Clinical and Laboratory Pearl

# Influence of Dietary Calcium Concentration on Body Size and Bone Composition in Rats During Recovery from Malnutrition

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**Key words: calcium, body size, bone composition, protein-energy malnutrition**

**Objective:** The purpose of our study was to assess the influence of different levels of calcium (Ca) in a diet containing 30% protein on the rehabilitated of the body size from protein-energy malnutrition (PEM) and to establish the optimal Ca/protein ratio for attaining a normal body composition.

**Methods:** Weanling female Wistar rats were fed with protein-free diet up to a weight deficit of 20±1%. Then they were arranged in groups (T0) and fed diets with 30% protein and 0.0, 0.2, 0.4, 0.6, 0.9 or 1.2% Ca for 28 days (T28). Food and deionized water were given ad libitum. Body weight and length were recorded every 3 days. At T28, the animals were sacrificed to determine femur composition.

**Results:** At T13, weight-for-age (W/A) was within the normal range for rats consuming ⩾0.6% Ca. At T28 all groups showed adequate W/A. Although length-for-age was adequate during rehabilitated period, rate of weight gain improved when Ca was ⩾0.6%. Femur length did not show significant difference between groups. Total femur Ca content and mg Ca/g of dry-weight tissue increased with increments in dietary Ca concentration and tended to plateau with 0.4% Ca. Ca/P ratio reached the highest value with 0.9% Ca.

**Conclusions:** Our findings indicate that at a dietary protein level of 30% the Ca/protein ratio is a limiting factor in attaining of normal body size; this is achievable when Ca concentration is 1.2% and the Ca/protein ratio is 0.04.

## INTRODUCTION

Protein-energy malnutrition (PEM) in children has been recognized as the most widespread nutritional problem around the world [1]. This disorder produces biochemical alterations leading to poor growth. The growth retardation varies in accordance with the severity and duration of the nutritional deficiency [2]. It may be associated with abnormalities in body size and composition in adulthood, as well as in bone length and skeletal mineral content [3,4,5].

During rehabilitation from infant PEM, weight gain and/or growth occur at an accelerated rate, independent of chronological age, when the dietary macro- and micronutrients allow satisfactory growth [6].

According to our experience, in children and in an experimental rat model, shortening of the recovery period can be obtained by using diets with a higher protein energy concentration (Pr:E) than usually consumed [7]. Our previous studies have also shown that undernourished children fed proprietary milk formulas show greater weight-gain rate (WGR) and higher levels of Ca retention, nitrogen (N) retention than normally growing children [8,9,10].

This study was performed to determine whether different levels of Ca in a diet containing 30% protein influence body size during the recovery of young rats from PEM and to determine the optimal Ca/protein ratio to normalize body composition.

## MATERIALS AND METHODS

### Animals

Forty-six female Wistar rats were supplied by the Central Animal Lab (School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina). Weanling rats were selected for study when they attained a body weight of 34±1 g. The

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animals were housed in individual suspended, galvanized cages, in a temperature-controlled room (21±1°C). A 12-hour light-dark cycle was maintained.

### Diets

The experimental diets contained the following per 100 g: 30 g protein as casein and six different concentrations of Ca (from 0.0 to 1.2 g/100 g) as described in Table 1. Diets were prepared to meet all other required nutrients for adequate growth [11]. The protein source was sodium caseinate (Nestle Argentina S.A.) with 85.07% protein and 0.095% Ca for the diets of 0.0 and 0.2% Ca, and calcium caseinate (Kasdorf S.A.) with 85.00% protein and 0.461% Ca for the other diets. A protein-free diet (PFD), containing 0.6% of Ca, given during the undernutrition period, was prepared by replacing protein with dextrin. Throughout the study, all rats were given food and demineralized water ad libitum. All diets were isocaloric.

### Anthropometry Measurements

For the longitudinal assessment of the growth patterns, the anthropometric data of the animals were processed as Z scores of the weight-for-age (WAZ), length-for-age (LAZ) and weight-for-length (WLZ) ratios. A Z-score expresses the value of a score relative to the mean and the standard deviation of a reference distribution. It has a mean equal to zero and a standard deviation of one. The reference distribution used in this study was the rat population norms for age and sex as previously published [12].

### Experimental Design

Weanling rats were fed the PFD for 7 to 10 days until they reached the anthropometric undernourished category according to WAZ. The mean weight loss from their initial weight was 20±1%. Animals were divided into six groups with a mean WAZ of −3.01±0.49. They were then randomly fed one of six experimental diets (Fig. 1) for 28 days. Body weight and length were recorded three times a week. Growth data were compared as WAZ, LAZ and WLZ over time. After 28 days of refeeding (T28), all animals were sacrificed under ether anesthesia and right femurs removed for chemical analysis.

**Table 1.** Composition of the Experimental Diets (g/100 g)

| Group | PFD | Ca 0.0 | Ca 0.2 | Ca 0.4 | Ca 0.6 | Ca 0.9 | Ca 1.2 |
|---|---|---|---|---|---|---|---|
| Protein | — | | | 30 | | | |
| Corn oil | | | | 4.50 | | | |
| Choline | | | | 0.15 | | | |
| Vitamins[1]: | | | | | | | |
| water soluble | | | | 0.25 | | | |
| fat soluble | | | | 0.50 | | | |
| Mineral mix[1] | | | | | | | |
| (Calcium free) | | | | 5.00 | | | |
| Calcium (Ca $CO_3$) | 0.6 | 0.0 | 0.2 | 0.4 | 0.6 | 0.9 | 1.2 |
| Dextrin up to[2] | | | | 100 | | | |
| Ca/Protein ratio | — | 0.00 | 0.01 | 0.01 | 0.02 | 0.03 | 0.04 |

[1] According to Harper, A.E. [11].

[2] Dextrin British Gum 90010/201/4 (Refinerias de Maiz, S.A.I.C., Argentina).

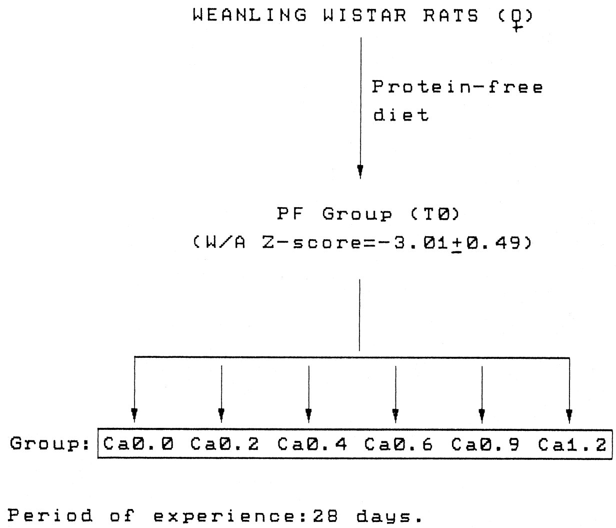


**Fig. 1.** Experimental design.

### Analytical Procedures

**Diets.** Dietary N was determined by Kjeldahl method [13] and protein calculated as N×6.25.

Ca and phosphorus (P) were determined after digestion with $HNO_3$:$HClO_4$ (1:1) mixture. Ca was measured by atomic absorption spectrophotometry (AAS) with $LaCl_3$ (6500 ppm) as an interference suppressor, using a Varian Spectrophotometer model SpectrAA-20, air-acetylene flame, a slit 0.5 nm, at a wavelength 422.7 nm. P was determined by Gomori colorimetric method [14].

**Bone.** The right femur was cleaned of any adhering soft tissue, dried at 100°C for 72 hours, and fat was extracted by immersion for 15 days in chloroform-methanol (3:1) that was removed every 3 days and dried for 48 hours at 100°C. Femur lengths were measured from the proximal junction of the femur neck and the greater trochanter to the midpoint of the condyles with Vernier calipers. The fat-free and dried femurs were weighed and ashed at 700°C until white and crystalline. Ashes were dissolved in HCl and diluted for Ca and P analysis. The amount of Ca was calculated as total content (mg Ca/bone) and as mg Ca/g of dried fat-free tissue. Ca/P ratio was also calculated.

### Statistical Analysis

The results were calculated as the mean±standard deviation or error of the mean. Groups were compared using one-way analysis of variance (ANOVA) [15]. When a statistically significant ratio was encountered, the difference between values was analyzed by the Student-Newman-Keuls multiple comparisons test ($p<0.05$). The INSTAT V2.02 computer software package was used to perform these statistical analyses.

## RESULTS

### Weight-for-Age Z-Score

WAZ scores in the six experimental groups showed a marked deterioration in body weight during the first 13 days of refeeding, remaining between −1.01 and −1.63 below the mean. In contrast, the mean WAZ scores of Ca 0.9% and Ca 1.2% groups were closer to 1 SD below the $50^{th}$ percentile after 28 days. At T28, there were no significant differences between mean WAZ scores of the groups with Ca 0.9% and 1.2%. However, the differences in mean WAZ scores between the two groups 0.9% or 1.2% and the other groups studied were significant at T28 ($p<0.01$) (Fig. 2A and 2B).

### Length-for-Age Z-Score

Length-for-age showed that stunting was not significant in this experiment. The linear growth was similar in the six groups of animals. Throughout the study, LAZ scores of the six groups remained close to the mean. There were no significant differences between groups at any time (Fig. 2C and 2D).

### Weight-for-Length Z-Score

The weight-for-length (W/L) deficit of the rats persisted until about T13. Thereafter the body W/L deficit decreased until it was normalized for groups Ca 0.0%, Ca 0.2% and Ca 0.4%. In contrast, the groups Ca 0.6%, Ca 0.9% and Ca 1.2% exhibited higher W/L ratios throughout the study, reaching the anthropometric category of lean and adequate which demonstrated the dinamic process of growing.

WLZ score was significantly different at all times (from T4 to T28) for groups Ca 0.6%, Ca 0.9% and Ca 1.2% ($p<0.01$). The differences in WLZ scores between the six groups of rats studied were not significant at T28 (Fig. 2E and 2F).

### Bone Composition

There were no statistically significant differences among groups for bone length because the short depletion phase did not affect either body length or bone length (Fig. 3A).

Femur Ca content (mg/bone) showed a significant increase from Ca 0.0% to Ca 0.9% groups and no further significant differences between Ca 0.9% and Ca 1.2% (Fig. 3B). When Ca was expressed per g of tissue, no significant differences between groups Ca 0.4% and higher were detected (Fig. 3C). Ca/P ratio was the lowest in Ca 0.0% and Ca 0.2%, relative to the other groups, but Ca 0.9% showed the highest value of Ca/P ratio (Fig. 3D).

## DISCUSSION

PEM in children, recognized as the most widespread nutritional problem around the world [1], is characterized by stunted growth (low height-for-age) and progressive wasting (low weight-for-age and/or low weight-for-height) [2].

In our experience, infants under 2 years of age, recovering from PEM with approximately 30% body weight deficit, had higher protein-energy requirements than normal infants of similar chronological age, sex and physical activity. In fact the Pr:E ratio should be higher for PEM infants than that of in human milk (Pr:E ratio of 8%) to achieve an adequate weight-for-age. Moreover, infants recovering from PEM were much more efficient in utilizing energy and protein than were clinically well-nourished infants [16], and they could achieve their protein-calorie needs when receiving ad libitum therapeutic diets with a Pr:E ratio between 11 and 17% [6,7,12,16,17].

Acutely undernourished infants, during the nutritional supportive therapy period, regulated energy intake according to the demands of the actual weight-gain velocity, and they reached 51.33 g/kg/day for a protein intake equal to 6.2 g/kg/day [6].

We have proposed that undernourished infants may reach a growth rate higher than the normal for age and animal species [6] if the dietary Pr:E ratio allows such increment. Under this transient situation, individuals have behaviors and requirements similar to other animal species with high growth rate velocity [18].

Moreover, there is an interdependence of dietary needs of protein and Ca for bone matrix and mineralization [19,20]. Protein is necessary for allowing a bone mineral accretion adequate for skeletal growth and maturation. Therefore, undernourished infants have not only reduced weight-gain velocity and poor growth but decreased bone mineralization [3,5], since protein deficiency affects both body mass and bone organic matrix synthesis and mineralization. Therefore, in order to reach a normal body composition and bone size during PEM recovery, Ca and P requirements should increase simultaneously with Pr:E for proper bone mineralization [20,21].

In fact, we have found that our undernourished infants fed ad libitum with proprietary cow's milk formulas containing a Pr:E ratio of 17% and a Ca/protein ratio in the range 0.036 to 0.050 had increased Ca absorption ($72\pm12.9\%$) and four times higher Ca retention than normal infants of their age. Although urinary Ca excretion may be enhanced by increasing protein intake, Ca urinary excretion by PEM infants in our study was only 1 to 3% of the Ca intake. In our observation, Ca retention was linearly dependent on Ca absorption and strongly related to N Balance and the dietary Ca/protein ratio [8,9]. These findings indicate that the great nutritional demands of undernourished infants during the catch-up growth of recovery are associated with a degree of skeletal mineralization deficit. Thus, the Ca/protein ratio requirement would be higher than the value of human milk (0.03). This observation was also reported by Torun et al [22].

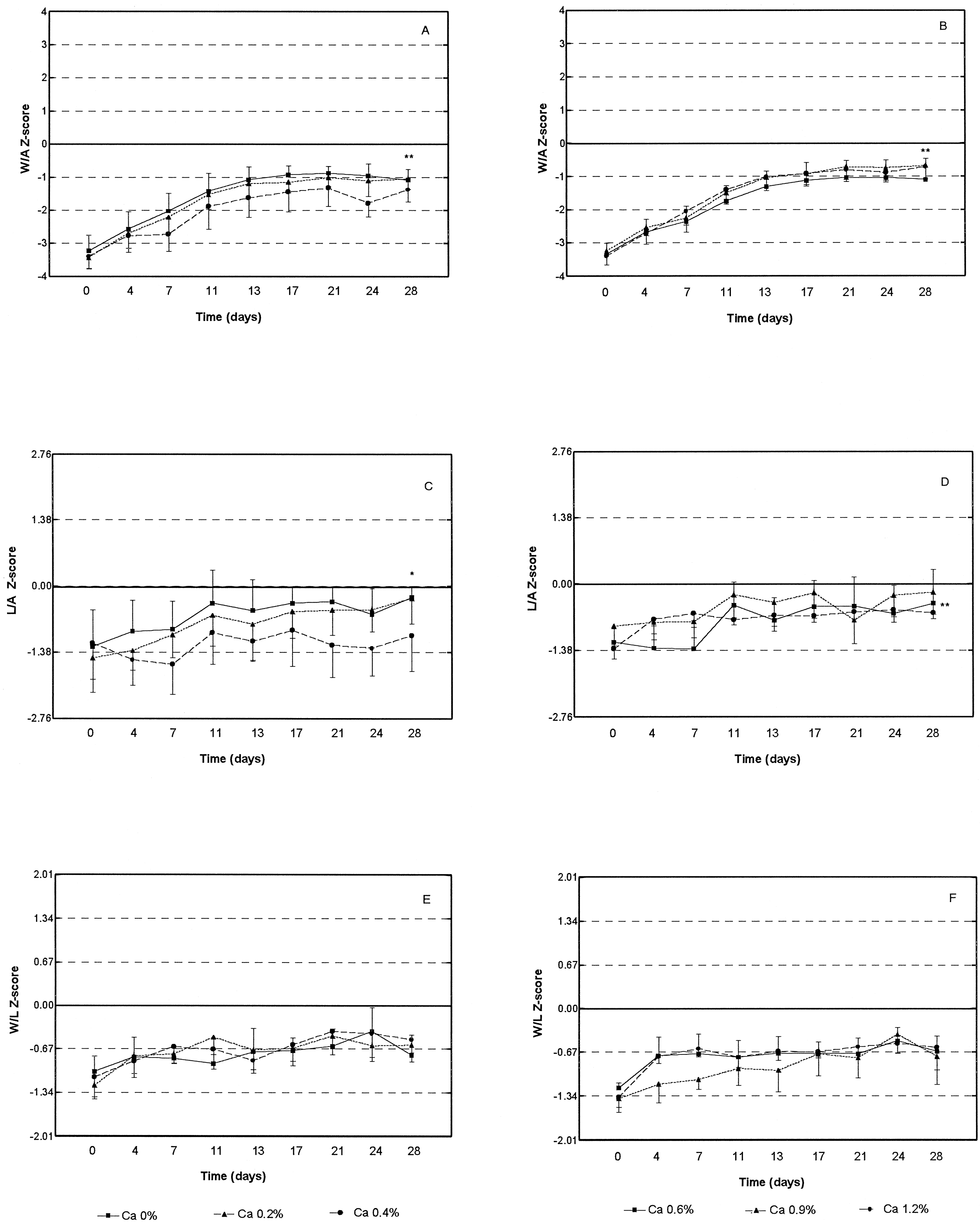


**Fig. 2.** Changes of the W/A Z-score (A,B), of the L/A Z-score (C,D) and of the W/L Z-score (E,F) during recovery from malnutrition.

The complexity of living organisms requires the use of simplified models which represent various aspects of the organism under study [23]. In the past, the lack of a suitable animal model for studying the consequences of PEM and the optimum composition of the diet for recovery has made this task very difficult. However, it has been demonstrated in this

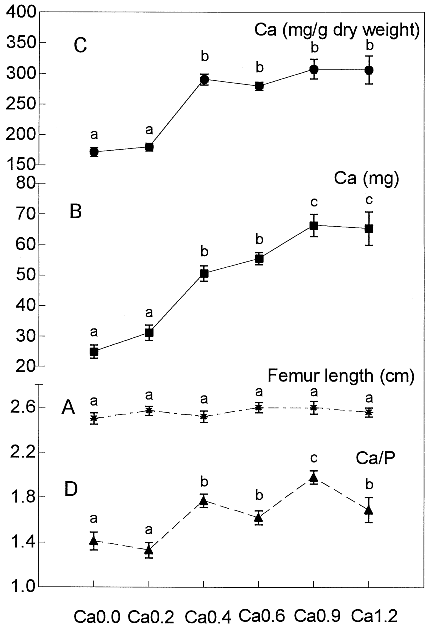


**Fig. 3.** A) Femur length (cm), B) Total femur calcium content (mg), C) Calcium (mg/g dry weight), D) Bone Ca/P ratio, attained at the end of the experimental period. Each point represents the mean±SD. Equal letters indicate no statistically significant difference between them.

laboratory that the growing rat can be an adequate experimental model for study of several nutritional aspects related to PEM recovery [7].

Weanling rats, growing at a normal rate for their age and sex, consume rat milk that contains 0.27% of Ca and 8.8% of protein (Pr:E 23% and a Ca/protein ratio of 0.03). Interestingly, weanling rats fed with protein-free diets until a loss of 20±1% of their initial body weight respond to a therapeutic diet similar to infants recovering from PEM in that their growth rates improved by increasing the protein content of their diets to a Pr:E of 30% [24]. However, in our study, following up the recovery phase by standard scores of W/A and W/L showed that the rats fed with 30% Pr:E remained underweight for their chronological age and lean for their actual length, during the first stage (13 days). Stunting was not evident, and length growth, expressed as standard deviation for norms in age and sex, remained close to the 50th percentile throughout the depletion-repletion phases.

This experimental rat model reproduces acute undernutrition in a short time. In addition, stunting, evaluated as length-for-age, is not expected to differ significantly. Moreover, during the second stage of recovery (13 to 28 days) the rats fed diets containing 0.9% and 1.2% of Ca (Ca/protein ratio 0.03 and 0.04, respectively), could reverse the growth delay (W/A), but those fed diets containing Ca below 0.9% (Ca/protein ratio below 0.03), decreased their rate of body weight gain and showed inadequate W/A. Therefore, an appropriate evaluation of nutritional status should consider the three anthropometric parameters simultaneously, since one parameter may fail to distinguish a normal growth from decreased length and weight progression. Using these multiple criteria, we can see that only animals with 1.2% of Ca (Ca/protein ratio of 0.04) reached an adequate anthropometric category at T17.

These results show that, in the undernourished rat, a normal body size is not achievable with a recovery diet containing less than 0.04 of Ca/protein ratio, in spite of a Pr:E ratio of 30%. A normal body size is achievable at T17 when Ca concentration is 1.2% and Ca/protein ratio is 0.04 (Fig. 2A and 2B); however, if Ca concentration is 0.9% and Ca/protein is 0.03, normal body size is achievable at T21, but the rat remains lean according to W/L (Fig. 2E and 2F).

Furthermore, study of bone composition at the end of the recovery period indicated that mineral content was compromised to a great extent by low Ca levels. Fig. 3B and 3C showed a decreased mineral content in femur with diets containing Ca 0.0 and Ca 0.2. The mean bone Ca value increased significantly from Ca 0.4 to Ca 1.2. However the Ca/P ratio of the Ca 0.9 group reached the maximum value ($p<0.05$) (Fig. 3D). The bone Ca/P ratio in Ca 1.2 showed a marked decrease compared to the Ca 0.9 group, probably because of an inadequate dietary Ca/P ratio.

During the period of rapid growth, our experimental rats and the infants previously studied had a low urinary excretion of Ca, demonstrating that, during rapid growth, Ca excretion is unrelated to Ca intake because rapidly growing individuals retain most absorbed Ca in the skeleton rather than excreting it in the urine [8,9,22,25].

Additionally, these results show that the proposed rat model is reliable for explaining the previous findings in infants, confirming the importance of an adequate dietary Ca concentration during nutritional recovery for attaining normal bone composition and size.

These findings indicate that young rats, recovering from acute PEM, as in the case of PEM infants, were able to recover fully normal body size and composition only if the increase in dietary Pr:E ratio and Ca/protein were adequate to meet the needs imposed by the accelerated catch-up growth and by the mineral-deficient bone.

Furthermore, although the therapeutic diet for PEM provides an adequate Pr:E ratio above the requirements of normally growing individuals, the dietary Ca/protein ratio is the limiting factor for attaining a normal body size [10], and this is achievable with a Ca concentration of 1.2% and a Ca/protein ratio of 0.04.

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