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Estimation of dietary calcium utilization in rats using a biomechanical functional test

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Three-point bending of femora was evaluated as a biomechanical functional test of calcium utilization in male Wistar rats fed 20% casein and soy protein diets containing different levels of dietary calcium (2·0, 0·5 and 0·05% w/w), respectively. Rats were meal-fed for 10 weeks after which animals were sacrificed and both femora removed. Femur mineral composition and biomechanical parameters: bioyield, peak force, bending failure energy and a normalized force parameter, maximum bending stress were determined. Femur calcium content and ash weight were significantly ($p < 0·05$) decreased in animals fed low calcium diets compared to those fed medium and high calcium diets. Femur biomechanics were not related to animal growth parameters. Femur biomechanical parameters were correlated with dietary calcium intake and femur composition in both casein- and soy-fed animals. The bioyield and peak force parameters identified were significantly ($p < 0·001$) correlated with femur calcium content in casein- and soy-fed rats. Calcium utilization was indicated by regression equations relating bone calcium content and ash weight to bioyield and peak force, respectively, for casein- and soy-fed animals. Bending failure energy could not be reliably modelled using these same variables, suggesting that it is influenced by not only bone mineralization but possibly also the collagen matrix component. These results indicate that three-point bending can be useful in assessing calcium utilization from the diet. Bone biomechanics were not influenced by casein and soy dietary protein sources.

INTRODUCTION

Nutrient bioavailability has been determined in the past by a variety of techniques including in-vitro solubility assays, balance studies, nutrition evaluations, model systems and lastly, functional tests (Allen, 1982; Greger, 1988). These tests vary in their value as predictive indicators of calcium absorption and utilization. In-vitro studies, simulating the conditions of the gastrointestinal tract have been used to investigate mineral availability in various foods (Miller *et al.*, 1981; Platt *et al.*, 1987; Walsh *et al.*, 1989). It has recently been debated, however, that in-vitro determinants of calcium solubility may not accurately reflect true calcium absorbability in vivo (Heaney *et al.*, 1989, 1990). Balance studies, widely used in the study of nutrient bioavailability, are subject to collection and adaptation errors (Allen, 1982; Pak & Avioli, 1988) but, nevertheless, are valuable in assessing calcium absorption from a test diet. Monitoring nutritional status as determined by plasma calcium profiles is of limited value due to the strong hormonal regulation of circulating levels of this nutrient (Aurbach, 1988). Bone calcium content is a useful measurement of calcium utilization, but requires the necessary adaptation of animals to diets (Greger, 1988). Isotopic (stable and radioactive) tracer techniques are widely used (Weaver, 1990), but require compartmental modelling for quantitative analysis. Functional tests have also been designed to measure in vivo endpoint determinants of calcium utilization (Currey, 1969; Yuan *et al.*, 1991*a*). These tests are fundamentally perfect in that they reflect many physiological host factors, in addition to nutrient status and absorbability of a food, in a final evaluation of nutrient utilization. They can be difficult to standardize, however, because a system of interest is seldom isolated, but rather is confounded by associated dietary or physiological factors such as dietary protein or lactose intolerance (Yuan *et al.*, 1991*b*).

Previous investigators have studied the compressive

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or bending strength of vertebral and long bones, respectively, in relation to bone mass (Mosekilde *et al.*, 1987; Ortoff & Oxlund, 1988) and the development of osteoporosis (Oyster & Smith, 1988). However, there are few studies that have attempted to evaluate bone biomechanical parameters as an index of utilization of absorbed calcium from foods. Previous studies from the authors' laboratory, using bone strength parameters as an index of calcium utilization from diets differing in protein sources and calcium bioavailability, reported a significant correlation between femur calcium content and bone strength in normal animals (Yuan *et al.*, 1991*a*). The objectives of the present study were, first, to evaluate additional specific biomechanical test parameters for assessing dietary calcium utilization and, secondly, to determine their usefulness in studies with animals fed diets potentially differing in calcium bioavailability.

MATERIALS AND METHODS

Animals and diets

Four-week-old male Wistar rats (Charles River, Montreal, PQ) were divided into six experimental groups (six animals per group). Dietary groups included 20% casein or soy protein isolate (ICN Biochemicals, Cleveland, OH) containing a high level of dietary calcium (2·0% w/w), a medium level of calcium (0·5% w/w), and a low level of calcium (0·05% w/w), respectively. Diets contained (in g/100 g): casein or soy protein isolate, 20·0; DL-methionine, 0·3; cornstarch, 15·0; fibre, 5·0; vegetable oil, 5·0; Ca-free mineral mix, 3·5; vitamin mixture, 1·0; choline bitartrate, 0·2; calcium carbonate, 4·98, 1·23 or 0·11 for the 2·0%, 0·5% and 0·05% Ca diets, respectively; and sucrose to 100 g.

Animals were fed *ad libitum* until they reached 100 g body weight, after which meal-feeding was initiated. Over a two-week period, animals were trained to consume the diets within a six-hour period daily (9 a.m. to 3 p.m.). Deionized water was made available to animals *ad libitum*. Daily feed intakes and weekly body weight gains were recorded throughout the experiment.

Animals (14 weeks of age) were sacrificed by exsanguination and both femora removed. Following dissection, adhering soft tissue and epiphyses were removed from the femora.

Analyses

Femur calcium and phosphorus contents were determined using the right femur. The femora were dried at 100°C for three days, then ashed at 550°C for 24 h. Bone ash was solubilized in 3 ml 4 N HCl for bone mineral content analysis. An aliquot was taken and diluted with 0·5% $LaCl_3$ for calcium analysis by atomic absorption spectrophotometry (Perkin Elmer-306, Perkin Elmer, Norwalk, CT). A further aliquot was diluted with deionized water and the phosphorus content determined by the colorimetric method of Chen and coworkers (1956).

Bone biomechanics

Bone biomechanical parameters were measured on the left femora using an Instron Universal Testing Machine (Model 1122, Instron Corp., Canton, MA) in three-point bending. The femur was placed unsupported on supporting points (1 mm width), but resting on the greater trochanter, to prevent the specimen from revolving during the test. Femora were bent until failure occurred by lowering a centrally placed point (1 mm width) at a constant speed (1·0 mm/min).

The time–force deformation data were monitored using the JCL 6000 Chromatography Data System (Jones Chromatography Ltd, Littleton, CO) which was interfaced with the Instron, through an IBM AT compatible personal computer. Sample run time was 3 min, at a sampling rate of five signals per second. The Instron signal was calibrated using 1·0 and 2·0 kg known weights. Data were analyzed by transforming the millivolt signal output into kg force.

This test allows a number of whole bone properties to be determined from the time–force deformation curve (Fig. 1). Bone biomechanical parameters identified were:

(1) *Bioyield (N)*: the force at which there occurs the first damage to the bone tissue, indicated by a break in the initial slope;
(2) *Peak force (N)*: the maximum force obtained during the bending procedure resulting in the initiation of bone failure which is propagated to the point where the bone breaks and the force reading drops to zero;
(3) *Bending failure energy (J)*: the work energy (area under the time–force deformation curve) required to achieve failure of the bone in bending;
(4) *Maximum bending stress (σ, N/mm²)*: a normalized, calculated force value that takes into consideration bone size.

This parameter was calculated as follows:

$$\sigma = \frac{8 \times \text{maximum bending load}(L - 1)D}{\pi(D^4 - d^4)}$$

where L is the distance between the supporting points (13 mm); D and d are the outer and inner diameters of the bone (mm) (Ortoff & Oxlund, 1988). The three-point bending test of femora used in this study is based on a well-defined engineering test involving the application of a pure model of deformation to measure the bending strength of tubes with a circular cross-section and uniform wall thickness (Ortoff & Oxlund, 1988). Testing

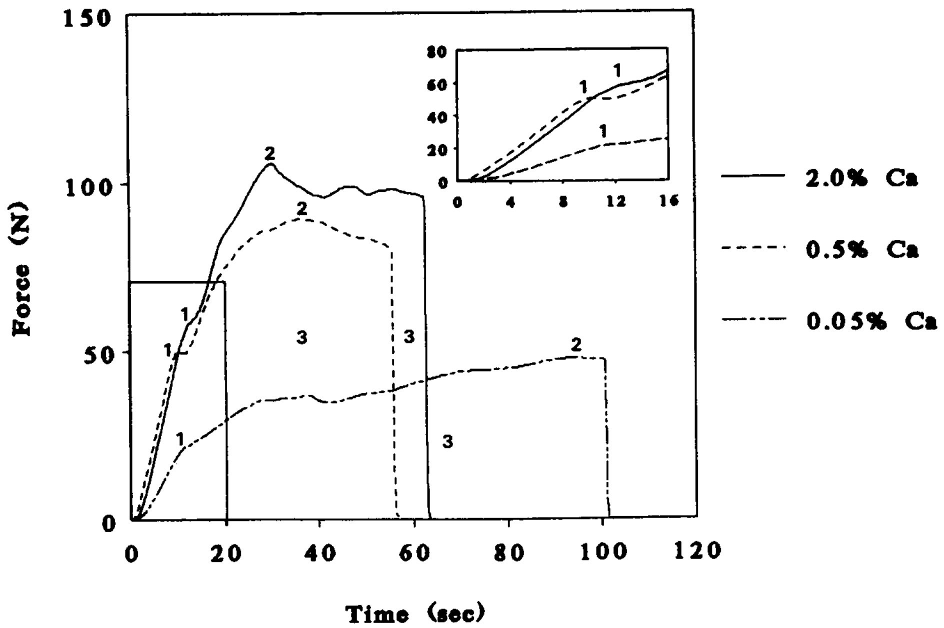

Fig. 1. Typical time–force deformation curves of three-point bending of femora from rats fed high, medium and low calcium diets. 1, Bioyield (N); 2, peak force (N); 3, bending failure energy (area under the curve) (J). Insert shows enlargement for increased detail of bioyield inflection point in the initial slope.

of complex biological materials, such as femora, can be performed assuming that the mid-diaphyseal bone configuration is constant (Kusy *et al.*, 1987; Segars & Kapsalis, 1987; Ortoff & Oxlund, 1988).

Statistical analyses

All data are expressed as mean ± SEM. Differences between treatments were tested for by one-way analysis of variance (ANOVA; SPSS Inc., Chicago, IL). Where differences did exist, the Student–Newman–Keuls multiple range test (SPSS) was used to identify the source of the differences at a $p < 0.05$ level of significance. Linear and multiple regression analyses (SPSS) were also performed on bone biomechanical and experimental parameters.

RESULTS

Animals fed the low Ca casein diet exhibited a lower final body weight ($p < 0.05$) than counterparts fed the medium and high Ca casein diets (Table 1). Dietary protein source influenced animal growth characteristics at the 2·0% dietary Ca level only; soy-fed animals consumed a significantly ($p < 0.05$) lower amount of diet which corresponded to a lower Ca intake and final body weight than observed in casein-fed counterparts.

Femur calcium content was significantly ($p < 0.05$) decreased in animals fed the low calcium diets in comparison to animals fed the medium and high calcium diets (Table 1). Soy-fed animals exhibited a significantly ($p < 0.05$) lower femur Ca content than those fed casein at the 2·0% Ca level, which was in agreement with the Ca intake data. Conversely, at the 0·05% dietary Ca level, soy-fed animals had a greater ($p < 0.05$) femur Ca content than counterparts fed casein.

A representative time–force deformation curve of three-point bending of femora from animals fed 2·0, 0·5 and 0·05% Ca diets, respectively, is presented in Fig. 1. The individual bioyield and peak force values are indicated on these sample curves. The femora of animals fed the 0·05% Ca diets were observed to bend markedly before breaking, resulting in the greater time course for testing these bones.

Table 1. Calcium intake and bone calcium of experimental animals[a]

Diet	Final body weight[b] (g)	Ca intake[c] (g)	Femur Ca (mg/bone)
2·0% Ca			
Casein	309 ± 11[a]	6·25 ± 0·34[a]	86·25 ± 4·02[a]
Soy	257 ± 8[ab]	5·58 ± 0·21[b]	71·55 ± 5·01[b]
0·5% Ca			
Casein	259 ± 8[ab]	1·48 ± 0·03[c]	64·50 ± 2·71[b]
Soy	252 ± 14[b]	1·42 ± 0·06[c]	68·62 ± 2·36[b]
0·05% Ca			
Casein	249 ± 9[b]	0·13 ± 0·01[d]	27·98 ± 2·31[d]
Soy	253 ± 9[b]	0·14 ± 0·01[d]	43·11 ± 3·01[c]

[a] Data are expressed as mean ± SEM; $n = 36$.
[b] Fourteen weeks of age.
[c] Cumulative intake over experimental period.
The superscrips [a–d] are significantly ($p < 0.05$) different.

Table 2. Femur biomechanics correlations for casein-fed animals

		Animal growth[a]				Femur composition			
		FBwt (g)	FI (g)	FER	Ca_i (g)	Dry wt (g)	Ash wt (g)	Ca (mg/bone)	Ca/P
Biomechanical parameter:									
Bioyield (N)	*r*	0·579	0·551	0·553	0·793	0·962	0·974	0·957	0·639
	p	0·038	NS	NS	0·001	0·001	0·001	0·001	0·019
Peak force (N)	*r*	0·534	0·521	0·536	0·761	0·959	0·979	0·949	0·635
	p	NS	NS	NS	0·002	0·001	0·001	0·001	0·020
Bending failure energy (J)	*r*	0·745	0·514	0·478	0·594	0·632	0·590	0·618	0·524
	p	0·004	NS	NS	0·032	0·028	0·044	0·024	NS
Normalized biomechanical parameter:									
Maximum bending stress (N/mm²)	*r*	0·396	0·385	0·524	0·640	0·865	0·911	0·858	0·516
	p	NS	NS	NS	0·018	0·001	0·001	0·001	NS

[a] FBwt, Final body weight; FI, feed intake; FER, feed efficiency ratio; Ca_i, Ca intake.
NS, Not significant.

The results of correlations between femur biomechanical parameters with animal growth and femur composition parameters for casein and soy fed animals are presented in Tables 2 and 3, respectively. Femur biomechanical parameters, bioyield, peak force, bending failure energy and maximum bending stress, were not related to animal growth parameters such as final body weight (FBwt), feed intake (FI) or feed efficiency ratio (FER) in both casein- and soy-fed groups of animals. Femur bending failure energy was significantly correlated with animal final body weight in the casein (r = 0·745, p = 0·004), but not the soy-fed animals (Tables 2 and 3). This result reflects the lack of significant treatment differences in the final body weights of animals fed the soy diets with varying calcium levels observed above. However, femur biomechanical parameters were correlated with dietary calcium intake (Ca_i) for both casein- and soy-fed animals. The highest correlation was between peak force and Ca_i for the soy-fed group (r = 0·805, p = 0·001).

Strong correlations were found to exist between femur biomechanical parameters and femur composition parameters such as bone dry weight, ash weight, calcium content and calcium : phosphorus ratio in both protein groups (Tables 2 and 3). The strongest correlations were found between biomechanical force measurements (bioyield and peak force) and dry weight (range r = 0·816 to r = 0·962) and ash weight (range r = 0·963 to r = 0·979). Similarly, strong correlations existed between biomechanical force measurements and bone calcium content in both casein- and soy-fed groups. Femur bending failure energy was significantly correlated with calcium content for casein-fed animals only, similar to the result with final body weight above. The absence of

Table 3. Femur biomechanics correlations for soy-fed animals

		Animal growth[a]				Femur composition			
		FBwt (g)	FI (g)	FER	Ca_i (g)	Dry wt (g)	Ash wt (g)	Ca (mg/bone)	Ca/P
Biomechanical parameter:									
Bioyield (N)	*r*	0·245	0·161	0·013	0·773	0·816	0·963	0·822	0·781
	p	NS	NS	NS	0·001	0·001	0·001	0·001	0·001
Peak force (N)	*r*	0·174	0·254	0·058	0·805	0·841	0·968	0·825	0·744
	p	NS	NS	NS	0·001	0·001	0·001	0·001	0·002
Bending failure energy (J)	*r*	0·009	0·094	0·240	0·572	0·620	0·560	0·197	0·345
	p	NS	NS	NS	0·026	0·014	0·030	NS	NS
Normalized biomechanical parameter:									
Maximum bending stress (N/mm²)	*r*	0·210	0·312	0·057	0·626	0·671	0·889	0·817	0·767
	p	NS	NS	NS	0·013	0·006	0·001	0·001	0·001

[a] FBwt, Final body weight; FI, feed intake; FER, feed efficiency ratio; Ca_i, Ca intake.

Table 4. Use of composition parameters as factors in bone biomechanics

Bioyield (N)	
Casein:	Bioyield = −2·07 + 0·16 Ca + 237·47 ash wt (r = 0·976; SE = 4·61; $p < 0{\cdot}001$)
Soy:	Bioyield = −16·95 + 0·12 Ca + 334·30 ash wt (r = 0·965; SE = 4·58; $p < 0{\cdot}001$)
Peak force (N)	
Casein:	Peak force = −8·90 − 0·09 Ca + 602·26 ash wt (r = 0·979; SE = 8·17; $p < 0{\cdot}001$)
Soy:	Peak force = −14·60 + 0·18 Ca + 535·38 ash wt (r = 0·970; SE = 6·77; $p < 0{\cdot}001$)

a correlation in the soy-fed animals is likely a result of the significantly ($p < 0{\cdot}05$) decreased femur calcium content of 2·0% Ca fed animals in comparison to counterparts fed casein with 2·0% dietary Ca. Femur biomechanical parameters were moderately correlated with bone Ca/P ratios (range $r = 0{\cdot}345$ to $r = 0{\cdot}781$).

Multiple regression analysis was used to determine if predictive equations could be generated relating bone composition to biomechanical parameters. Femur calcium content and ash weight were selected as the independent variables and biomechanical parameters, bioyield, peak force and bending failure energy as the dependent variables for the casein and soy groups, respectively. Predictive equations could be generated to model femur bioyield and peak force using bone calcium content and ash weight for both casein- and soy-fed animals (Table 4). However, femur bending failure energy could not be reliably modelled using these bone composition parameters (range $r = 0{\cdot}590$ to $r = 0{\cdot}715$).

DISCUSSION

Previous investigators have used a number of different bone types in biomechanical testing, including vertebrae, metatarsal and metacarpal bones, as well as long bones (femur and tibia). Vertebrae, metatarsals and metacarpals contain mainly spongy trabecular bone (reflecting their function of skeletal support), which is best suited to compression testing. Long bones, on the other hand, are comprised of compact cortical bone tissue, especially in the diaphyseal region, in order to withstand bending, compression and torsional forces in movement. This characteristic lends itself to biomechanical testing in a bending model.

Femur biomechanical force and animal growth parameters measured in this study, were not related in either casein- or soy-fed animals. Femur bending failure energy, however, could be correlated to animal final body weight, but this observation occurred only in casein-fed animals. This result may be explained by the reduced feed intake and, therefore, lower body weight gain by high calcium, soy-fed animals. Dietary calcium intake was strongly correlated with all femur biomechanical parameters for both casein- and soy-fed animals. Thus, dietary calcium level is of significance to bone strength, since animals fed the low calcium diets exhibited significantly lower bioyield and peak force values. Other studies, with pigs fed diets containing two levels of calcium, have reported that animals fed a higher calcium diet had stronger bones (Crenshaw *et al.*, 1981). Moreover, our results from studying growing animals agree with, and extend those of epidemiological studies which indicate that bone health and density is a function of adequate calcium intake based on past calcium and milk consumption (Odland *et al.*, 1972; Sandler *et al.*, 1985). In the present study, highly significant correlations were observed between femur biomechanical force and composition parameters. Femur bending failure energy, on the other hand, was less strongly correlated with bone composition parameters. Bone calcium content was correlated with bending failure energy in casein-fed animals, but not soy-fed counterparts. The relationship between bone calcium and bending failure energy was weakened, due to the decreased feed and calcium intake of high calcium, soy-fed animals, relative to those fed casein. Previous reports have also indicated that bone mineral content and ash weight may be related to bone strength (Crenshaw *et al.*, 1981; Crenshaw, 1986), but the effects of specific dietary constituents other than calcium have not been examined. Other workers have reported similar correlations of bone strength and toughness, equivalent herein to peak force and bending failure energy, with bone ash weight (Kusy *et al.*, 1987). In addition to these measurements, the bioyield parameter, identified in our study, was also shown to be highly correlated with bone composition, the strongest correlation being with ash weight for casein- ($r = 0{\cdot}963$) and soy-fed ($r = 0{\cdot}974$) animals. Furthermore, our results corroborate studies with human bone which reported that bone mineral content, normalized for bone width, was significantly correlated with the force value at the break in the initial slope of the time–force deformation curve, or bioyield as defined herein (Oyster & Oxlund, 1988).

Multiple regression analyses were successful in generating highly significant predictive equations to model femur biomechanical force parameters, such as bioyield and peak force, with bone calcium content and ash weight as independent variables in both casein- and soy-fed animals. However, bending failure energy could not be reliably modelled using bone calcium content and ash weight as the independent variables. These results may reflect the dual nature of bone, namely the inorganic mineral component as well as the organic collagen matrix. The mineral composition enables bone to have hardness and rigidity, whereas the organic composition confers toughness and elasticity to the bone tissue (Gray, 1974). A possible reduction in the organic component in bone of soy-fed animals is suggested by the noted reduction in protein digestibility and nitrogen absorption from the small intestine in soy-fed animals, compared to counterparts fed animal protein sources (Sandström *et al.*, 1986). Our results, however, do not indicate a difference in bone biomechanical parameters between soy- and casein-fed animals and support previous findings from this laboratory that no differences in calcium utilization occur between normal animals fed dietary proteins differing in digestibility (Yuan *et al.*, 1991*a*). Rather, the mineral component of bone, as influenced by dietary calcium content, appeared to be the sole factor in this study to affect femur biomechanical strength. For example, femora from animals fed both casein and soy low-calcium diets, exhibited considerable flexibility in bending prior to breaking. The net result was that while less force was needed to break the bone, energy (area under the time–force deformation curve) was not similarly affected, due to the longer period of time required to break the bone. This observation suggests that bending failure energy does not accurately reflect calcium utilization as indicated by the fact that demineralized bone is highly flexible (Gray, 1974).

CONCLUSION

In summary, biomechanical three-point bending of bone, as applied in this study, has been shown to be a useful technique to assess calcium utilization from the diet. Of the test parameters identified in this study, the biomechanical force values, bioyield and peak force, were highly responsive to dietary calcium level and intake. Utilization of dietary calcium was indicated by the predictive equations generated by multiple regression analysis relating bone calcium and ash weight to bioyield and peak force. The bending failure energy parameter could be observed to be influenced not only by bone mineralization but possibly also by the organic matrix component. Thus, this method provides a relatively effective way of assessing calcium utilization from dietary sources. The method represents the net response to factors influencing calcium bioavailability prior to absorption, as well as the homeostatic mechanisms of the body that control calcium metabolism and deposition following absorption.

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