Eggshell fractions containing different particle sized affect mineral absorption but not bone mineral retention in growing rats

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ABSTRACT
The purpose of this study was to compare the calcium (Ca) bioavailability from eggshell fractions containing different particle size to purified CaCO3 in male growing rats. Mineral absorption, bone mineral concentration, and biomechanical properties were evaluated. Mean Ca absorption of rats fed with eggshell diets amounted to 56.2% of the ingested Ca, which is considered high. However, we observed lower Ca absorption in large-sized particle eggshell fraction (ES L) and small-sized particle eggshell fraction groups but similar Ca absorption in intermediate-sized particle eggshell fraction (ES M) compared with the CaCO3 group. Rats that received ES M and ES L had higher P and Mg absorption than the CaCO3 group. No changes were observed in the bone mineral deposition, weight or mechanical resistance. We conclude that eggshell Ca is well absorbed by the intestine and retained in bones of growing rats, being a low cost alternative to achieve adequate Ca ingestion.

Introduction
Calcium (Ca) is an essential mineral required for bone development, neuromuscular functioning, coagulation, cell permeability, enzyme activation, hormone secretion, and many other extracellular and intracellular functions (Pu et al. 2016). However, Ca dietary intake is often insufficient (Bauer 2013), reducing Ca deposition in bones and increasing the risk of chronic diseases such as osteoporosis (Pu et al. 2016).

Adequate dietary Ca levels are associated to the regular consumption of milk and dairy products, which are the main sources of this mineral (Caroli et al. 2011). Nevertheless, these foodstuffs are too expensive for low-income people, especially in developing countries (McLeod 2013). In addition, common problems as lactose intolerance and milk protein allergies also limit dairy consumption (Martorell-Aragonés et al. 2015; Lule et al. 2016).

Eggshell powder is a low-cost alternative to dairy products in order to provide adequate Ca intake. It has been demonstrated to be a good Ca source in growing (Schaafsma & Beelen 1999), adult (Ascar et al. 1993; Brun et al. 2013) and ovariectomized (Hirasawa et al. 2001) animals and also in humans (Schaafsma & Pakan 1999; Schaafsma et al. 2002; Fina et al. 2016). However, it has been scarcely used in human nutrition despite being a natural, abundant and renewable product, which is easily available at home and possesses lower levels of toxic metals than other natural Ca sources (Rovenský et al. 2003; Brun et al. 2013; Oliveira et al. 2013).

The method of processing eggshell destined to human consumption was patented in Slovakia and lately in many European countries and the United States, in the 1990s (Rovenský et al. 2003). Eggshell powder is also used in multimixture, a dietary supplement prepared with low-cost ingredients and food byproducts, that is distributed for low-income population in Brazil and other countries to counteract malnutrition (Callegaro et al. 2010). Moreover, the inclusion and acceptance of eggshell fortified food is the subject of several studies, including products as sausage, cake, bread, pasta, fermented milk, and others (Daengprok et al. 2002; Naves et al. 2007; Salem et al. 2012; Brun et al. 2013; Fina et al. 2016).
Eggshells are a matrix of interwoven protein fibers (about 2%) and calcified zones of calcium carbonate crystals (CaCO₃, ranging between 95 and 98%) (Masuda & Hiramatsu 2008; Oliveira et al. 2013). Ca is nearly 40%, whereas magnesium (Mg) and phosphorus (P) are found at concentrations near to 0.5 and 0.1%, respectively (Schaafsma et al. 2000; Milbradt et al. 2015). Moreover, eggshell contains small amounts of other bone health-related minerals, such as strontium (Sr) and selenium (Se), besides low levels of hormones as calcitonin, transforming growth factor-β1 and progesterone (Schaafsma et al. 2000; Milbradt et al. 2015). The eggshell matrix protein is composed of soluble and insoluble fractions with Ca-binding properties including proteoglycans, glycoproteins and protein acid mucopolysaccharides (Guru & Dash 2014).

Before human consumption, eggshell must be washed, dried, ground, and hygienized (Naves et al. 2007; Masuda & Hiramatsu 2008; Milbradt et al. 2015). Grinding is an important step since greater particle size may impair sensory acceptance due to texture changes (Brun et al. 2013). Domestic grinding may be achieved using a rolling pin or a domestic blender followed by sieving to separate coarse particles (Naves et al. 2007; Brun et al. 2013) but the particle size will be greater than that obtained in laboratory mill or in factory scale (Brun et al. 2013). In fact, the reduction of particle size was precisely the strategy used to improve Ca absorption from carbonate in previous studies (Shahnazari et al. 2009; Elble et al. 2011).

Eggshell Ca bioavailability was previously reported to be similar or even higher than purified CaCO₃ (Schaafsma & Beelen 1999; Schaafsma & Pakan 1999; Hirasawa et al. 2001; Schaafsma et al. 2002; Brun et al. 2013). Nevertheless, the influence of eggshell granulometry on Ca absorption and bone properties has not been evaluated.

Our hypothesis is that the smaller eggshell particle size will increase the gastrointestinal absorption and retention of Ca. The purpose of this study was to compare the Ca bioavailability from eggshells containing different particle size to purified CaCO₃, by evaluating mineral absorption, bone mineral concentration and biomechanical properties. Growing rats were used to simulate Ca absorption in the infancy-adolescence period, since these stages of life are critical to reach adequate bone mass and prevent or retard osteoporosis (Uusi-Rasi et al. 2013). This study is important to elucidate a factor that may interfere in the bioavailability of eggshell Ca which is a low-cost, safe, alternative Ca source.

Materials and methods

Eggshell processing and characterization

Different eggshell varieties from Santa Maria region (RS, Brazil) were used to obtain the powder. Eggshells were washed with tap water and superficial sediment was removed. To separate inner membranes, the eggshells were roughly triturated with tap water in a blender (1:10, w/v) and the liquid containing membranes was discarded. This step was repeated until obtaining an eggshell apparently free of membranes (indicated by clear liquid). Then, samples were oven dried (50°C for 24 h) and milled in a knife mill (Marconi, MA 630). The mix of eggshell powder was fractioned using a vibratory sieving machine (Servylab, Porto Alegre, RS, Brazil) attached to sequential 420, 212, and 106 μm-sieves to yield three eggshell fractions with decreasing particle size: large (between 420 and 212 μm; ES L), intermediate (between 212 and 106 μm; ES M), and small (< 106 μm; ES S) particle fractions. The fraction retained in the 420 μm-sieve was discarded.

The eggshell fractions and the purified CaCO₃ (used in the control diet) were evaluated in a multi-wave length laser diffraction analyzer (Beckman Coulter LS13 320) to determine the particle size. Figure 1 illustrates the particle size distribution of the Ca sources used to formulate rat diets. Median particle size (d₅₀ ± SD) was 333 ± 78 μm for ES S, 172 ± 63 μm for ES M, 47 ± 40 μm for ES S, whereas CaCO₃ had the smallest particle size (6.6 ± 5.4 μm). Particle size analysis also showed that ES M and ES L had a narrower range of particle dimension than ES S (Figure 1).

Diets

Four diets were formulated: CaCO₃ (standard), ES S, ES M, and ES L. Diets’ ingredients and composition are shown in Table 1. Diets were prepared according to the rodent diet from the American Institute of Nutrition (AIN-93) (Reeves 1997). The standard Ca source (CaCO₃; 99% purity; Dinâmica Química, Diadema, São Paulo Brazil) was completely replaced by the different eggshell powders to provide the same Ca concentration in all diets (500 mg/100 g). The amount of eggshell powder and CaCO₃ to be used in each diet was calculated after Ca measurement in each Ca source separately. Ca concentration was 40.20 ± 0.17% in CaCO₃, 38.05 ± 0.90% in ES S, 39.09 ± 1.41% in ES M, and 39.92 ± 0.25% in ES L.

Moisture (method 925.10), ash (method 923.03), fat (method 945.39), and crude protein (method 960.52, N × 6.25, micro-Kjeldahl method) of diets were
evaluated as stated by the Association of the Official Analytical Chemists (AOAC 1995). The total dietary fiber content was determined by the enzymatic-gravi-metric method (985.29) (AOAC 1995). The available carbohydrates (nitrogen-free extract, NFE fraction) were calculated as follows: 

\[
\text{NFE fraction (\%)} = \frac{100}{\text{moisture} + \text{crude ash} + \text{crude fat} + \text{crude protein} + \text{dietary fiber}}.
\]

Energetic value was calculated using the factors 37.6 kJ/g for lipids and 16.7 kJ/g for protein and NFE fractions. Mineral content in diets was determined as described in Minerals content and calculation section.

Crude protein was also determined in triplicate in eggshell fractions. ES L had 1.72 ± 0.18% protein, while ES M and ES S had 1.83 ± 0.18% and 2.21 ± 0.10% protein, respectively.

Eggshell powders did not add important amounts of P, Mg, protein, or other macronutrients to the diets (Table 1). Calcium concentrations in the experimental diets were similar to control, as expected. However, Ca content of all diets was about 9% higher than the amount added to the diets (average content of 545 mg% versus expected 500 mg%). It possibly occurred due to the presence of Ca in other diet ingredients, mainly casein.

### Animals and experimental protocol

Thirty-two male Wistar rats (3 weeks-old; weighing 62 ± 6 g) from our own breeding colony were acclimated on the feeding system with AIN-93 diet during 5 days, and further randomly divided into four experimental groups (n = 8 per group). The animals were individually housed in metabolic cages, under controlled room temperature (22 ± 2°C) and humidity (55 ± 5%) with 12:12 h light and dark cycle, and had free access to drinking water and diet during the experimental period of 28 d. The feed intake was recorded daily and the body weight of animals was obtained every 5 d. These data were used to determine the feed efficiency ratio (g body weight gain/g food intake). Feces were quantitatively collected for each animal from days 15 to 25 of the experiment, and subsequently dried, milled and stored. After 4 weeks feeding period, animals were anesthetized with an intramuscular injection of xylazine (10 mg/kg body weight) and ketamine (75 mg/kg body weight) and euthanized by decapitation. Legs were collected and frozen intact (−20°C) to prevent bone desiccation. All animals survived the experimental period but data from one rat of the ES L group was excluded because of outlier results for dietary intake and weight gain.

All animal procedures were conducted according to the Brazilian guidelines for care and use of animals for scientific and educational purposes (CONCEA 2016) and were approved by the Ethics and Animal Welfare Committee from the Federal University of Santa Maria (protocol 038/2011).

### Table 1. Ingredients and proximate composition of diets fed to growing rats.

| Ingredient | CaCO₃ | ES S | ES M | ES L |
|------------|-------|------|------|------|
| Casein (%) | 20.0  | 20.0 | 20.0 | 20.0 |
| Cornstarch (%) | 53.0 | 53.0 | 53.0 | 53.0 |
| Sucrose (%) | 10.0  | 10.0 | 10.0 | 10.0 |
| Soybean oil (%) | 7.0  | 7.0  | 7.0  | 7.0  |
| Cellulose (%) | 5.0  | 5.0  | 5.0  | 5.0  |
| BHT* (%) | 0.0014 | 0.0014 | 0.0014 | 0.0014 |
| Vit-mixb (%) | 1.0  | 1.0  | 1.0  | 1.0  |
| L-Cystine (%) | 0.3  | 0.3  | 0.3  | 0.3  |
| Choline bitartrate (%) | 0.25 | 0.25 | 0.25 | 0.25 |
| Ca free min–mixc (%) | 2.25 | 2.25 | 2.25 | 2.25 |
| CaCO₃ (%) | 1.25 | 1.32 | 1.29 | 1.26 |
| NFE fraction (%) | 57.6 | 57.4 | 57.5 | 58.6 |
| Energy (kJ/100 g) | 1559 | 1555 | 1557 | 1561 |
| Moisture (%) | 8.8  | 8.8  | 8.9  | 8.6  |
| Crude ash (%) | 2.7  | 2.9  | 2.7  | 2.8  |
| Crude fat (%) | 7.7  | 7.7  | 7.7  | 7.4  |
| Crude protein (%) | 18.4 | 18.4 | 18.4 | 18.2 |
| Total dietary fiber (%) | 4.8  | 4.8  | 4.8  | 4.4  |
| NFE fraction (%) | 57.6 | 57.4 | 57.5 | 58.6 |
| Ca (mg/100 g) | 54.5 | 54.5 | 53.9 | 54.4 |
| Mg (mg/100 g) | 324.2 | 334.3 | 335.6 | 325.3 |
| Mg (mg/100 g) | 49.7 | 52.2 | 52.6 | 52.1 |

*ES S: small-sized particle eggshell fraction; ES M: intermediate-sized particle eggshell fraction; ES L: large-sized particle eggshell fraction.

*Calcium free AIN-93 mineral mixture.

**AIN-93 vitamin mixture.

Diet composition is shown as g/100 g except for minerals, shown as mg/100 g.

NFE (nitrogen-free extract).
Minerals content and calculations

The left legs were thawed and tibias were dissected, cleaned and dried overnight at 110 °C, weighed and stored for mineral analysis. Diets and feces and the whole left tibias were wet-digested with nitric and perchloric acids (Merck, Darmstadt, Germany) (Tedesco et al. 1995). Ca and Mg concentrations were assessed by atomic absorption spectrometry using an Atomic Absorption Spectrophotometer (SpectrAA-20 Plus, Varian) after adequate dilutions with La2O3 solution (1%, Merck, Darmstadt, Germany). A range of Ca (Titrisol, Merck, Germany) and Mg (metallic, Merck, Germany) standards were used to obtain the calibration curves. P concentrations were measured spectrophotometrically after reaction with ammonium–molybdate and ammonium–metavanadate (Quinlan & DeSesa 1955). The intra- and the inter-assay coefficient of variation for Ca were 2.4 and 8.7%, for Mg were 1.4 and 5.6%, and for P were 0.9 and 1.5%, respectively. Every analysis was processed at least in triplicate.

Mineral parameters (ingestion, fecal excretion, and apparent absorption) were obtained based on the mineral content of diets and feces. The mineral absorption was calculated using the following equation:

Relative apparent absorption (%) = (intake − fecal excretion) × 100/intake

Tibia biomechanical testing

Right legs were slowly thawed and tibias were dissected, cleaned and wrapped in saline-soaked gauze at 10 °C overnight. Before testing, bones were acclimated at room temperature, while kept wet using saline.

Tibial 3-point flexural bending tests were conducted using a TA.XT plus texture analyzer operated with the Exponent software 6.1.3.0 (Stable Micro Systems, Orlando, FL) and a 16 mm support span (HDP/3PB probe) attached to a 50 kg load cell, using crosshead speed set at 0.6 mm/min. Bones were consistently oriented with the load applied on the posterior side at mid-diaphyseal region. Load–displacement curve of each tibia was used to determine yield point and ultimate load (N), displacement at the ultimate load (mm), and stiffness (N/mm). The method was based on previous publication of Wray et al. (2011).

Statistical analysis

A power analysis was performed based on the Ca absorption data from a previous study of our group in male growing Wistar rats (Callegaro et al. 2011).

A sample size of eight rats per group was required to detect a 15% difference in apparent mineral absorption among the groups with 86% significance power, at α = .05 level. Statistical analyses were performed using Statistica 7.0 for Windows (Tulsa, OK). Data were analyzed using 1-way analysis of variance (ANOVA) except for the body weight data along the experimental period that was analyzed by 2-way factorial ANOVA (4 groups x 7 time points). An effect was considered significant at p ≤ .05. For all parameters with significant p values, intergroup differences were determined with Tukey unequal test. The results were presented as the means ± SEM of eight animals per group, except for group ES L that had n = 7.

Results

Feed intake, growth, and feed efficiency ratio

The growth parameters of rats are shown in Table 2 and Figure 2. Rats fed with eggshell or with CaCO3 as Ca source had similar feed intake, body weight gain, and feed efficiency ratio (Table 2 and Figure 2). Body weight significantly increased over the experimental period (p < .05) but no Ca source effect or a Ca source × time effect was observed (Figure 2).

Apparent mineral absorption

Table 3 presents data on the ingestion and excretion of Ca, P and Mg, while Figure 3 presents data on relative apparent absorption (%) of these minerals.

All experimental groups had similar Ca ingestion during the absorption-measuring period, but Ca fecal excretion was higher in rats fed with the ES L and ES S diets compared to the CaCO3 diet (Table 3). Ca absorption was high for all groups and ranged between 52.4 and 62.0%. Besides, it was similar for rats fed with ES M and the CaCO3 diet, whereas rats fed with ES S and ES L diets had lower Ca absorption than the CaCO3 diet (p < .05; Figure 3).

P ingestion was similar for all groups, whereas Mg ingestion was greater in rats fed the ES M diet than the CaCO3 diet (p < .05). P excretion was lower in rats fed ES M and ES L than the CaCO3 diet, whereas the Mg excretion was lower in rats fed ES L than the CaCO3 diet (p < .05; Table 3). As the dietary concentration of P and Mg was similar among all groups (Table 1), the lower excretion found was due to the increased P and Mg absorption of the eggshell groups compared with the CaCO3 group. Animals fed with ES M and ES L diets had greater P and Mg absorption than CaCO3 diet (p < .05; Figure 3).
In fact, P and Mg absorption were strongly correlated \((r = .75; p < .05)\).

**Bone assays**

Table 4 presents the weight, mineral content and mechanical properties of tibias. Rats that received eggshell powder as the unique dietary source of Ca had similar bone Ca, P and Mg contents regardless of the particle size, and these contents were also similar to the CaCO\(_3\) diet. In addition, the weight and the biomechanical properties of tibias were also similar among all treatments (Table 4).

**Discussion**

Growing rats receiving eggshell as Ca source achieved the same body weight gain and feed efficiency ratio as the control group that received CaCO\(_3\), regardless of the particle size. Previous studies evaluating Ca bioavailability from different sources in growing rats also did not find differences in these parameters, despite unequal Ca absorption (Toba et al. 1999; Mora-Gutierrez et al. 2007). In fact, when Ca is provided in sufficient amounts, changes in Ca absorption may not alter growth parameters. In addition, Schaafsma and Beelen (1999) did not observe variation on the feed intake or growth of piglets receiving eggshell compared to CaCO\(_3\) (Schaafsma & Beelen 1999).

Mineral bioavailability is influenced by many factors, including those related to the mineral source, namely speciation, molecular bonds, food matrix components, and disintegration; those related to the person, namely age, gender and nutritional state; and others, namely mineral dietary intake and interaction with dietary components (Cozzolino 1997; Emkey & Emkey 2012; Slupski et al. 2014). The major determinant of bioavailability is the portion which is absorbed by the gastrointestinal tract.

Table 2. Body weight gain, feed intake, and feed efficiency ratio of rats fed with eggshell fractions containing different particle size as the Ca source compared with CaCO\(_3\).

| Parameters                  | CaCO\(_3\)          | ES S  | ES M  | ES L  |
|-----------------------------|---------------------|-------|-------|-------|
| Feed intake (g/day)         | 18.17 ± 0.39        | 18.36 ± 0.45 | 18.35 ± 0.32 | 17.66 ± 0.62 |
| Body weight gain (g/day)    | 6.68 ± 0.18         | 6.71 ± 0.08 | 6.70 ± 0.30 | 6.46 ± 0.20 |
| Feed efficiency ratio*      | 0.37 ± 0.01         | 0.37 ± 0.01 | 0.37 ± 0.01 | 0.37 ± 0.01 |

The results were expressed as means ± SEM (n = 8 for all groups, except for ES L that had n = 7). No significant effect of diet was observed on the evaluated parameters (ANOVA, \(p > .05\)). ES S: small-sized particle eggshell fraction; ES M: intermediate-sized particle eggshell fraction; ES L: large-sized particle eggshell fraction.

 ag body weight gain/g feed intake.

**Table 3. Ingestion and excretion of Ca, P, and Mg of rats fed with eggshell fractions containing different particle size as Ca source compared to CaCO\(_3\).**

| Parameters                  | CaCO\(_3\)          | ES S  | ES M  | ES L  |
|-----------------------------|---------------------|-------|-------|-------|
| Ca Ingestion (mg/day)        | 122.1 ± 3.2         | 126.9 ± 3.1 | 128.6 ± 3.0 | 120.2 ± 4.8 |
| Fecal excretion (mg/day)     | 47.6 ± 2.6          | 58.1 ± 1.8  | 48.9 ± 2.2  | 57.1 ± 2.4  |
| P Ingestion (mg/day)         | 72.6 ± 1.9          | 77.8 ± 1.8  | 80.0 ± 1.8  | 71.9 ± 2.8  |
| Fecal excretion (mg/day)     | 15.3 ± 1.3          | 13.5 ± 0.9  | 10.0 ± 0.8  | 6.5 ± 1.2   |
| Mg Ingestion (mg/day)        | 11.1 ± 0.3          | 12.1 ± 0.3  | 12.5 ± 0.3  | 11.5 ± 0.5  |
| Fecal excretion (mg/day)     | 3.3 ± 0.3           | 3.4 ± 0.1   | 2.8 ± 0.1   | 2.5 ± 0.1   |

The results were expressed as means ± SEM (n = 8 for all groups, except for ES L that had n = 7). Means that have no common superscript letter within the same line are different (Tukey’s test; \(p < .05\)). ES S: small-sized particle eggshell fraction; ES M: intermediate-sized particle eggshell fraction; ES L: large-sized particle eggshell fraction.
compared with CaCO3 but the effect of the particle lar or even superior Ca absorption from eggshells in a rat model of osteoporosis (Hirasawa et al. 2001). Piglets had similar Ca absorption from eggshell and CaCO3 when casein was the protein source of diet, and higher Ca absorption from eggshell than from CaCO3 when the protein source was soybean isolate (Callegaro et al. 2010). Previous papers indicated similar Ca absorption of rats fed eggshell diets (56.2% of the ingested Ca) was similar to other study using AIN-93 diet containing conventional Ca sources (Schaafsma & Beelen 1999). Thus, we suppose that the methodology used to obtain the eggshell fractions in the present study (milling and sieving) could differentially separate the components of eggshell layers generating particles with similar Ca content but different Ca bioavailability. Supporting this proposal, we observed that the protein content was different among the eggshell fractions; the protein content of ES S was 28% higher than ES L fraction and 20% higher than ES M fraction. On one hand, the interlacement of proteins with Ca crystals could hamper Ca biological disintegration and dissolution in the ES S during digestion, and, consequently, its intestinal absorption. On the other hand, Ca absorption impairment in group ES L could be mainly explained by its greater size, which would delay its disintegration during the intestinal transit. In fact, we could observe eggshell particles in the feces of ES L group rats.

In this work, although only rats fed with ES M diet had the same Ca absorption as the CaCO3 group, the average Ca absorption of rats fed eggshell diets (56.2% of the ingested Ca) was similar to other study using AIN-93 diet containing conventional Ca sources (Callegaro et al. 2010). Previous papers indicated similar or even superior Ca absorption from eggshells compared with CaCO3 but the effect of the particle granulometry had not been previously evaluated. Ca absorption from eggshell was similar to that from CaCO3 in adult male rats (Brun et al. 2013) and in a rat model of osteoporosis (Hirasawa et al. 2001). Piglets had similar Ca absorption from eggshell and CaCO3 when casein was the protein source of diet, and higher Ca absorption from eggshell than from CaCO3 when the protein source was soybean isolate (Schafsma & Beelen 1999).

In this study, we had special care to separate only eggshell, eliminating the adhered inner membranes by a simple and fast domestic method based on the differential flotation of shell fragments and membrane particles in water. However, the development of economically viable and environmentally safe procedure to separate calcium carbonate from membranes at industrial scale remains a challenge (Oliveira et al. 2013).

We hypothesized that smaller particle size would enhance surface area facilitating salt dissolution and improving calcium absorption from eggshell. Indeed, rats receiving the ES L had lower Ca absorption than those receiving ES M and CaCO3. However, animals that received the ES S also had lower Ca absorption than ES M and the CaCO3 groups. Eggshell is a complex tissue composed of multiple layers which differ in the size and form of carbonate crystals (Hincke et al. 2012; Guru & Dash 2014). Eggshell layers also have protein matrix differences in interlacement and in chemical composition and solubility (Guru & Dash 2014). Thus, we suppose that the methodology used to obtain the eggshell fractions in the present study (milling and sieving) could differentially separate the components of eggshell layers generating particles with similar Ca content but different Ca bioavailability.

### Table 4. Weight, mineral content, and biomechanical properties of tibias from rats fed with eggshell fractions containing different particle size as Ca source compared to CaCO3.

| Parameters                        | CaCO3 | ES S | ES M | ES L |
|----------------------------------|-------|------|------|------|
| Tibia dry weight (g)             | 0.215 ± 0.004 | 0.214 ± 0.007 | 0.212 ± 0.002 | 0.203 ± 0.007 |
| Mineral content                  |       |      |      |      |
| Ca (% dry weight)                | 27.25 ± 0.29 | 26.94 ± 0.33 | 27.05 ± 0.29 | 26.65 ± 0.27 |
| P (% dry weight)                 | 11.07 ± 0.14 | 11.05 ± 0.14 | 10.96 ± 0.38 | 10.99 ± 0.42 |
| Mg (% dry weight)                | 0.55 ± 0.01  | 0.56 ± 0.01  | 0.54 ± 0.01  | 0.54 ± 0.01  |
| Biomechanical properties         |       |      |      |      |
| Yield point (N)                  | 33.4 ± 1.9  | 35.0 ± 1.5  | 32.4 ± 1.6  | 32.2 ± 1.6  |
| Ultimate load (N)                | 42.6 ± 1.6  | 42.4 ± 1.3  | 40.2 ± 1.4  | 39.1 ± 1.8  |
| Displacement at ultimate load (mm)| 0.75 ± 0.04 | 0.86 ± 0.05 | 0.75 ± 0.04 | 0.84 ± 0.06 |
| Stiffness (N/mm)                 | 75.7 ± 2.1  | 71.8 ± 4.0  | 74.4 ± 1.6  | 67.9 ± 3.1  |
| Mg (% dry weight)                | 0.55 ± 0.01  | 0.56 ± 0.01  | 0.54 ± 0.01  | 0.54 ± 0.01  |
| P (% dry weight)                 | 11.07 ± 0.14 | 11.05 ± 0.14 | 10.96 ± 0.38 | 10.99 ± 0.42 |
| Ca (% dry weight)                | 27.25 ± 0.29 | 26.94 ± 0.33 | 27.05 ± 0.29 | 26.65 ± 0.27 |

The results were expressed as means ± SEM (n = 8 for all groups, except for eggshell L that had n = 7). No significant effect of diet was observed on the parameters evaluated (ANOVA; p > 0.05). ES S: small-sized particle eggshell fraction; ES M: intermediate-sized particle eggshell fraction; ES L: large-sized particle eggshell fraction.

D-dependent, transcellular movement in the duodenum; and a passive, non-saturatable, paracellular transport through the tight junctions between mucosal cells of distal small intestine (Pu et al. 2016). Since paracellular transport is independent of nutritional and physiological regulation, its mechanism accounts for most calcium absorption when calcium intake is adequate or high (Bronner & Pansu 1999; Pu et al. 2016).

Throughout this study, we observed that the ES M group had similar Ca absorption to the purified CaCO3, although its particle size was 26-fold larger. However, the ES L group, which had particle size 50-fold larger than CaCO3, had lower Ca absorption than that of the purified CaCO3 group. Nevertheless,
the average Ca absorption rate found in the ES L group, 52.4%, is not negligible as it was similar to that found for growing rats feed an AIN diet containing CaCO₃ at adequate Ca levels (Callegaro et al. 2010). These findings are in agreement with previous studies, which suggested that eggshell has good dissolution even at large granulometry, possibly due to its very porous physical structure (Masuda & Hiramatsu 2008).

Furthermore, the amount of Ca ingested is the major determinant of Ca absorption. Although Ca absorption efficiency decreases with increasing Ca load, the total amount absorbed still increases by paracellular route (Weaver & Heaney 2006). In this sense, extending the use of eggshell as Ca source may be a good strategy in order to achieve daily positive Ca balance.

P is an essential bone-forming element that is found in hydroxyapatite and plays an important role in maintaining skeletal mechanical strength (Peacock 2010). Depletion of serum phosphate leads to impaired bone mineralization and compromised osteoblast function (Prentice 2004; Peacock 2010). Mg is also involved in bone health. Bone contains between 50 and 60% of body Mg, which is a surface constituent of hydroxyapatite that regulates crystal growth and stabilization (Prentice 2004; Rude & Gruber 2004).

Rats that received eggshell had higher P and Mg absorption than the CaCO₃ group, which was statistically different only from the ES M and ES L groups. Since mineral absorption was expressed as relative absorption, this effect of eggshell could not be explained by the slightly higher amount of P and Mg found in the eggshell-containing diets (Table 1) or by the higher Mg ingestion that occurred only in the ES M group (p < .05; Table 3). We suggest that this eggshell effect could be related to some eggshell organic constituent that may have facilitated P and Mg absorption. Furthermore, there was a direct relationship between the absorption of these minerals ($R = .75, p < .05$; Figure S1).

It has been demonstrated that soluble eggshell proteins improve Ca transport across Caco-2 cell monolayers by 64% (Daengprok et al. 2003) but their effect on P and Mg absorption has not been evaluated yet. The only study that evaluated Mg absorption from an eggshell-containing diet in piglets did not find differences compared to the absorption from a diet that had CaCO₃ as the Ca source (Schaafsma & Beelen 1999). P and Mg absorption was similar to previous studies with growing rats taking into account the daily animal ingestion (Callegaro et al. 2010; Callegaro et al. 2011). Since one of the primary functions of Ca is acting as a structural component of bone, we have examined Ca bioavailability by measuring the degree of bone mineralization and the mechanical properties of bones. Bone mineralization is at least partially regulated by Ca availability (Garcia-Lopez & Miller 1991; Cashman 2007). Since the percentage of both P and Mg in bone are significantly influenced by the dietary Ca level (Garcia-Lopez & Miller 1991), we investigated whether changes in absorption could lead to changes in bone accumulation of these minerals.

Although the ES L and ES S groups had significantly lower Ca absorption, whereas the ES M and ES L groups had higher P and Mg absorption than the CaCO₃ group, bone mineral content, weight, and mechanical properties were not changed.

Ca deficiency clearly impairs bone mass, morphology and biomechanical properties in growing rats but a previous study has shown that these parameters were stabilized when Ca dietary intake reaches 250 mg/100 g diet and had no further improvement with additional dietary Ca (Hunt et al. 2008). Another study evaluated eggshell as Ca source at 1% in SAMR1 mice, finding similar bone weight, mineral content and mechanical properties compared to CaCO₃ group (Maehira et al. 2009).

Conclusions

This study shows that eggshell Ca is well absorbed by intestine and retained in bones of growing rats. Although Ca absorption was reduced in groups fed large- and small-sized eggshell fractions, whereas P and Mg absorption were increased with the increase in the eggshell particle size, no changes were observed in the bone mineral deposition, weight or mechanical resistance. Our results indicate that eggshell fractions containing intermediate-sized particles (106–212 µm) have Ca absorption similar to that of CaCO₃.

Disclosure statement

The authors report that they have no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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