate constituents of the extract again played a role in reducing bone iron levels compared to the commercial phytate as the addition of zinc to the diet of animals fed phytate extract, resulted in the introduction of the mineral for which phytate has the highest affinity thus leaving the available iron for incorporation into bones. The situation observed upon addition of zinc to the diets of rats fed commercial phytic acid showed an opposite trend and this was not expected.

Assessment of faecal minerals

Faecal magnesium levels were higher in the test groups fed diet supplemented with phytic acid extract plus zinc and diet supplemented with phytic acid extract (D + Zn + PE and D + PE), respectively. These values were significantly higher than those displayed by all other groups (Table 4). This suggests that phytic acid extract was very effective in reducing the amount of dietary magnesium available for normal metabolism and may have negative effects on metabolic processes that require this mineral as an essential component. This increase in faecal magnesium was not reflected at the level of the bones as the groups which had the highest levels of faecal magnesium output (D + Zn + PE) and (D + PE) also had the highest level of bone Mg. This suggests that the increase in faecal magnesium output did not

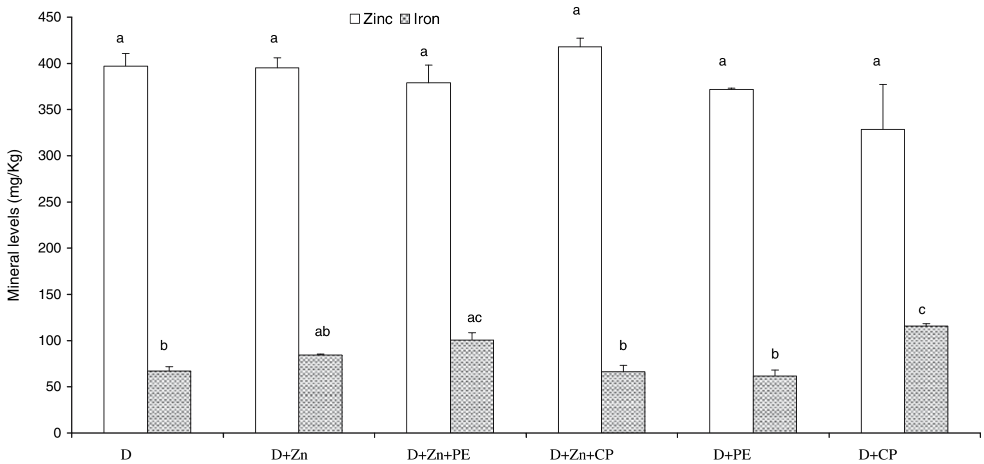

Fig. 1 Levels of zinc and iron in the femur of rats fed phytic acid supplemented diets. *Bars* that display different letters are significantly different at the level where $P < 0.05$. Mean ± SEM, $n = 3$. Bars that display different letters are significantly different at the level where P < 0.05. Mean ± SEM, n = 3

Table 4 Faecal Calcium and Magnesium levels in rats fed diets supplemented with or without phytic acid extract

Groups	Diets	Magnesium (g/kg dry weight)	Calcium (g/kg dry weight)
I	D	5.01 ± 0.25^{a}	39.92 ± 1.15^{a}
II	D + Zn	9.82 ± 0.005^{a}	35.17 ± 0.73^{a}
III	D + Zn + PE	16.90 ± 2.40^{b}	61.31 ± 7.44^{a}
IV	D + Zn + CP	6.45 ± 0.35^{a}	53.57 ± 14.86^{a}
V	D + PE	17.10 ± 0.10^{b}	51.35 ± 9.34^{a}
VI	D + CP	3.36 ± 0.38^{a}	52.20 ± 13.66^{a}

Figures that share different letter superscripts are significantly different ($P < 0.05$). Mean ± SEM, $n = 3$

affect the levels within the bones. It could be that consumption of phytic acid may be contributing to the depletion of magnesium from other sources in the body. It may also further suggest that the increase in bone magnesium levels in these two groups may be as a result of the body trying to compensate for the increased output being experienced at the level of the faeces.

Faecal calcium levels did not vary significantly among the groups (Table 4); however, the values indicate that both phytic acid extract and commercial phytic acid contributed to increased faecal calcium levels observed in all the test groups. The groups fed formulated diet or formulated diet + Zn only, had lower faecal calcium levels confirming the role of phytic acid in the removal of calcium from the gastrointestinal tract which eventually ends up in the faeces. The results show that phytic acid may be working closely with its e extra-phytate components and collaboratively may be responsible for the significantly higher levels of fecal magnesium output in rats which consumed the extract compared to rats which consumed the commercial phytate. A similar trend was not observed for faecal calcium as all rats fed phytic acid had higher faecal calcium levels compared to those without phytate diets.

Previous studies have shown that consumption of phytic acid from sweet potato may contribute to greater faecal zinc output and may eventually lead to zinc deficiency if not properly managed (Dilworth et al. 2004). These results also show that calcium intake need to be monitored in diets rich in phytic acid as the resultant high faecal output may contribute negatively to the amount of calcium available for metabolic purposes.

Scanning electron microscopy (SEM) assessment of rat femur

The trabecular region of bone is a latticework of thin bone plates oriented along lines of stress. Excess loss of minerals (especially calcium) as seen in conditions like osteoporosis, will result in thinning of the plates within the trabecular matrix which may eventually compromise the overall mechanical strength of the bone. In this case the thinning of the bone will increase the risk of breakage with even trivial injuries (Davidson et al. 1975). For the sake of comparison, the femurs highlighted are derived from animals in the two groups which recorded the highest and lowest values for trabecular plate thickness, respectively (Fig. 2). All the animals which had their diets supplemented with phytic acid (groups D + Zn + PE, D + CP, D + PE and D + Zn + CP), had significantly thinner trabeculae compared to the control groups which were fed a formulated diet only or a formulated diet plus zinc, i.e. groups D and D + Zn (Table 5). Although significant differences existed in the thickness of trabecular plates between control groups (D and D + Zn) compared to all other groups, this difference is not apparent upon visual examination of the figures presented. With accurate measurements however, statistical analysis confirmed that trabecular plates were indeed thicker in the femur of animals belonging to groups D and D + Zn compared to the femur of all other test animals. These test animals with thinner trabecular plates were all fed diets supplemented with phytic acid extract or commercial phytic acid, hence establishing the role of this supplement in reducing the availability of dietary calcium, a key mineral in bone metabolism and formation. The trend seen in these test groups of increased faecal calcium levels and a contrasting reduction in the femur, reiterates the fact that sufficient dietary calcium was not made available to the rats fed phytic acid supplemented diets.

In older animals that have higher rates of calcium loss we would expect the rate of trabecular thinning to be increased and a corresponding acceleration in the onset of osteoporosis. It is not known however, if the reduction in bone calcium would have any detrimental effects on the younger animals which normally have a much lower rate of bone loss under normal conditions. For older animals, a regime of adequate calcium supplementation along with reduced phytic acid intake is advised in order to minimize the onset of bone loss, which is a precursor to some bone diseases (Fig. 3).

In conclusion, the consumption of diets supplemented with phytic acid, did not significantly alter the levels of calcium, magnesium or zinc, in the bones of Wistar rats. The changes in femur iron levels did not show a significant reduction compared to the control group. Although the assessment of faecal minerals

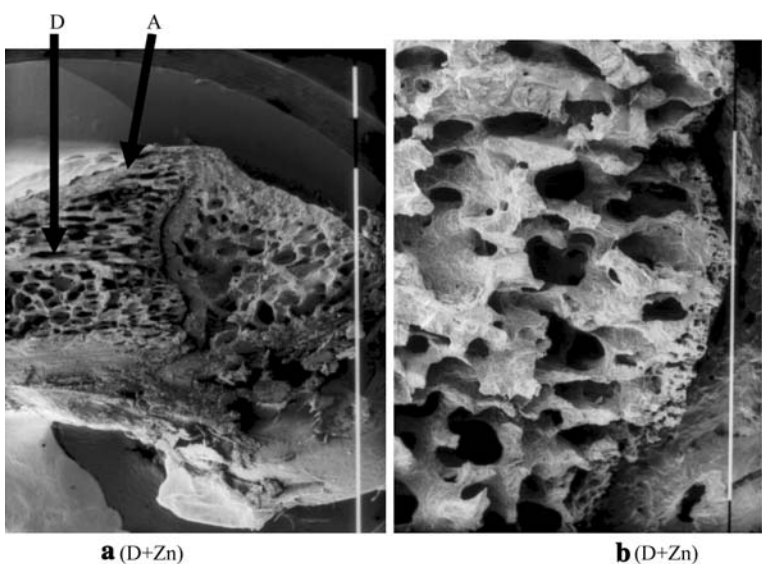

Fig. 2 Scanning electron microscopic micrographs of the femur of Wistar rats highlighting different regions. * For all figures, *Scale bar* ≡ 1 mm, *Spot size* ≡ 100 nm. (**a**) (D + Zn), Magnification = ×16, A = Cortical bone of the shaft, D = Region of trabecular bone. (**b**) (D + Zn) magnification = ×85 Detailed view of the trabecular bone region

Table 5 Assessment of rat femur showing average width of the trabeculae

Diets administered	Width of trabecular plates (μm)
Formulated diet (D)	69.886 ± 10.67[a]
Formulated diet + Zinc (D + Zn)	66.304 ± 12.17[a]
Formulated diet + Zinc + phytic acid extract (D + Zn + PE)	55.978 ± 17.63[b]
Formulated diet + Zinc + commercial phytic acid (D + Zn + CP)	57.279 ± 12.72[b]
Formulated diet + phytic acid extract (D + PE)	58.854 ± 10.73[b]
Formulated diet + commercial phytic acid (D + CP)	54.02 ± 6.07[b]

Figures that share different letter superscripts are significantly different ($P < 0.05$)

Values: mean ± Standard Error of the means, $n = 10$

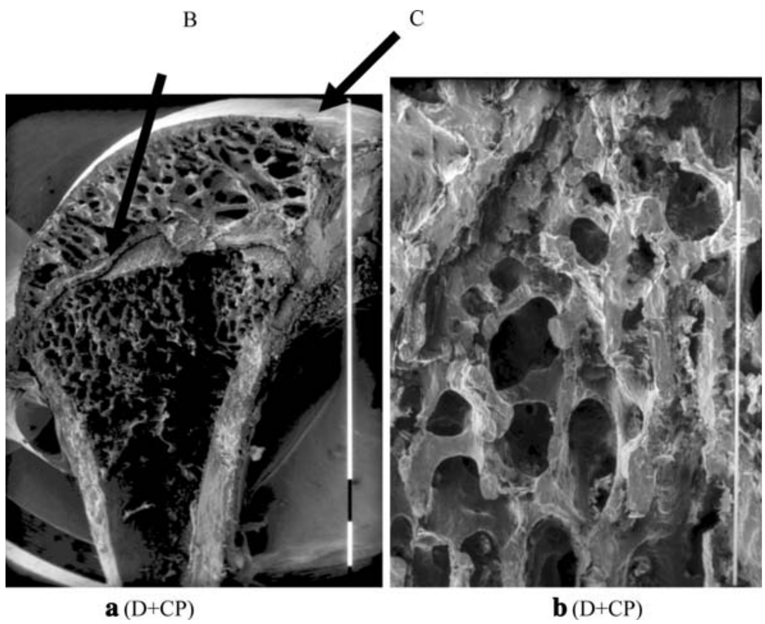

Fig. 3 (**a**) (D+CP) Magnification = ×16 B = Hyaline cartilage C = Distal end of the bone *For all figures, *Scale bar* ≡ 1mm, *Spot size* ≡ 100nm. (**b**) (D+CP) High power view of trabecular bone Magnification = ×85

may be used to assess the amount of minerals not absorbed, it is not a comprehensive method as we acknowledge that urinary mineral levels also have to be taken into consideration. The ultrastructure of the femur was significantly altered in rats that consumed phytic acid supplemented diets. Our findings may not be conclusive, as other minerals like phosphates also play a role in the maintenance and alteration of the bone trabecular region. Other parameters like bone density and bone breaking strength are also important in painting a complete picture of the effect of phytic acid supplementation of bone structure in mammals.

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