In vivo rat assay: bone remodeling and steroid effects on juvenile bone by pQCT quantification in 7 days

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The 21-day-old rat represents a juvenile model of bone growth. The juvenile bone is particularly sensitive to corticosteroids. Corticosteroids are prescribed for severe asthma, juvenile rheumatoid arthritis, or dermatologic disease and are known to reduce bone turnover, stunt growth, and decrease bone mineral density (BMD). The juvenile bone is particularly sensitive to corticosteroids. Corticosteroids are prescribed for severe asthma, juvenile rheumatoid arthritis, or dermatologic disease and are known to reduce bone turnover, stunt growth, and decrease bone mineral density (BMD) (5, 6, 9, 15, 23). An average daily dose of 5 mg·kg⁻¹·day⁻¹ will cause loss of BMD in the prepubertal child, and monitoring of bone density is recommended even for adults taking 7.5 mg·kg⁻¹·day⁻¹ for ≥6 mo (17). In their retrospective review of 212 patients, Hougardy et al. (10) showed that the median daily dose was 10 mg prednisolone equivalent and the median duration of oral corticosteroid treatment was 50 wk, which is well above the dosage capable of eliciting bone loss. Moreover, at an average dose of 0.67 mg·m⁻²·day⁻¹ of inhaled steroids, there is a reduction in the acquisition of bone mineral in prepubertal children that compromises their peak bone mass and predisposes them to osteoporosis and a higher fracture risk as an adult (2). Therefore, we also wanted to determine whether we could quantify by pQCT with this model negative effects of corticosteroids on juvenile bone.

Although pQCT scanning cannot replace the information obtained from histostaining, immunostaining, or histomorphometric analysis, it allows for the rapid and non-invasive evaluation of potential therapeutic anti-resorptive compounds in a shorter amount of time.

MATERIALS AND METHODS

These studies were conducted under a protocol approved by the Schering-Plough Research Institute Animal Care and

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Corticosteroid treatment to growing rats. Twenty-five rats were randomly assigned to one of five groups (n = 5/group): a low-dose group (methylprednisolone 3.5 mg·kg\textsuperscript{-1}·wk\textsuperscript{-1} sc), a standard-dose group (methylprednisolone 7 mg·kg\textsuperscript{-1}·wk\textsuperscript{-1} sc), two high-dose groups (methylprednisolone 10.5 and 14 mg·kg\textsuperscript{-1}·wk\textsuperscript{-1} sc), and a control group that received vehicle only (saline + methanol sc).

High-dose corticosteroid treatment. Eighteen rats were randomly assigned to one of two glucocorticoid-treated groups (one group received double the highest dose of our previous study (methylprednisolone 28 mg·kg\textsuperscript{-1}·wk\textsuperscript{-1} sc, n = 5), and a control group that received vehicle only (saline + methanol sc, n = 8). For measurement of femoral length and weight, the left femur was removed and cleaned free of muscle and nonbone tissue. Because of the rat’s quadruped stance, the femur is not the head but the greater trochanter. The length of the femur was measured from the highest point on the rat femur to the lateral condyle using a digital caliper (Pro-max, Japan Micrometer Manufacturing). The bones were then weighed (wet weight) on an Ohaus Voyager balance (Ohaus, Pine Brook, NJ).

pQCT measurements. All groups were treated in the same manner. On day 1, we took baseline BMD of the proximal tibia by pQCT (XCT Research, Stratec Medizintechnik, Pforzheim, Germany). The rats were given their supplements or vehicle daily on days 1–7. On the 8th day, the final BMD measurements were taken. The settings for pQCT scanning included research SA collimation at a voxel size of 0.1 mm\textsuperscript{3}; therefore, the slice width was 0.1 mm. The voxel is equivalent to a pixel with three-dimensional volume. This small voxel size minimizes “partial volume effect” errors (i.e., including voxels that are not completely filled with bone). Before the pilot study, multiple slice scans were performed on the weanling to examine variations in slice density at the proximal tibia. The slice placement that gave the most consistent density with least variability between adjacent slices at baseline was determined and is shown by the low variability of the baseline data in Fig. 1. Comparable placement of slices was ensured by measuring a slice in the metaphysis 2 mm from the reference line, which was placed at the proximal edge of the growth plate.

pQCT analysis. All bone slices were analyzed with the same parameters by use of Stratec software (Stratec Medizintechnik). The analysis parameters of the Stratec software employed in the present study were an automatic Contour-Mode 1, which was used to define the outer edge of the cortical bone, and PeelMode 20 (an adaptation of PeelMode 2 that determines the threshold to be used by evaluating the BMD at a predefined percentage of total bone), which was used to define the inner edge of the cortical bone and the beginning of the trabecular bone. For determining trabecular BMD, the percent option was used, with trabecular area defined at 30% with a threshold of 280 mg/cm\textsuperscript{3}. For cortical bone analysis, cortical bone was defined as any density >710 mg/cm\textsuperscript{3} within the defined region of interest. Thresholds were determined by using the “profile” function of the CT scanner to visualize the density at the edges of the cortical bone. Statistical analysis of data was assessed using ANOVA, with Dunnett’s test for treatments with a control, simple linear regression, and Pearson product-moment correlation coefficient (SigmaStat, SPSS Software, Chicago, IL). All data are reported as means ± SE.

RESULTS

Initial study. Baseline trabecular BMD data as measured by pQCT scans were highly consistent among groups (Fig. 1, baseline). After 7 days of treatment, the final pQCT measurements (BMD) were significantly different from their own baseline (P < 0.001), a finding characteristic of young, growing animals. A comparison of final trabecular BMD measurements showed that there was a significant difference between the treatment groups. Both of the treated groups had a significantly higher trabecular BMD than the untreated controls (P < 0.001). Also, alendronate pro-

![Fig. 1. Bone mineral density (BMD) in the growing rat—effect of bisphosphonate treatment. The 1st experiment shows effect of bisphosphonate treatment (alendronate sodium 10 and 20 µg·kg\textsuperscript{-1}·day\textsuperscript{-1}; n = 5/group) on BMD in the growing rat. Trabecular BMD values at baseline were consistent between groups. After 7 days of bisphosphonate treatment, there was significant increase in trabecular BMD that was dose dependant. *Significant vs. baseline, P < 0.001; †significant difference from control final measurement, P < 0.001; ‡significant difference between 2 doses of alendronate sodium, P = 0.03. Data are means ± SE.)

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duced a dose-related increase in trabecular BMD at 20 vs. 10 μg/kg (Fig. 1, final) that was statistically significant (P = 0.03). The final area measurements of cortical bone (volume) were insignificant among groups (0.18 ± 0.04 mm² in control vs. 0.31 ± 0.07 mm² and 0.27 ± 0.05 mm² in the 10 and 20 μg/kg groups, respectively).

**Alendronate dose-response study.** Baseline BMD data as measured by pQCT were not significantly different among groups. After the 7 days of treatment, final trabecular BMD measurements were significantly different from those at baseline. Moreover, when we compared the groups on the basis of longitudinal trabecular bone density measurements (change from baseline), all doses showed a significant difference from control (P < 0.001, Fig. 2). The final measurements of cortical bone area (volume) demonstrated that, at the highest dose, the increase in cortical bone was significantly higher than control (P = 0.002, Fig. 3).

**Corticosteroid treatment to growing rats.** Baseline pQCT scans were not significantly different among groups. After 7 days of treatment, each rat’s final trabecular BMD measurement was significantly greater than its respective baseline values. When we compared the final BMD (trabecular) measurements among all three groups, there were no significant differences. Because glucocorticoids (GC) have effects on cortical bone as well, we analyzed the bone slice for cortical bone content. The slices from the GC-treated animals had significantly higher areas of cortical bone than untreated controls (Fig. 4, A and B). The group receiving the highest dose showed a trend toward having a lower trabecular BMD than the midrange doses. When we repeated the study with high doses of GC, we observed a diminished growth rate in the GC-treated rats vs. control that was significant (P = 0.002). The final mean weight of the control rats was 92 ± 2 g vs. 76 ± 3 g and 61 ± 15 g in the 28 and 42 mg·rat⁻¹·wk⁻¹ GC-treated rats, respectively (Fig. 5A). The left femur (long bone) was removed to determine whether longitudinal bone growth was also affected. There were no significant differences in the length of the femur among groups; however, there was a trend for the weight of the GC-treated femurs to be lower (Fig. 5B). This decrease in femur weight correlated positively with the diminished final mean weights of the rats (Pearson correlation coefficient 0.528, P = 0.024). Baseline trabecular BMD data as measured by pQCT scans were not significantly different among groups. After 7 days of treatment, each rat’s final trabecular CT BMD measurement was significantly different from its own baseline. An ANOVA comparing the final trabecular BMD measurements among all three groups showed no significant difference, although there was a trend toward a lower BMD in trabecular bone in the groups treated with high doses of GC (Fig. 6A). A comparison of cortical area showed that, again, the slices from the GC-treated animals had a significantly higher volume of cortical bone than those from untreated controls (Fig. 6B).

**DISCUSSION**

In this study, we demonstrated that pQCT analysis can be utilized to quantitate changes in bone in the growing rat model. The data were consistent, repeatable, and statistically relevant. We observed a clear dose-response effect with small increments in the drug. Although the growing rat bone is not a disease model of osteoporotic bone, it can be used to detect resorption/anti-resorption efficacy. The growing 21-day-old rat is an attractive model for the prescreening of bone density effects of anti-resorptive drugs. The relatively small body weights of these animals mean that less compound is needed, and the ability to detect dramatically significant changes in a relatively short period of time is an invaluable tool in studies on bone. Employing pQCT scanning techniques allows for quicker evaluation, especially as a prescreening tool vs. histology or histomorphometry.
An important limitation of using the growing rat is the difficulty in finding the initial scanned area after a period of growth. We circumvented this problem by utilizing rats that were 44 ± 5 g in weight. The rate of growth over the 7-day period still permitted identification of the original site. The young Sprague-Dawley rat has growth spurts. We found that if we used rats that had an initial weight between 80 and 100 g, the rate of bone growth at this stage was prohibitive to obtaining consistent measurements (personal observation). The changes in bone are again quantifiable at weights between 120 and 145 g, but the dosages required at these weights negate the benefits of using a growing rat.

To our knowledge, this is the first report to use pQCT technology for assessing bone density in the growing rat. Our study investigates the use of the CT scanner to quickly assess bone changes in the growing rat for purposes of early drug discovery; however, this model has many potential applications in other areas of bone research. The advantages of using this model include observation of faster bone turnover due to the age of the animals, earlier quantification of changes to bone, and a noninvasive technique that allows for measurements in the same animals over multiple time points. For example, Barou et al. (3) used micro CT scanning techniques to quantify bone loss in a disuse rat model of bone loss (3). This group observed significant changes in 13 days. By using a weanling rat model, the study time could thus be halved. Furthermore, weanling rats are useful models for studying effects of chronic alcohol consumption (20) and binge drinking (19) (two major concerns of teenage alcohol consumption) on the growing bone. The use of pQCT scanning techniques would offer a noninvasive and quick way of assessing changes to bone in these studies. In our study, we killed the rats at 7 days, when significant changes were observed in trabecular bone density; however, this noninvasive technique can also be employed to scan animals multiple times until significant changes are observed. This feature would be helpful in studies such as those that evaluate effects of dietary changes on bone density (22). Our model also allows each animal to serve as its own control, rather than killing animals at different time points during a study.

Because the weanling rat is a model of growing bone and there is a great deal of concern regarding the affects of anti-inflammatory steroids on juvenile bones, we attempted to simulate the effects of GC on the growing bone by use of this in vivo assay. The effects of GC on human bone are multifactorial. Not only do steroids inhibit the secretion of gonadal hormones (7, 14), thereby eliminating the bone-sparing effects of....
Fig. 6. Trabecular BMD and cortical bone area—effect of glucocorticoid treatment. A: a trend demonstrated toward a lower trabecular BMD in longitudinal measurements (difference from baseline); with high doses of glucocorticoids, however, significance was not achieved. B: effects of glucocorticoid treatment on final 7-day measurement of cortical bone area. Both doses showed significant increases in cortical area compared with control (28 mg·kg⁻¹·wk⁻¹, P < 0.001; 42 mg·kg⁻¹·wk⁻¹, P = 0.005). Data are means ± SE.

The decrease in available circulating calcium stimulates secondary hyperparathyroidism. In the growing child, these side effects are magnified by the fact that peak bone mass has not yet been achieved, and the decreases in bone-promoting hormones result in smaller bones (5). The decrease in raw materials and bone turnover leads to a lower bone mass. Moreover, growth during toddler and prepuberty years is predominantly dependent on growth hormone (GH). Oral GC excess has direct and indirect effects on GH secretion. Endogenous GCs downregulate the hypothalamic relay mechanisms that are responsible for pituitary pulsatile GH secretion (1). GCs act directly to downregulate expression and binding of the GH receptor and interfere with the bioactivity of IGF-I, the primary second messenger of GH (1). Furthermore, GH therapy appears to counteract the adverse effects of GC therapy in children with juvenile chronic arthritis (4).

Whereas the major effect of steroids on the adult skeleton is in the axial skeleton and femoral neck (24, 25), in the growing preadolescent the effects on longitudinal bone growth and achieving peak bone mass are of concern. Using lower therapeutic doses of methylprednisolone, we observed no significant decreases in trabecular BMD in the growing rat model. We did, however, observe significant changes to cortical bone area in doses greater than 7 mg·kg⁻¹·wk⁻¹, suggesting an effect on trabecular-to-cortical bone ratios. At the higher doses, we observed significant changes in bone size and body size/weights similar to the stunted growth observed in the preadolescent on severe steroidal regimens. Although there was a trend toward lower trabecular BMD in the animals subjected to higher doses, statistical significance was not achieved. The decrease in body weight may be explained by high-dose GC decreases in ad libitum food intake (18); however, even though animal size decreased in our study, the area of cortical bone (as defined by >710 mg/cm³) increased in a dose-dependent manner, suggesting that cortical bone growth/metabolism was enhanced. These data do not preclude the fact that there may be differential effects of GCs on other bones in the rat and bone loss occurrence in sections of the skeleton that were not presently assessed for BMD. We cannot ascertain on the basis of present data that extending the study beyond the 7-day period would yield different results on bone density; however, there are conflicting data regarding the effects of steroids on the BMD of the rat bone. Ferretti et al. (8) observed a dose-dependent decrease in femoral cortical BMD by pQCT scanning and a decrease in load-bearing capacity in older female Sprague-Dawley rats that were exposed to lower doses of steroids (dexamethasone) over 4 wk. By using an extremely sensitive (smaller) voxel size (0.0219 mm³; Stratec), they observed an increase in cortical porosity that compromised bone strength. In contrast, King et al. (11) observed an increase in bone, by histomorphometry, with GC treatment, and the biochemical markers of bone turnover (osteocalcin and deoxypyridinoline cross-links) were reduced. Differences in quantification techniques may account for the conflicting results. The increases in bone observed in the study by King et al. may reflect the larger cortical area that we observed. The increased porosity as measured by Ferretti et al. with the three-dimensional analysis by pQCT was not observed by King et al., who used histomorphometric techniques. Had porosities been present, they should have been visible by histomorphometry despite the fact that these techniques are not comparable, and histomorphometry cannot measure the true physical BMD of bone tissue. In the present study, we were unable to observe increased cortical porosity with our 0.1-mm³ voxel size compared with their voxel size of 0.0219 mm³. Alternatively, the differences may be due to the differences in ages of the animals. Our 21-day-old rats double their weight during the 7 days of the study and can be compared with humans at the GH-dependent stage of growth. Animals studied by Ferretti et al. were slightly older than those of the present study, and rats used in the study by King et al. were ~3 mo old.

Although we anticipated simulating adult human GC-induced osteoporosis at the trabecular level in this growing rat, this was not the case, possibly because GCs in rats do not inhibit absorption of calcium from...
the intestine as the human on steroid therapy does, and therefore they may not have the same bone loss effects on trabecular-rich areas that humans have (12, 26). Our findings did show, however, that normal weight gain (growth) and cortical bone growth (area) were significantly affected with GC treatment in the growing rat. We thus propose that the 7-day-rat model relates more to the effects of GCs on the juvenile bone growing rat. We thus propose that the 7-day-rat model significantly affected with GC treatment in the weight gain (growth) and cortical bone growth (area) were signifi-
cantly affected with GC treatment in the 7-day-rat model.

In summary, although the relevance and appropri-
ateness of this model to adult human disease may be limited because of the differences just mentioned that we have observed with steroid therapy, we propose the use of the weanling rat and pQCT techniques for early/first assessment of compounds on bone remodeling. Moreover, our data with alendronate demonstrate that the weanling rat may provide a useful model for mechanistic evaluation of nonglucocorticoid therapeutic modalities or efficacy assessment to help guide dose selection for chronic long-term osteoporosis models.

REFERENCES