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Glucocorticoid-Induced Osteoporosis in Growing Mice Is Not Prevented by Simultaneous Intermittent PTH Treatment

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Abstract Glucocorticoids (GCs) are widely used in medicine for treatment of chronic diseases. Especially in children, prolonged treatment causes growth retardation and early onset of osteoporosis. Human parathyroid hormone (PTH) has an anabolic effect on bone when administrated intermittently. The aim of the present study was to examine whether a combined therapy of dexamethasone (DEX) and PTH could prevent the detrimental effects of GC on cortical and trabecular bone in the femur and vertebrae of growing mice. Three-week-old female FVB mice were treated with control, DEX, PTH, or a combination of DEX and PTH by daily subcutaneous injections. After 4 weeks, animals were killed and the femur and L5 vertebra were isolated. Cortical and trabecular bone parameters and relative calcium density were measured by high-resolution X-ray micro-computed tomography (micro-CT). In the femur, PTH can reverse the effects of DEX on bone volume to control. However, it cannot reverse the undermineralization, which most likely is a strong contributor to bone fragility. In the vertebra, PTH improves bone volume to some extent but does not restore the values to normal. It augments the negative effect of DEX on mineralization. We conclude that the detrimental effects of DEX in the growing skeleton cannot be prevented by simultaneous PTH treatment.

Keywords Bone architecture/structure · Osteoporosis · Animal model · Peptide hormone · Parathyroid hormone · Parathyroid hormone-related peptide

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Glucocorticoids (GCs) are often used for treating chronic diseases such as rheumatoid arthritis and asthma. Especially in young children, this therapy has serious side effects, such as growth retardation and early onset of osteoporosis, particularly after prolonged treatment. Administration of the synthetic GC dexamethasone (DEX) can cause growth retardation, which is due to the induction of chondrocyte apoptosis in the growth plate [1], inhibition of chondrocyte proliferation [2], and matrix synthesis [3]. GC-induced osteoporosis is one of the most common causes of drug-related osteoporosis [4] and is observed in patients chronically exposed to excessive amounts of GC. Multiple mechanisms are involved in GC-induced osteoporosis. They impair the replication, differentiation, and function of osteoblasts and induce the apoptosis of mature osteoblasts and osteocytes. These effects lead to a suppression of bone formation [5–7]. Management is mainly based on vitamin D or calcium supplements and, when the risk of fracture is high, on antiresorptive agents such as bisphosphonates [8–11]. These therapies preserve or slightly increase bone mass, but the main lesion of GC-induced osteoporosis, decreased bone formation due to suppression of osteoblast function [9], is not prevented.

A large number of studies in experimental animals and in humans have indicated that intermittent administration of low doses of parathyroid hormone (PTH) by daily

subcutaneous injections has anabolic effects on bone by stimulating cancellous and sometimes cortical bone formation. The mechanism by which intermittent PTH treatment elicits an anabolic response is complex. It involves, among other things, activation of lining cells, induction of expression of pro-osteoblastic growth factors like IGF-I and FGF-2, inhibition of the expression of osteoblast repressors such as the Wnt antagonist sclerostin, and inhibition of osteoblast apoptosis. All these effects can increase osteoblast number and, hence, stimulate new bone formation [12, 13]. In many aspects, the actions of intermittent PTH on bone are opposite to the actions of GCs. Consequently, intermittent PTH has been postulated as a treatment for GC-induced osteoporosis. Indeed, intermittent PTH treatment following a period of GC use induces new bone formation and improves bone mineral density in animal models and in patients [14, 15]. At present, it is unclear whether intermittent PTH treatment can reverse the devastating effects of GCs on bone in a mouse model of GC-induced growth retardation.

Previously, we showed that simultaneous treatment with intermittent PTH could not reverse the decrease in body length gain induced by DEX in young growing mice. In this experiment 3-week-old mice entering sexual maturation were treated for 4 weeks with daily injections of PTH and DEX. This treatment period covers almost the whole period of sexual maturation in mice and is characterized by rapid growth [16].

In the present study, we evaluated in more detail the effects of DEX on various bone parameters by means of high-resolution micro-computed tomography (micro-CT) [11, 17, 18] using this established mouse model for GC-induced growth retardation [16, 19, 20]. We addressed the question of whether simultaneous treatment with DEX and intermittent PTH could reverse the negative effects of DEX on bone.

Materials and Methods

DEX was obtained from Merck Sharp & Dohme (Haarlem, The Netherlands). Human PTH(1–34), hereafter "PTH," was from Bachem (Basel, Switserland) and was dissolved in 0.1 mM acetic acid and 0.01 mM β-mercaptoethanol. The pH value of the PTH solution was adjusted to 7.4 with HCl and diluted in phosphate-buffered saline (PBS, pH 7.4) containing 0.2% bovine serum albumin (BSA). DEX was also dissolved in PBS (pH 7.4) with 0.2% BSA.

Animals, Experimental Design, and Tissue Preparation

Three-week-old female FVB mice were kept under standardized conditions as described earlier [16] in accordance with the NIH guidelines for the care and use of laboratory animals. The experimental protocol was approved by the Committee for Animal Experiments of the University Medical Center Utrecht, The Netherlands.

Animals were divided into four groups of five, ensuring equal means and SEM for body length and weight in each group at the start of the experiment. They were subcutaneously injected in the neck with 0.1 mL DEX, 0.1 mL PTH solution, vehicle, or a combination once a day, 5 days/week for 4 weeks: group I, control, vehicle (PBS, pH 7.4); group II, PTH (0.14 μg/g/day); group III, DEX (20 μg/day); group IV, PTH+DEX (0.14 μg/g/day + 20 μg/day, respectively). The dose of PTH was based on a dose–response study of the anabolic effects of PTH in a mouse model of comparable age and has been used by others to induce an anabolic effect in growing mice [21, 22]. The dose of DEX has been previously shown to induce a maximal effect on growth retardation [19]. All animals were weighed and the total length was measured under anesthesia once a week as previously reported [16]. After 4 weeks of treatment, the mice were killed by decapitation after anesthesia, 2 hours after the last injection. The femur and vertebra (L5) were carefully dissected and cleared from adjacent muscle tissue. Bones were immediately fixed in buffered 3.8% formalin for 24 hours and stored in 70% alcohol until further processing.

Micro-CT Procedure

A high-resolution X-ray micro-CT desktop system (Skyscan 1072, Kontich, Belgium) was used to evaluate the effects of PTH and DEX on bone after 4 weeks of treatment. In this instrument an air-cooled point X-ray source (focal spot size approximately 8 μm in diameter, peak X-ray energy 80 kV/100 μA) illuminated the object with a cone beam. Shadow pictures were detected by a two-dimensional 14-bit CCD camera. Virtual cross sections were reconstructed by the Feldkamp cone-beam algorithm [23].

Total bone volume and cortical bone parameters were measured. For this purpose, femurs and L5 vertebrae were scanned with 19 μm and 8 μm pixel sizes, respectively. An aluminum filter (1 mm) was essential to reduce beam hardening. To perform trabecular bone analysis with more accuracy, the distal top of the femurs was scanned a second time with a pixel size of 5 μm.

Bone Parameters

Initially, whole-bone parameters, including total femur volume, total vertebral volume, diameter, and thickness of the shaft, were studied. For the analysis of the shaft, the central part of the femur was chosen (one-third of the bone length). To compare geometric properties of the shafts, a

center of mass was found in every cross section and an average diameter determined. Shaft thickness was calculated as an average path of the beam crossing the bone perpendicular to every point of its surface.

The relative amount of calcium per unit of volume (calcium density) of femurs and vertebrae was calculated as reported previously [24]. Relative density was expressed in Hounsfield units in the histograms resulting from image analysis. Calcium density was expressed as a relative parameter. The density of the control group was taken as a reference and set to 1 (Table 1).

In the metaphysis of the femurs the following parameters were measured: trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular bone volume (BV). To increase reproducibility, a reference point in the growth plate was chosen (e.g., the boundary of the growth plate and the primary spongiosum) and an offset of 100 virtual slices (corresponding to approximately 500 μm) was taken into account. Over a distance of 150 slices (approximately 750 μm) trabecular bone was analyzed. For calculations of bone parameters, commercial software (CTAnalyser, Skyscan) was used.

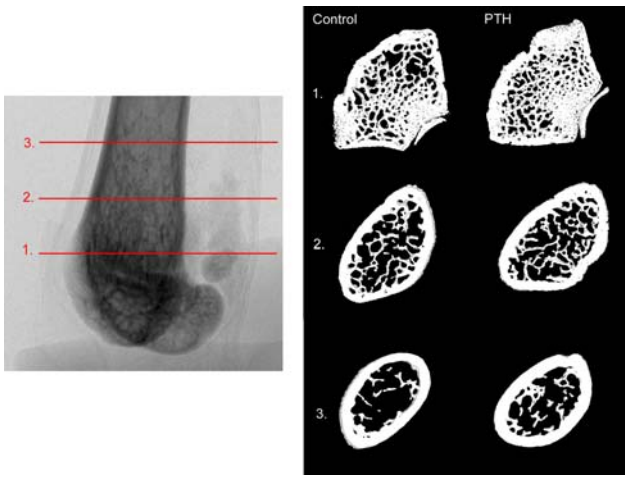

Fig. 1 Representative example illustrating analysis by micro-CT. **a** A shadow image of the femur indicating the different locations in the bone that were studied: (1) metaphysis, (2) combination of trabecular and cortical bone, (3) cortical bone. **b** 3D models (reconstructed from 20 virtual slices, each 5 μm thick) at the different levels in a representative control (*left*) and a PTH-treated femur (*right*). Notice that at levels 1 and 2 there is an increase in trabecular bone in the PTH group and at level 3 the thickness of the cortical bone was increased. A bone from the PTH group was chosen because here the changes were most pronounced and visually detectable

Statistical Analysis

Most parameters were represented as average ± standard deviation (SD). Mutual comparison between groups was evaluated using ANOVA with the least significant difference post-hoc test with the statistical software SPSS 13.0 (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

Figure 1 shows micro-CT results obtained from a representative femur in control conditions and after treatment with PTH. This example was chosen to illustrate the changes in bone as observed by micro-CT, which are the most prominent in the PTH group. In Fig. 1a, the location where the femur was virtually cut is indicated. In Fig. 1b, 3D models of 20 cross sections (each 5 μm thick) are shown at the three different levels. Level 3 contains mainly cortical bone, whereas cortical and trabecular bone are present at levels 2 and 1. In the bone treated with PTH, trabecular bone volume was increased at all three levels compared to control. The increase was most pronounced at level 1, representing the metaphysis. This region was chosen for subsequent analysis of trabecular bone. At level 3 an obvious thickening of the cortical bone can be observed in the PTH-treated femur compared to control.

Figure 2 shows the length of the femurs in the different groups. Femurs of DEX-treated animals were 3.6% shorter than those of controls, indicating DEX-induced growth retardation. PTH did not have an effect on bone length and did not restore the length of DEX-treated bones to normal.

Table 1 Comparison between femur and vertebra

	Control	PTH	DEX	DEX+PTH
Femoral bone volume (mm^3)	28.21 ± 2.82	33.61 ± 0.97[a,c]	26.85 ± 0.65[b,c]	29.10 ± 1.98[b]
Relative Ca density femur	1 ± 0.067	0.971 ± 0.024[c]	0.894 ± 0.033[a,b]	0.897 ± 0.024[a,b]
Vertebral bone volume (mm^3)	3.75 ± 0.15	3.96 ± 0.31[c]	2.73 ± 0.39[a,b]	3.03 ± 0.31[a,b]
Relative Ca density vertebra	1 ± 0.021	0.947 ± 0.033[a]	0.950 ± 0.039[a]	0.902 ± 0.035[a]

Total bone volume and Ca density in the femur and vertebra. Numbers represent mean ± SD. Statistical differences are indicated: [a] $P < 0.05$ vs. control group, [b] $P < 0.05$ vs. PTH group, [c] $P < 0.05$ vs. PTH+DEX group

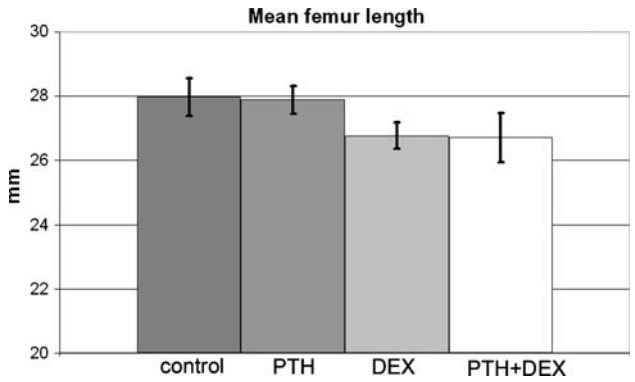

Fig. 2 Bar graph showing the length of the femur. Notice that PTH could not compensate for the effect of DEX

Figure 3a, b summarizes the results of the image analysis of the entire data sets of the whole femur and of the shaft only. In these histograms the volume taken by voxels with a given gray value is plotted vs. the gray value or color, the latter being expressed in Hounsfield units. The area under the curves, both in the whole bone and in the shaft, is much larger for the PTH group, whereas the area under the curve for the DEX and DEX+PTH groups is much smaller, although there is not so much difference with the control group. These results indicate that there is a large difference in the amount of bone present, which is most likely reflected in a larger bone size in the PTH group and a smaller bone size in the DEX and DEX+PTH groups. These observations are confirmed by the quantitative data on bone volume in Table 1. PTH-treated bones have gained 20% in bone volume, while DEX-treated bones have 5% less bone volume compared to control. Intermittent PTH treatment restored bone volume to control levels, but the effect of PTH was blunted by DEX (DEX+PTH group).

The curves in Fig. 3a are shifted to the left for DEX and DEX+PTH compared to control. This can be taken as an indication that these bones are less dense. This is also confirmed by the calcium content measurement in Table 1, showing reductions in relative Ca density of 10.6% and 10.3%, respectively, indicating that the effects of DEX on bone mineralization could not be counteracted by intermittent PTH treatment. Compared to control, the curve for the PTH-treated group is shifted upward, indicating that there is more bone with the same calcium content. This is also clear from Table 1. From Fig. 3b, summarizing the results of the shaft only, it can be concluded that there is more cortical bone in the PTH group. This is also illustrated by the representative example in Fig. 1 (level 3). The volume occupied by bone is smaller in the DEX and DEX+PTH groups with a diminished mineral content.

In cortical bone, mean shaft volume, diameter, and thickness were measured as shown in Fig. 4a–c. Mean

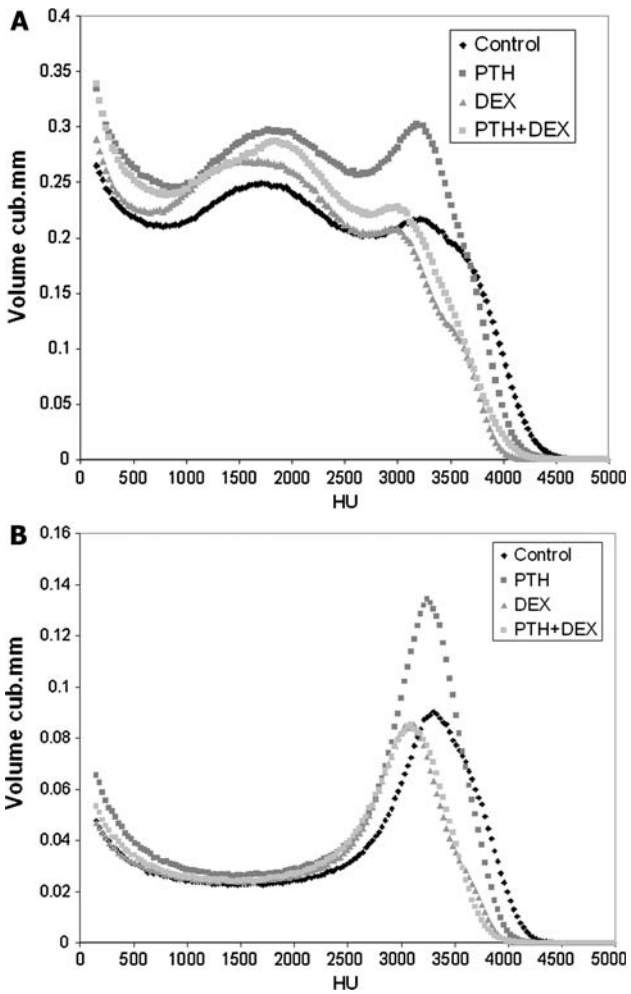

Fig. 3 Histograms representing the volume taken by bone with a given gray value vs. the gray values expressed as Hounsfield units (HU). **a** Analysis of the whole bone. **b** Shaft only

shaft volume as well as mean shaft thickness and shaft diameter of the DEX-treated group were smaller compared to the control group, although the differences for diameter and thickness of the shaft were not statistically significant vs. the control group. In contrast, PTH treatment increased shaft volume, diameter, and thickness significantly. In the PTH-DEX group shaft volume was restored to normal. However, the intermittent PTH treatment could not restore calcium content in DEX-treated bones to normal (Table 1).

Trabecular architecture was studied in a selected region of the metaphysis (Fig. 1), the results of which are illustrated in Fig. 5. PTH treatment for 4 weeks significantly increased trabecular bone volume (Fig. 5a) due to an increase in trabecular number (Fig. 5c), while trabecular thickness did not change (Fig. 5b). Trabecular bone volume did not change after DEX or DEX+PTH treatment. A tendency toward a decreased trabecular thickness and an increased trabecular number was observed in the

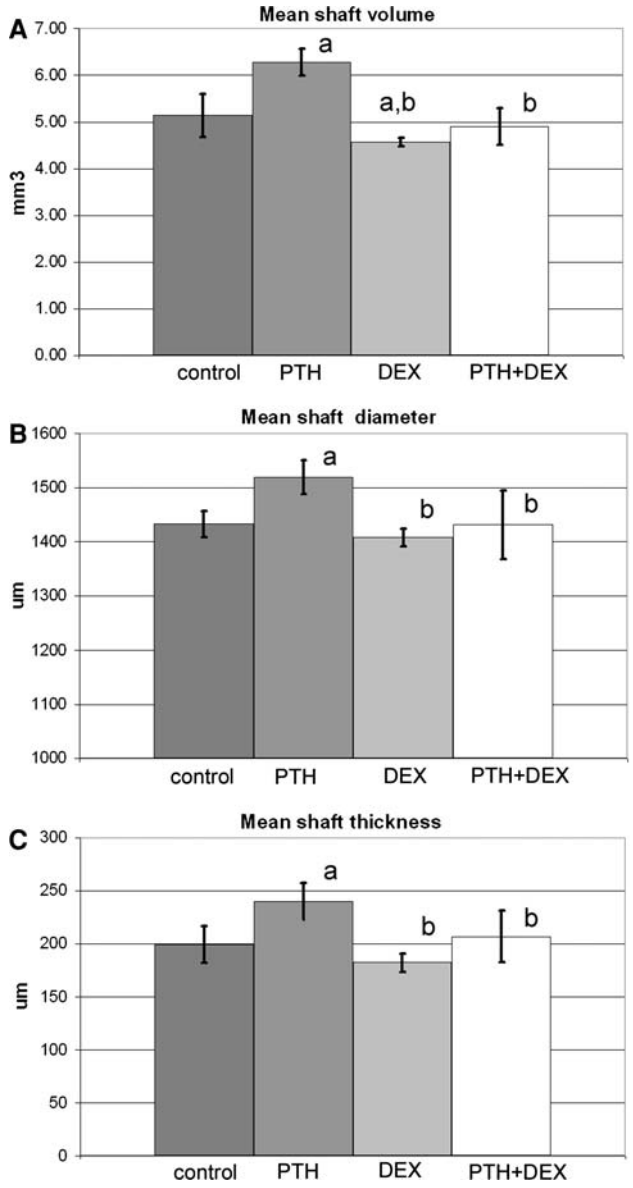

Fig. 4 Bar graphs of **a** shaft volume, **b** shaft diameter, and **c** shaft thickness in the femur. [a] $P < 0.05$ vs. control group, [b] $P < 0.05$ vs. PTH group, [c] $P < 0.05$ vs. PTH+DEX group

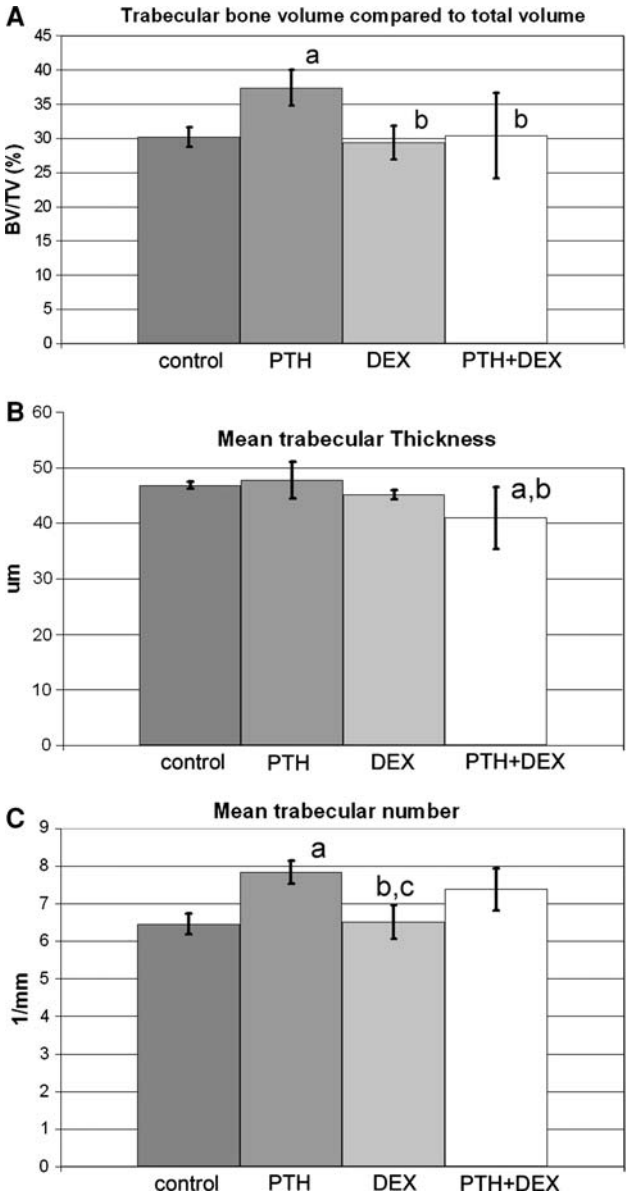

Fig. 5 Bar graphs showing the analysis of trabecular bone in a selected region of the metaphysis corresponding to level 1 in Fig. 1: **a** trabecular bone volume vs. total volume (BV/TV), **b** trabecular thickness, **c** trabecular number. [a] $P < 0.05$ vs. control group, [b] $P < 0.05$ vs. PTH group, [c] $P < 0.05$ vs. PTH+DEX group

DEX+PTH group compared to control and DEX-treated bones.

In addition to the femur, we analyzed the L5 vertebra. Bone volume and relative calcium density are summarized in Table 1. In contrast to the femur, the increase in bone volume by PTH was less dramatic and did not reach significance. Compared to the 5% decrease in bone volume in the femur, DEX treatment reduced bone volume of the L5 vertebra by 28%. The combined treatment of DEX and PTH only marginally improved the volume of the vertebra compared to DEX treatment alone. All treatments resulted in a reduction of mineral content compared to control. This latter effect was the most pronounced in DEX+PTH treatment.

Discussion

The aim of the present study was to evaluate whether simultaneous treatment with intermittent PTH can protect from the devastating effects of GCs on cortical and trabecular bone in the growing skeleton. We have used an established mouse model for DEX-induced growth retardation. In this model, treatment was started at 3 weeks of age, around the onset of sexual maturation. The treatment lasted 4 weeks, which covers almost the whole period of sexual maturation in mice. We chose a simultaneous treatment with

DEX and intermittent PTH, mimicking a clinical situation in which patients require long-term treatment with corticosteroids to cope with daily living. Previously, we showed [16] that the DEX-induced decrease in body length and body weight gain cannot be restored by simultaneous intermittent PTH treatment. PTH treatment did not change bone length gain of the tibia and did not change growth plate width. PTH at the specific dose used could not protect from the GC-induced growth retardation in mice during sexual maturation, probably because of the broad action pattern by which DEX affects growth. Besides the well-known effect on growth, long-term use of corticosteroids in children is associated with early onset of osteoporosis.

In the present study, we used high-resolution micro-CT, a noninvasive method that is well-suited for studying the internal structure and architecture in bones, to analyze the femur and L5 vertebra of growing mice treated with DEX, intermittent PTH, or the combination of both [11, 17, 18].

As expected, administration of DEX caused a decrease in bone volume of the entire femur compared to control. Furthermore, the femur contained up to 10.6% less mineral. These depressing effects of DEX could also be seen in trabecular bone in the metaphysis, where the volume and number of trabeculae were reduced, although trabecular thickness was not reduced significantly, compared to control bones. This is also in line with previously reported results [25–27]. Similar effects of DEX were also seen in the vertebrae, although less pronounced. The effects of DEX on bone volume can be explained by inhibiting bone modeling by repressing osteoblast activity. Alternatively, the effects can be explained by DEX-induced bone loss or a combination of both. The design of our study does not allow discrimination between these two possibilities.

In line with previous observations, intermittent treatment with PTH alone increased bone volume of the entire femur [12, 13, 28–30]. Relative mineral content was similar compared to control. The most prominent effects were found in cortical bone, in which both shaft thickness and shaft diameter were increased, indicating enhanced periosteal and endosteal bone acquisition. Others also reported an increase in periosteal bone acquisition due to intermittent PTH treatment; however, these effects seem less pronounced compared to the effects we report in this study [30]. This may be explained by the fact that we performed our study in growing mice, in which bone modeling prevails, compared to studies in adult mice, where remodeling prevails. In the metaphysis an increase in trabecular bone volume was observed, which was due to an increase in trabecular number with unaltered trabecular thickness. This may indicate that PTH either induced the formation of new trabeculae or prevented the remodeling and loss of trabeculae formed earlier in the growing bone. The design of our study does not allow discrimination between these possibilities. In the vertebrae, the effect of PTH showed a similar trend but was less pronounced.

Despite the fact that intermittent PTH treatment has opposite effects on various bone parameters compared to DEX treatment, simultaneous treatment with PTH did not completely counteract the deteriorating effects of DEX. Although DEX+PTH could counteract the loss in bone volume in the femur due to DEX, it could not restore the decreased mineralization. In the vertebra some compensation of bone volume was seen but bone volume did not fully recover to the control values. In the femur, a slight increase in trabecular number with, on average, a decreased thickness was observed in the combined treatment compared to DEX treatment. Such an effect was not seen in the L5 vertebrae. A possible explanation for the different effects on femurs and vertebrae can be found in a different bone turnover rate at both sites in the skeleton. Bone turnover is higher in the femur compared to vertebrae. Since new trabeculae form faster when bone turnover is high, the treatment period was likely too short to allow development of new trabeculae in the vertebrae. Furthermore, the ratio of cortical vs. trabecular bone in femur and vertebra is different. This may also be a cause of the discrepancy between femur and vertebra in our study. In addition, it has been shown that DEX-induced growth retardation differentially affects the growth of long bones compared to the vertebra. For example, Rooman et al. [19] showed that body length gain, mainly caused by growth of the vertebral column, is reduced 33% by DEX treatment. In contrast, the length gain of the tibia was not affected. Also in our study, we found a very modest effect of DEX treatment on the length of the femur, which was decreased by 3.6% compared to control. In line with this, DEX treatment affected bone volume more dramatically in the L5 vertebra than in the femur (28% vs. 5% decrease compared to normal).

From our findings, we cannot draw definitive conclusions about the underlying cause of the decrease in vertebral volume. Given the dramatic effect of DEX on body length gain, it is likely that the large decrease can be explained by a negative effect on growth of the vertebral body.

One of the most prominent effects of DEX on bone was the reduction in relative calcium content with 5% and 12% in vertebra and femur, respectively, compared to control. PTH itself did not have an effect on relative mineral content compared to control in the femur but slightly decreased mineral content in the L5 vertebra. In combination with DEX, bone mineral content was even further decreased to 90% of control. Our data suggest that in the growing skeleton a decreased mineralization of the skeleton as well as a reduction in bone volume contribute to DEX-induced bone fragility. Decreased bone mineralization induced by GCs has also been reported by others [14, 27].

Yao et al. [14] recently reported that simultaneous treatment of mice with a slow-release pellet of prednisolone and PTH was able to restore the deteriorating effects of prednisolone on bone to normal. The discrepancy with our study can be explained by the use of a different GC (prednisolone vs. DEX), the treatment dose, and the age of the animals. Compared to 5-month-old mature animals in the study of Yao et al. [14], we used very young, rapidly growing animals aged 4 weeks. At this age, longitudinal bone growth and bone turnover are exceptionally high and bone modeling prevails over remodeling. Since lower concentrations of PTH were able to ameliorate the effects of GC-induced osteoporosis in aged mice [31] and in postmenopausal women [4], it would be worth studying the effects of other doses of PTH as well. Most of the studies on GC-induced osteoporosis start the anabolic PTH treatment after discontinuation of the treatment with GCs. The combination of DEX and PTH treatment as it is used in our study is unique: We started both treatments at the same time point, mimicking a clinical situation in which growing children are dependent on continuous corticosteroid treatment due to, e.g., chronic inflammatory diseases. In our model representing the growing skeleton, the effect of DEX seems to be dominant over that of PTH. Perhaps if the treatment strategy were sequential instead of combined, PTH could reverse the GC-induced bone loss in the growing skeleton as well.

In summary, our experiments show that simultaneous treatment with intermittent administration of PTH in a mouse model for GC-induced growth retardation cannot reverse DEX-induced growth retardation both in long bones and in the vertebral column. It only marginally improves DEX-induced reduction in bone volume in the L5 vertebra but restores bone volume to control levels in the femur. However, intermittent PTH does not prevent the decrease in bone mineral content induced by DEX in the femur and exaggerates the decrease in bone mineral content in the L5 vertebra. PTH alone had a significant anabolic effect on bone volume but did not increase bone length. The increase in bone volume was predominantly caused by the stimulation of periosteal bone formation and an increase in trabecular numbers. These effects were most pronounced in the femur relative to the vertebra, indicating regional differences in skeletal responses to PTH. We conclude that simultaneous treatment with intermittently administered PTH cannot be used to prevent the deteriorating effects of GC on the growing skeleton.

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