Additive Effect of Vitamin K₂ and Risedronate on Long Bone Mass in Hypophysectomized Young Rats

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Abstract: Hypophysectomy (HX) arrests bone growth and induces osteopenia in the long bones of rats. The present study investigated the combined effect of vitamin K₂ and risedronate on long bone mass in HX rats, in order to determine whether treatment with these two agents had an additive effect. Forty female Sprague-Dawley rats were hypophysectomized at 6 weeks of age by the supplier, and were shipped to our laboratory at three days after surgery along with ten intact rats that served as age-matched controls. The study was started on the day when the rats were received. Three HX rats were excluded from the study because of the failure of HX. Forty-seven rats (6 weeks old) were assigned to the following 5 groups by the stratified weight randomization method: intact controls, HX alone, HX + vitamin K₂ (30 mg/kg, p.o., daily), HX + risedronate (2.5 µg/kg, s.c., 5 days a week), and HX + vitamin K₂ + risedronate. The dosing period was 4 weeks. HX resulted in a decrease of the femoral bone area, bone mineral content (BMC) and bone mineral density (BMD), as well as a decrease in the cancellous bone mass of the proximal tibial metaphysis and the total tissue and cortical areas of the tibial diaphysis. These changes were associated with a marked reduction in the serum level of insulin like growth factor (IGF)-I and with elevation of serum alkaline phosphatase (ALP) and pyridinoline. Administration of vitamin K₂ increased the serum ALP level in HX rats, but did not affect any of the other parameters. On the other hand, risedronate ameliorated the decrease of femoral BMD and cancellous bone mass at the proximal tibial metaphysis in HX rats without affecting the serum IGF-I level, as a result of not causing a significant elevation of serum pyridinoline. Vitamin K₂ and risedronate combined had an additive effect on the femoral bone area, BMC and BMD, and the combined treatment group did not show any significant reduction of the total tissue and cortical areas at the tibial diaphysis, as well as a reduced serum pyridinoline level compared with untreated rats and an increased serum ALP level compared with untreated or risedronate-treated rats. These results suggest that risedronate had a positive effect on the BMD and cancellous bone mass of long bones in HX rats. Despite the lack of a significant effect of vitamin K₂ on bone mass parameters, it had an additive effect with risedronate on the BMC, BMD and cortical bone mass of long bones in HX rats.

Key words: BMD, hypophysectomy, long bone, risedronate, vitamin K₂

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Introduction

Hypophysectomized rats have been used as a model for evaluating the effects of pituitary hormone deficiency on the bones. Hypophysectomy (HX) results in the deficiency of various hormones, such as growth hormone, follicle-stimulating hormone, adrenocorticotropic hormone, and thyroid-stimulating hormone, all of which have an effect on bone metabolism. After HX is performed in rats, bone growth ceases and osteopenia occurs in the long bones [4, 39].

We previously found in HX young rats that growth hormone therapy could alleviate the decrease of tibial and femoral length and dry weight, as well as tibial bone mass, while prostaglandin E2 prevented a reduction of tibial density, but did not have any significant effect on tibial length, wet weight, or bone volume [5, 6]. These results suggest that anabolic agents only partially prevent a reduction in the length and mass of the long bones in rats after HX. Therefore, it may be important to investigate whether antiresorptive agents can prevent the loss of long bone mass after HX and whether combined administration of two agents has an additive effect on the long bones in young HX rats.

Vitamin K2 is known to have an anabolic action on bone. Regulation of bone formation by vitamin K2 may involve the γ-carboxylation of osteocalcin or may be mediated via the steroid and xenobiotic receptor (SXR) [13, 27, 34–36]. However, it has also been reported that vitamin K2 inhibits bone resorption via a mechanism that is independent of γ-carboxylation and related to its side chain [11]. Vitamin K2 has been reported to regulate bone metabolism and to improve bone mass, structure and strength in various rat models of osteopenia [1, 2, 10, 12, 17, 19, 20, 22–26, 29, 37, 38]. These results suggest that vitamin K2 has both anabolic and antiresorptive actions on bone in various in vivo and ex vivo studies. Up to date, the effect of vitamin K2 on long bone mass in HX rats has not been studied, and it remains unclear whether this vitamin acts on the long bones independently of pituitary hormones.

It is also known that risedronate is a potent antiresorptive agent that inhibits osteoclast-mediated bone resorption [9]. Thus, the mechanism for bone resorption seems to differ between vitamin K2 and risedronate. Because risedronate and vitamin K2 have different effects on bone metabolism, it would also be of interest to compare the combined influence of these two agents on long bone mass in HX rats. Accordingly, the present study was performed to examine the effects of vitamin K2 and risedronate on long bone mass in HX rats, and to determine whether combined treatment with these agents had a more beneficial effect on the long bones than either agent alone.

Materials and Methods

Animals

Forty female Sprague-Dawley rats were hypophysectomized at 6 weeks of age by the supplier (Hilltop Lab Animals, Inc., Scottdale, PA, USA), and were shipped to the Animal Facility of Winthrop University Hospital at three days after surgery along with ten intact rats that served as age-matched controls. The study was started on the day when the rats were received. The HX rats were assigned to four groups of 10 animals each by the stratified weight randomization method: HX group, HX + vitamin K2 group, HX + risedronate group, and HX + vitamin K2 + risedronate group. Treatment with vitamin K2 and/or risedronate was started just after the rats were grouped. Vitamin K2 (menatetrenone; Eisai Co., Ltd., Tokyo, Japan) was suspended in 0.1 ml of a solution of 1,2-propanediol and glycerol at a concentration of 12 mg/0.1 ml and was administered by gavage once daily at a dose of 30 mg/kg (body weight). Risedronate (Aventis Pharma, Tokyo, Japan) was dissolved in 10 ml of PBS to make a working stock solution with a concentration of 200 µg/ml and was administered subcutaneously at a dose of 2.5 µg/kg, 5 days per week. The selected dose of vitamin K2 was effective according to the results of a previous study [29], although the influence of this vitamin on the bones seems to be modest. The dose of risedronate was set in accordance with previously published data [28]. Throughout the study, all HX rats (with or without risedronate and/or vitamin K2 treatment) were treated with implanted pellets of corticosterone (100 mg/pellet) and implanted pellets of L-thyroxine (15 mg/pellet). HX rats were also given access to 3% sucrose water ad libitum, while the intact pituitary rats were given normal tap water ad libitum. All animals were housed in the local vivarium at a temperature of 23.8°C with a 12-h light/dark cycle, and
were allowed free access to a standard pellet diet that contained 0.93% calcium, 0.65% phosphorus, and 3 IU/g of vitamin D3 (Robert Laboratory Chow 5001, Ralston Purina, Madison, WI, USA). The body weight of the rats was monitored weekly and the dosing period was 4 weeks. Successful HX was confirmed at the end of the study by observing a significant reduction of the serum insulin-like growth factor (IGF)-I level and the daily longitudinal growth rate (LGR/day) of the proximal tibial metaphysis. Two rats in the HX group and one rat in the HX + risedronate group were excluded from the study because there was no significant difference of serum IGF-I and LGR/day compared with intact controls, indicating the failure of HX. The study was carried out at Winthrop University Hospital, and the animals were maintained according to the National Institutes of Health (NIH) Guidelines for Care and Use of Laboratory Animals. The animal protocol was approved by the Laboratory Animal Care Committee of Winthrop University Hospital.

Preparation of specimens

The animals were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (12 mg/kg), and were sacrificed by exsanguination. The serum, the right femur and the right tibia were collected.

Serum was stored at –20°C until it was subjected to analysis as described below. The femur was used for the measurement of bone length, bone area, bone mineral content (BMC) and bone mineral density (BMD), as described below. The tibia was used for the measurement of bone length and for static and dynamic bone histomorphometric analyses. The bones were fixed overnight in 40% cold ethanol, and then cut into three parts using an Isomet saw (Buehler, Lake Bluff, IL, USA). The proximal tibial metaphysis and tibial diaphysis with the tibiofibular junction were stained with Villanueva Osteochrome Bone Stain (Polyscience, Warrington, PA, USA) for 5 days. Then the specimens were dehydrated in an ethanol (70%, 95%, and 100%) and xylene series and embedded in methyl methacrylate (EM Science, Gibbstown, NJ, USA) at 4°C according to the method of Erben [7]. Cross-sections of the tibial diaphysis just proximal to the tibiofibular junction were cut at a thickness of 40 μm using a diamond wire Histo-Saw machine (Delaware Diamond Knives, Wilmington, DE, USA), and the thickness of each section was confirmed with an Inspectors’ Dial Bench Gauge (L.S. Starrett, Athol, MA, USA). Frontal sections of the proximal tibial metaphysis were cut at a thickness of 5 μm using a microtome (Leica RM2155; Leica Inc., Nussloch, Germany), transferred to chromium/gelatin-coated slides, dried overnight under pressure at 42°C, and coverslipped with Eukitt mounting medium (Calibrated Instruments, Hawthorne, NY, USA) for static histomorphometric analysis.

Histomorphometric analysis of the tibia

A digitizing morphometric system was used to measure bone histomorphometric parameters. The system consisted of an epifluorescence microscope (Nikon E-400, OsteoMetrics, Atlanta, GA, USA), an Osteomeasure High Resolution Color Subsystem (OsteoMetrics, Atlanta, GA, USA) coupled to an IBM computer, and a morphometry program (OsteoMetrics, Atlanta, GA, USA). The parameters of cancellous bone measured were the total tissue volume (TV) and bone volume (BV), which were used to calculate the cancellous bone volume (BV/TV) in accordance with the method of Parfitt et al. [32]. In the present study, the region of cancellous bone measured was located 1–4 mm distal to the lower margin of the growth plate in the proximal tibial metaphysis, which consists of secondary spongy bone. The parameters of cortical bone measured were the total tissue area (Tt Ar) and cortical bone area (Ct Ar), which were used to calculate the marrow area (Ma Ar).

Femoral bone area, BMC, and BMD

After the bone length of the right femur was measured with a dial caliper, the bone area, BMC, and BMD of the whole right femur were determined by dual energy X-ray absorptiometry (DXA) using a Hologic QDR-4500 A with Delphi upgrade (Hologic Inc., Bedford, MA, USA). The instrument was set in the ultra-resolution mode, with a line spacing of 0.0254 cm, a resolution of 0.0127 cm, and a collimator of 0.9 cm in diameter. The bone was placed in a Petri dish, and tap water was added to a depth of 1 cm to simulate soft-tissue density. After the BMC and bone area were measured, the BMD was calculated as BMC divided by bone area. The coefficient of variation of these measurements at our laboratory was less than 1.0% [33].
Serum biochemistry
Serum levels of insulin-like growth factor (IGF)-I were measured with a commercial RIA kit after acid-ethanol precipitation (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). Serum total alkaline phosphatase (ALP) levels were measured according to the method of Hoffmann et al. [14]. Serum levels of pyridinoline were measured by radioimmunoassay using a commercial kit that was specific for rats (Diagnostic Systems Laboratories Inc., Webster TX, USA).

Statistical analysis
Data are expressed as the mean and standard deviation (SD). Multiple comparisons among the groups were performed by analysis of variance (ANOVA) with the Tukey-Kramer test. All statistical analyses were performed using the Stat View J-5.0 program on a Macintosh computer, and P<0.05 was taken as the level of significance.

Results

Effects of HX
HX resulted in loss of weight (corporeal growth) and a decrease of tibial length, femoral length, femoral bone area, BMC and BMD compared with the intact controls (Fig. 1). HX also resulted in a marked reduction of the serum IGF-I level and an increase in the serum levels of bone turnover markers (ALP and pyridinoline) compared with the intact controls (Fig. 2). Furthermore, HX caused the loss of cancellous bone mass (BV/TV) and cortical bone mass (Tt Ar and Ct Ar) compared with the intact controls, although there was no significant change of Ma Ar (Fig. 3).

Effect of vitamin K2 in HX rats
Administration of vitamin K2 did not alter the body weight, tibial length, femoral length, femoral bone area, BMC and BMD, or the serum IGF-I and ALP levels, but it ameliorated the decrease of femoral BMD compared with that in untreated HX rats (Figs. 1 and 2). However, risedronate prevented elevation of the serum pyridinoline level in HX rats (Fig. 2). Risedronate ameliorated HX-induced loss of cancellous bone (BV/TV), but did not affect cortical bone mass (Tt Ar and Ct Ar) compared with that in the intact controls and untreated HX rats (Fig. 3).

Effect of vitamin K2 + risedronate in HX rats
Combined administration of vitamin K2 and risedronate had an additive effect on femoral BMC and BMD in HX rats compared with either agent alone (Fig. 1). Femoral bone area was greater in HX rats treated with vitamin K2 plus risedronate than in HX rats given risedronate alone (Fig. 1). Combined administration of vitamin K2 and risedronate reduced the serum pyridinoline level compared with that in untreated rats and increased serum ALP compared with that in untreated rats given risedronate alone. Furthermore, cortical bone mass (Tt Ar and Ct Ar) was not decreased by combined treatment with vitamin K2 and risedronate compared with that in untreated HX rats (Fig. 3).

Discussion

This study was performed to clarify the effects of vitamin K2 and risedronate on long bone mass in HX rats, and to determine whether combined treatment with these two agents has a more beneficial effect on long bone mass in HX rats than treatment with either agent alone. Risedronate had a positive effect on BMD and on the cancellous bone mass of long bones in HX rats, while vitamin K2 did not have any significant effect on bone mass parameters. Vitamin K2 plus risedronate had an additive effect on BMC, BMD and the cortical bone mass of long bones in HX rats.

The serum levels of ALP and pyridinoline were increased after HX, suggesting elevation of bone turnover. Thus, not only anabolic agents but also antiresorptive agents are expected to be useful for at least ameliorating the decrease of long bone mass in HX rats.

Vitamin K2 appears to regulate both bone formation and bone resorption. This has been supported by numerous studies using various rat models of osteopenia created by ovariectomy, orchidectomy, sciatic neurec-
Fig. 1. Final body weight, bone length, femoral bone area, BMC and BMD. Data are expressed as mean ± SD. ANOVA with the Tukey-Kramer test was used to compare the data among the groups. a: significant vs Intact; b: significant vs HX; c: significant vs HX+K₂; d: significant vs HX+Ris. HX: hypophysectomy; K₂: vitamin K₂; Ris: risedronate; BMC: bone mineral content; BMD: bone mineral density.

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tomy, tail suspension, calcium deficiency and magnesium deficiency [1, 2, 10, 12, 17, 19, 20, 22–26, 29, 37, 38]. In general, however, the effect of vitamin K₂ on bone mass seems to be modest. In the present study, vitamin K₂ did not have any significant effect on bone mass parameters in HX rats, being overwhelmed by HX-induced cessation of corporeal and bone growth and the marked loss of long bone mass. However, vitamin K₂ increased the serum ALP level independently of pituitary hormones, suggesting an anabolic effect of vitamin K₂ on the bone in HX rats, although no significant change of bone mass was observed. Thus, more potent anabolic agents such as growth hormone and prostaglandin E₂ or possibly potent antiresorptive agents like bisphosphonates may be needed to overcome the reduction of long bone length or mass in HX rats.

Risedronate is a potent antiresorptive agent that is known to inhibit osteoclast-mediated bone resorption [9]. Several preclinical studies have demonstrated the efficacy of risedronate for preventing cancellous bone loss in rat models of osteopenia. Risedronate suppresses bone resorption and prevents cancellous bone loss in ovariectomized rats [8, 31], while it prevents loss of cancellous bone mass, bone density and bone strength without affecting cortical bone parameters in sciatic neurectomy rats or hindlimb-immobilized rats [18, 30]. The absence of any adverse effect of risedronate on longitudinal and radial bone growth has been confirmed [18]. In the present study, risedronate had a positive effect on the BMD and the cancellous bone mass of long bones without affecting longitudinal bone growth in HX rats. Thus, risedronate may have a positive influence on cancellous bone in rat models of osteopenia, a conclusion that is supported by evidence suggesting that osteoclasts located on the trabecular surface (cancellous bone) may be more responsive to
Fig. 2. Serum biochemical markers. Data are expressed as mean ± SD. ANOVA with the Tukey-Kramer test was used to compare the data among the groups. a: significant vs Intact; b: significant vs HX; c: significant vs HX+K2; d: significant vs HX+Ris. HX: hypophysectomy; K2: vitamin K2; Ris: risedronate; IGF-I: insulin-like growth factor; ALP: alkaline phosphatase.

Fig. 3. Histomorphometric analysis of cancellous and cortical bone of the tibia -Structural parameters-. Histomorphometric analysis was performed for cancellous bone of the proximal tibial metaphysis and cortical bone of the tibial diaphysis. Data are expressed as mean ± SD. ANOVA with the Tukey-Kramer test was used to compare the data among the groups. a: significant vs Intact; b: significant vs HX; c: significant vs HX+K2. HX: hypophysectomy; K2: vitamin K2; Ris: risedronate; BV/TV: bone volume/total tissue volume; Tt Ar: total tissue area; Ct Ar: cortical area; Ma Ar: marrow area.
bisphosphonates than those residing on the endocortical surface (cortical bone) [3].

A beneficial effect of combined treatment with vitamin K$_2$ and bisphosphonates on cancellous bone mass or bone structure has been reported in ovariectomized rats or tail-suspended rats [15, 21]. The present study showed that vitamin K$_2$ had an additive effect on that of risedronate on femoral bone area, BMC, BMD and cortical bone mass of the tibial diaphysis; i.e., it promoted radial growth of long bones in HX rats. This combined effect was attributable to the suppression of bone resorption and stimulation of bone formation by vitamin K$_2$ in HX rats treated with risedronate. Thus, vitamin K$_2$ may improve bone metabolism in various conditions. We demonstrated that combined treatment with vitamin K$_2$ and risedronate had a more beneficial effect on long bone mass than either agent alone in HX rats.

Risedronate or vitamin K$_2$ alone did not prevent the HX-induced decrease of corporeal growth (body weight), longitudinal bone growth (tibial and femoral length), and radial bone growth (Tt Ar). Thus, even though risedronate with or without vitamin K$_2$ had a positive skeletal effect in HX rats, agents that can further stimulate corporeal and bone growth may be needed to prevent HX-related cessation of skeletal growth.

The present study examined the effect of vitamin K$_2$ and/or risedronate on bone length, BMC and BMD of the long bones, as well as bone turnover markers, in HX rats. Histomorphometric analysis has indicated that HX induces cancellous bone loss in young rats as a result of suppression of longitudinal growth-dependent increase of bone mass as well as a reduction in tissue-based bone formation rate (BFR/TV) as an index of bone turnover. HX also reduces the radial growth-related increase of bone mass [4, 39]. Thus, there seems to be a discrepancy between serum bone markers and bone histomorphometric parameters. This is probably because metabolism in localized areas of cortical and cancellous bone in the tibia is not always correlated with overall skeletal metabolism as evaluated by bone markers. Further studies are needed to confirm the effect of these agents on various bone turnover markers and histomorphometric bone formation and resorption parameters in HX rats.

In conclusion, the present study has confirmed that risedronate has a positive effect on BMD and cancellous bone mass of long bones in HX rats. Also, despite having no significant effect on bone mass parameters, vitamin K$_2$ had an additive effect to risedronate on the BMC, BMD and cortical bone mass of long bones in HX rats.

References


