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^3H -tetracycline as a proxy for ^{41}Ca for measuring dietary perturbations of bone resorption

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Abstract

Our group is interested in evaluating early effects of dietary interventions on bone loss. Postmenopausal women lose bone following reduction in estrogen which leads to increased risk of fracture. Traditional means of monitoring bone loss and effectiveness of treatments include changes in bone density, which takes 6 months to years to observe effects, and changes in biochemical markers of bone turnover, which are highly variable and lack specificity. Prelabeling bone with ^{41}Ca and measuring urinary ^{41}Ca excretion with accelerator mass spectrometry provides a sensitive, specific, and rapid approach to evaluating effectiveness of treatment. To better understand ^{41}Ca technology as a tool for measuring effective treatments on reducing bone resorption, we perturbed bone resorption by manipulating dietary calcium in rats. We used ^3H -tetracycline (^3H -TC) as a proxy for ^{41}Ca and found that a single dose is feasible to study bone resorption. Suppression of bone resorption, as measured by urinary ^3H -TC, by dietary calcium was observed in rats stabilized after ovariectomy, but not in recently ovariectomized rats.

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1. Introduction

Osteoporosis, a disease that is characterized by low bone mass, warrants attention. In 2002, the estimated national direct expenditure for osteoporotic hip fractures was \$18 billion. It is projected that the number of people with osteoporosis will increase from 10,100,000 in 2002 to 12,000,000 and 13,900,000 in 2010 and 2020, respectively [1]. Consequences of osteoporosis are potentially fatal as an average of 24% of hip fracture in patients aged 50 and over usually die within a year following fracture [1]. Management of osteoporosis is crucial because there are treatments, but no cure, for the disease.

There are various ways to manage osteoporosis, including medications such as bisphosphonates and hormone therapy, as well as dietary means such as calcium and vitamin D supplementation. There is a large body of evidence to indicate that calcium supplementation has beneficial effects on bone during the entire life span. During childhood and adolescence, calcium increases bone acquisition that may ultimately protect against low bone mass and fracture later in life. It has been calculated from a metabolic balance study that an increase of calcium intake from an average of 920 mg/d to 1300 mg/d during adolescence would increase net calcium retention by 112 mg/d [2]. This translates to an additional 4% of skeletal mass accrual over 1 year if the increase in dietary calcium were maintained. During adulthood, dietary calcium supplementation reduces fractures and slows down age-related bone loss,

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possibly by replacing the daily excretory and cutaneous losses that would otherwise have drained skeletal calcium. The importance of adequate dietary calcium during adulthood has also been shown through an increase in bone mineral density when hormone therapy was administered in the presence of sufficient calcium [3].

The mechanism by which calcium exerts a positive effect on bone has been examined by kinetic studies. In a randomized, cross over, study of calcium kinetics in adolescent girls, the increased retention seen on a high calcium diet (47.5 ± 3.9 mmol/day) versus a low calcium diet (21.5 ± 2.0 mmol/day) was due to an increase in absorbed calcium and suppression of bone resorption without affecting bone formation [4]. This is in contrast to another kinetics study in normal adults that assessed the response in calcium metabolism to dietary calcium perturbation [5]. In this study, a high dietary calcium intake did not result in an increase in absorbed calcium for all the subjects. However, when absorbed calcium increased in some subjects, bone resorption was decreased, while renal and gastrointestinal calcium clearance were increased. A low calcium intake (0.2 g/day) resulted in a reduction of absorbed calcium, an increase in bone resorption rate, and a decrease in renal and gastrointestinal rate constants, possibly suggesting that an increase in parathyroid hormone (PTH) is the common mediating factor since PTH exerts its action on the kidney, intestine, and bone.

There is emerging evidence that calcium functions as an indirect regulator of remodeling of the skeleton [6]. During bone remodeling, the bone mineral that is being released after bone is resorbed is either recycled or used to counterbalance excretory losses. When dietary calcium intake is low for a prolonged period of time, bone remodeling continues to be high, resulting in an increase in bone fragility. Therefore, high dietary calcium reduces bone remodeling, which results in an immediate reduction in fracture risk before any changes in bone mass or bone balance are detected [6].

Calcium status is a crucial factor in determining bone health and fracture risk. Calcium status is difficult to assess because serum calcium levels are tightly controlled. Bone mineral content (BMC) measured by dual X-ray absorptiometry (DXA) is a proxy for cumulative calcium status because 99% of the body's calcium is in the bones. Indirect measures of bone metabolism which impinge on calcium status include bone biochemical markers of turnover that measure substances that are released by osteoblasts and osteoclasts, as well as substances produced during the formation or breakdown of collagen, a main protein found in bone. Although DXA is considered the gold standard for assessing bone status, it is limited by the long lag time that is required between measurements (6 months to years) in order to show changes in bone mineral density (BMD) and BMC. In addition, biochemical markers of bone turnover have huge variation. Therefore, there is a need for a more sensitive but yet direct method of assessing bone status in response to treatments. A direct method for assessing

release of calcium from the skeleton uses a calcium isotopic tracer to assess bone changes in response to treatments. However, some calcium isotopic tracers have limitations for use in humans due to their short half life or radioactive nature. Fortunately, ^{41}Ca has a long half life of $\sim 100,000$ years and is safe for use in humans due to the low level of radioactivity required. Recently, ^{41}Ca has been proposed as a tool in bone research [7]. However, there is much to learn about designing studies and interpreting data for optimizing ^{41}Ca technology as a tool for assessing bone metabolism. In order to further our understanding of ^{41}Ca technology, we utilized ^3H -tetracycline (^3H -TC) as a proxy for ^{41}Ca in an animal study, with the ultimate goal of developing the use of urinary ^{41}Ca appearance from bone to directly measure bone resorption in humans. Previous studies have shown that upon injection of ^3H -TC in animals, it chelates with calcium and is incorporated into the skeleton. During bone resorption, ^3H -TC is released in a form that cannot be reincorporated into the skeleton [8]. Thus, urinary ^3H -TC is thought to reflect bone resorption.

The aim of our study was to use a single ^3H -TC injection, as a proxy for ^{41}Ca , to understand timing of label incorporation and release when bone resorption was manipulated by dietary calcium.

2. Experimental

Sixty-four ovariectomized (OVX) 6-month-old rats were purchased from Harlan (Indianapolis, IN). All the rats were fed an AIN 93 M diet [9] (Dyets Inc., Bethlehem, PA) during the acclimation period at our animal research facility. This is a standard semi-purified diet that uses casein as the protein source and meets nutrient requirements except as adjusted for calcium. Each rat was dosed with 30 μCi of ^3H -TC. The early OVX rats were dosed with ^3H -TC within 1 month after OVX while the OVX stabilized rats were dosed 3 months after OVX. Rats were randomized to receive either 0.2% calcium or 0.5% dietary calcium treatment. The recommended calcium intake is 0.5% which is the level in the standard diet and serves as the control. The 0.2% calcium intake was selected to be marginally deficient without inducing weight loss. Within each dietary calcium level, rats were further randomized according to time of sacrifice (1 week, 1 month, 3 months, or 6 months). There was no difference in average baseline weight among groups. Urine and feces were collected for 2–4 days at 1 week, 1 month, 3 months, and 6 months post dose. Immediately after collection, urine samples were centrifuged at 4000 RPM for 20 min. The supernatants were stored in a -20°C freezer. After all the metabolic collections for the entire study were completed, urine samples were thawed at room temperature and centrifuged again at 3000 RPM for 20 min. The supernatants were analyzed for calcium and ^3H -TC content. Feces were ashed for 4 days at 600°C , diluted with 0.5% Lanthanum Chloride to 25 mL and analyzed for calcium. Whole skeletons were recovered

by using Dermastid beetles (provided by Dr. Alan York, Entomology Department, Purdue University) as described by Hefti et al. [10] after the rats were sacrificed. The skeletons were separated into cortical-rich midshaft femur, trabecular-rich proximal tibia and L1-4, and the rest of the skeleton. Bones were digested with nitric acid and brought up to volume with nitric acid for the assessment of percent $^3\text{H-TC}$ present and $^3\text{H-TC}$ labeling efficiency. Total calcium in bone, urine, and feces was measured by atomic absorption spectrophotometry as previously described [4] (A Analyst 300, Perkin Elmer). Urinary and skeletal $^3\text{H-TC}$ content were measured by scintillation counting (LS 6500, Beckman Coulter Inc., Fullerton, CA) using a single labeled disintegration per minute (DPM) program.

Calcium balance was determined as follows:

$$\text{Calcium balance} = \text{Dietary calcium intake} - (\text{urinary} + \text{fecal calcium excretion})$$

Percent calcium retention was determined as:

$$100\% \times \text{Calcium balance} / \text{Dietary calcium intake}$$

Percent $^3\text{H-TC}$ present in bone was calculated as:

$$100\% \times \frac{{}^3\text{H-TC in bone (dpm)}}{{}^3\text{H-TC dose given to rat (dpm)}}$$

$^3\text{H-TC}$ labeling efficiency in bone was calculated as:

$$\text{Percent } {}^3\text{H-TC in bone} / \text{Calcium content in bone (grams)}$$

Bone mineral density (BMD) was determined by dual energy X-ray absorptiometry (Lunar DPX IQ 5455, Madison, WI) at the start of the study and again before sacrifice at 1 week, 1 month, 3 months, and 6 months post dose to assess change in BMD and total bone calcium. This study was approved by the Purdue Animal Care and Use Committee.

SAS statistical software (version 8.0, SAS Institute, Cary, NC) was used for all analyses. Data were reported as mean \pm standard deviation, unless otherwise indicated. Urinary $^3\text{H-TC}$, calcium balance, and percent calcium retention were compared at all time points by repeated measures analysis of variance with multiple comparisons of the means using Tukey's test. Constant variance and normality assumptions were improved when $^3\text{H-TC}$ in bone and urine was transformed by taking the log of the original data. Therefore, all analyses for $^3\text{H-TC}$ in bone and urine were based on log transformed data. Tukey's test was used for multiple comparisons when the differences in mean were significant. p values less than 0.05 were considered statistically significant.

3. Results and discussion

The effect of calcium intake on calcium balance over time is shown in Fig. 1. In an overall statistical model, rats that were fed the 0.5% calcium diet had significantly higher

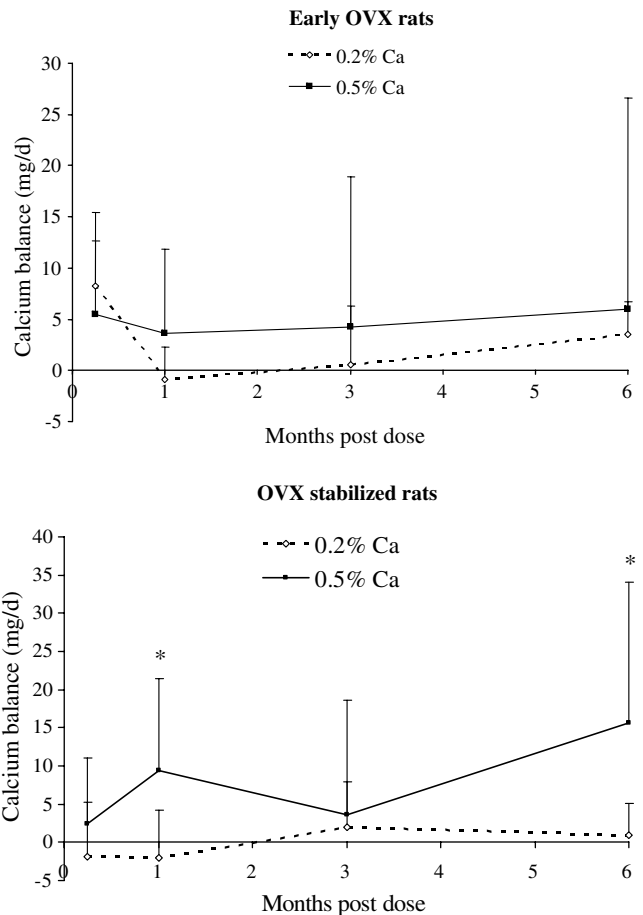


Fig. 1. Effects of the level of dietary calcium on calcium balance (mg calcium/day) in early OVX and OVX stabilized rats. Each line represents the mean \pm SD values of 4–16 rats, depending on the time of urine and feces collection. Asterisks indicate dietary calcium effect $*p < 0.05$.

calcium balance ($p < 0.005$) and percent calcium retention ($p = 0.052$) up to 6 months post dose, but this was driven by the rats stabilized to ovariectomy, as no time point was statistically significant in the early OVX rats. Rats fed adequate calcium were in net positive balance, whereas rats fed low calcium intakes were in negative balance at 1 month and in positive balance by 3 months. Rats fed the 0.5% calcium diet excreted more calcium in the urine and feces than rats fed the 0.2% calcium diet. Calcium excretion over time is shown in Fig. 2. Rats fed the 0.5% calcium diet consumed 2.5 times more calcium and excreted approximately 2.5 times more calcium in the feces and 1.4 times more calcium in the urine than rats fed the 0.2% calcium. Urinary calcium loss accounted for 3–6% of calcium intake in rats.

Bone resorption, as measured by urinary $^3\text{H-TC}$, is shown in Fig. 3. At 1 month post dose, higher calcium intakes significantly ($p = 0.013$) suppressed bone resorption in the OVX stabilized rats. The lack of a significant difference in urinary $^3\text{H-TC}$ due to calcium intake at 6 months ($p = 0.2$) despite a greater difference in means than at 1 month, which was statistically significant, was undoubtedly due to loss of power as rats were sacrificed

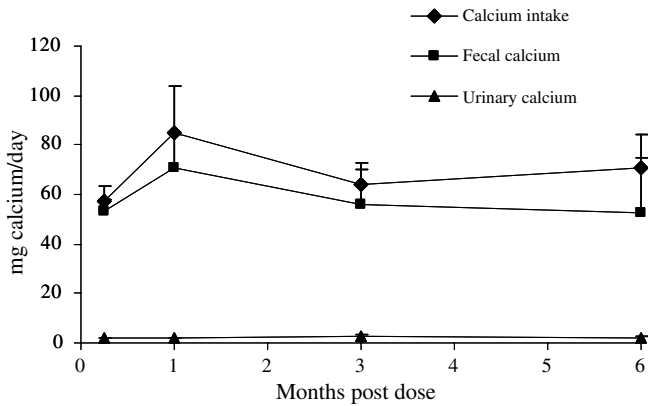


Fig. 2. Calcium intake, fecal and urinary calcium excretion of a representative group (OVX stabilized rats on 0.5% calcium) of rats over time ($n = 4$ per data point, mean \pm SD).

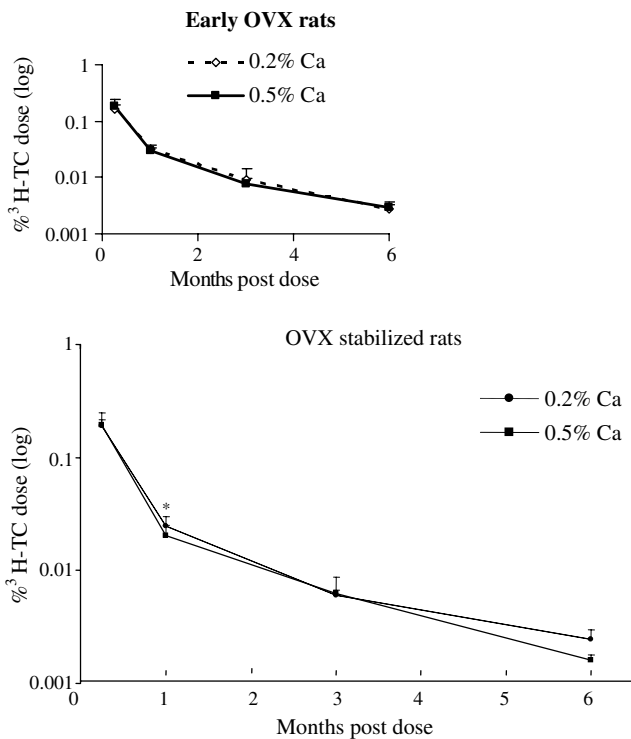


Fig. 3. Urinary ³H-TC of rats that received 0.2% Ca versus 0.5% Ca. Each line represents the mean \pm SD values of 4–16 rats (depending on urine collection time points). *0.2% Ca diet differs from 0.5% Ca diet, $p < 0.05$.

($n = 12$ rats/group at 1 month and $n = 4$ rats/group at 6 months). The lack of dietary effect at 3 months post dose for either ³H-TC excretion or calcium balance is inexplicable and inconsistent with the overall results. Because we know the total skeletal calcium content of the rats (3448 ± 302 and 3312 ± 358 mg Ca for early and stabilized OVX rats, respectively, at 1 month) and we know daily calcium loss, we can predict % skeletal calcium loss over time to determine clinical relevance. The calcium loss at 1 month post dose was 1.54 ± 0.73 mg Ca/day compared to 2.01 ± 0.68 mg Ca/day for rats fed 0.5% calcium diets. This

would lead to a difference of 5.6% skeletal loss per year due to the dietary calcium levels tested here, which is a substantial difference in loss of skeleton. The observation that higher dietary calcium suppressed bone resorption in rats stabilized to OVX, but not in the early OVX rats, is consistent with data in humans which show that dietary calcium does not affect bone density during peri-menopause when bone turnover is dominated by hormones [11].

Dietary calcium had no effect on ³H-TC retention or labeling efficiency at any bone site in all groups. BMD decreased from baseline to 1 week post dose for all rats ($p < 0.0001$) as expected for the first 3 months following ovariectomy due to estrogen deficiency. The reduction was more pronounced for rats on the 0.2% than the 0.5% calcium diet ($p = 0.03$) (Fig. 4). Overall, there was a cumulative effect of diet on BMD ($p = 0.0577$), with rats on 0.2% calcium diet having lower BMD. The amount of total bone calcium loss decreased over time as rats became stable to ovariectomy (Fig. 5). Rats that were fed the 0.2% calcium

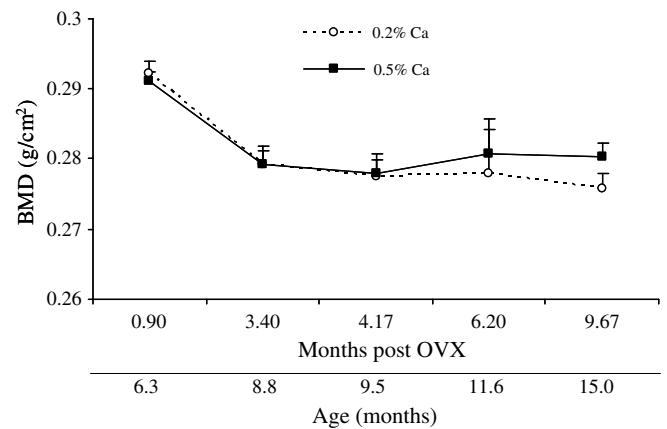


Fig. 4. Bone mineral density of rats over time, controlling for BMD at baseline. Each line represents the mean \pm SE values of 4 rats. There was a cumulative effect of diet on BMD ($p = 0.0577$), with rats on 0.2% calcium diet having lower BMD.

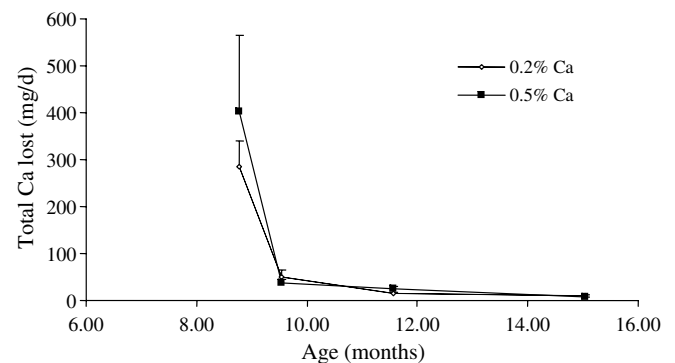


Fig. 5. Effects of dietary calcium levels on total bone calcium lost. Each line represents the mean \pm SD values of 16 rats. The effect of dietary calcium on bone calcium loss was significant at effect * $p < 0.05$. The rats are the same as those in Fig. 4; therefore, reference to months post OVX for a given age can be determined from Fig. 4.

diet had significantly higher total bone calcium loss than rats fed the 0.5% calcium diet as expected from urinary $^3\text{H-TC}$ and calcium balance.

Higher dietary calcium suppresses bone resorption through the parathyroid hormone/vitamin D axis of homeostatic control. High dietary calcium suppresses parathyroid hormone release which slows conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. This reduces bone resorption and active calcium absorption, and increases urinary calcium loss. Consequently, calcium retention differed between rats fed high and low calcium intakes in the rats stabilized to OVX, but in the early OVX rats, effects of loss of estrogen on bone resorption outweighed effects due to dietary calcium. Stabilized OVX rats were older animals than the early OVX animals and could have had lower ability compared to younger animals to adapt to changes in serum calcium in response to diet. Age and estrogen deficiency should combine to reduce the ability to adapt with time and would lessen differences between high and low calcium intake. However, Talbott et al. [12] showed little differences in response to dietary calcium with age. The effects of low dietary calcium-induced bone resorption have been shown using urinary $^3\text{H-TC}$ to occur as early as 6 hours after the initiation of a low calcium diet [8]. Interestingly, Talbott et al. [12] demonstrated an increase in urinary $^3\text{H-TC}$ levels only during the first 3 weeks of a 9 week period of dietary calcium restriction (0.1% calcium) in 20 and 48 week-old rats. These rats started receiving chronic $^3\text{H-TC}$ labeling over a 6 week period when they were 12 and 40 weeks old. Biochemical markers of bone resorption (pyridinoline and deoxypyridinoline) showed dietary effects up to 6 weeks. If the effect of diet is transient, the timing for measuring effects on bone resorption is crucial. Urinary $^3\text{H-TC}$ increased when the diets of 2 month-old male rats were changed from 1.1 g Ca/100 g dry weight to 0.2 g Ca/100 g dry weight in an acute setting [13]. Others [14,15] have also demonstrated changes in urinary $^3\text{H-TC}$ levels in an acute setting during dietary interventions that lasted for ~ 10 days. There is a paucity of data that examines urinary $^3\text{H-TC}$ in a chronic setting. In our study the effect of dietary calcium on calcium balance and bone calcium loss and BMD were significant at 6 months, but the effect of dietary calcium on urinary $^3\text{H-TC}$ excretion is equivocal because of lack of power beyond 1 month post dose. A further limitation is that in rodents, a relatively small proportion of dietary calcium is being excreted in the urine of rats (3–6% in rats versus 14% in humans) [4]. Thus, changes in urinary calcium in rats have minimal effect on calcium balance.

In this study, we are unable to resolve whether the effects of dietary calcium on bone turnover are transient or chronic. Biochemical markers of bone turnover have a sustained response to other interventions such as estrogen and bisphosphonate therapies that perturb bone turnover that result in differences in BMD [16,17]. It is unlikely that a lack of significant diet effect on urinary $^3\text{H-TC}$ beyond 1 month post dose was due to draining of $^3\text{H-TC}$ from the

skeleton as a result of calcium deficient diet, as proposed by Talbott et al. [12], because $^3\text{H-TC}$ could still be detected in the urine and skeleton up to 6 months post dose. It is also unlikely that a lack of diet effect on urinary $^3\text{H-TC}$ excretion beyond 1 month was due to the single $^3\text{H-TC}$ dosing regimen, because chronic pre-labeling [12] showed similar transient results. It has been reported that repeated exposure to the labeled material during early rapid skeletal growth period, such as in utero or shortly after birth, is required in order to study the normal behavior of calcium in adult rat skeleton [13,18]. Labeling the skeleton of older animals is not usually performed due to the lower rate of bone turnover. However, the present study demonstrates that labeling 6–9 month-old OVX rats with a single dose of 30 μCi of $^3\text{H-TC}$ is feasible to study bone resorption. Further research is needed with increased power to evaluate chronic effects of diet on urinary $^3\text{H-TC}$. It is logical that effects of dietary calcium on indices of bone turnover would parallel changes in calcium balance and bone mineral content.

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