Skeletal Effects of Parathyroid Hormone (1-34) in Ovariectomized Rats With or Without Concurrent Administration of Salmon Calcitonin

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ABSTRACT This study evaluated the effect of parathyroid hormone (PTH) infusion alone or in combination with salmon calcitonin (sCT) in ovariectomized (OVX) rats and compared it with daily PTH injections alone or in combination with sCT infusion. Female Sprague-Dawley rats were divided randomly into 6 groups and were either bilaterally ovariectomized or underwent a sham operation; they were then treated for 4 weeks, beginning the day after surgery. Each group of OVX rats received either PTH infusion (group 1), PTH + sCT infusion (group 2), sCT infusion + daily PTH injection (group 3), or daily PTH injection (group 4). One group each of OVX (group 5) and sham-operated rats (group 6) received daily injections of vehicle alone. PTH was injected at 80 µg/kg/day and infused at 40 µg/kg/day, whereas sCT was infused at 10 µg/kg/day. The animals were sacrificed 28 days after treatment, and cancellous bone volume was measured in the tibial metaphysis. Similar to daily PTH injections, continuous infusion of PTH alone increased cancellous bone volume significantly over that seen in vehicle-treated OVX and sham-operated rats. Although cancellous bone volume after continuous infusion of PTH + sCT was also significantly higher than that seen in vehicle-treated OVX and sham-operated rats, the increase was significantly lower than with the other 3 nonvehicle treatments. The increase in cancellous bone volume after administration of sCT infusion along with daily PTH injections was not different from that with daily PTH injections alone. Thus, at the doses tested, the beneficial effects of PTH injection were not apparently improved by PTH infusion or by combination with sCT.

KEYWORDS: Salmon calcitonin, human parathyroid hormone (1-34), infusion, ovariectomized rats, cancellous bone volume

INTRODUCTION

Parathyroid hormone (1-34) (PTH), when administered as a daily injection, stimulates bone growth in various species, including osteoporotic women¹⁻⁶. However, continuous administration of PTH as an infusion in rats with intact ovarian function has been repeatedly shown to result in a catabolic response in the skeleton⁶⁻¹⁰. The ovariectomized (OVX) rat model has been used to investigate the effects of antiresorptive agents as well as PTH fragments on bone¹¹⁻¹⁵. This model combines the easy manageability of rats with the main features of postmenopausal osteoporosis in women^{5, 14,15}. Bone changes in rats and women during the early stages of estrogen deficiency are qualitatively similar, both exhibiting rapid cancellous bone loss associated with increased bone turnover^{11-13, 16-20}. The OVX rat is also 1 of the 2 models required by the U.S. Food and Drug Administration for the preclinical assessment of drugs for treating osteoporosis²¹. Morley and colleagues²² reported a catabolic effect of PTH infusion on the skeleton in OVX rats, while Shen and colleagues²³ and Zhou and colleagues²⁴ showed PTH infusion, with or without estrogen repletion, to restore cancellous bone volume and architecture in the OVX rats. These latter researchers further showed that estrogen repletion enhanced bone volume to values substantially higher than that seen in sham-operated controls (normal rats). Also, infusion of PTH along with estrogen repletion in OVX rats resulted in significant improvements of bone mineral density (BMD) comparable to that seen in sham-operated rats, unlike PTH infusion alone, which resulted in loss of BMD. This finding raised the possibility that a continuous infusion of PTH could exert anabolic effects on skeletal tissue if its catabolic component could be minimized by coadministration of an antiresorptive agent such as salmon calcitonin (sCT).

The effect of estrogen repletion along with PTH infusion has been studied only in the late estrogen-deficiency stage and has been found to increase

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cancellous bone volume. Our study focused on the early stages of estrogen deficiency (because of both interest and time constraints) and the effects of concomitant administration of sCT, a recognized antiresorptive agent. This does not preclude a companion or follow-up study focusing on the late stages of estrogen deficiency, but that was not the intent of this research. The main objective of our study was to evaluate the effect of PTH infusion either alone or in combination with sCT in OVX rats and compare it with the effect of daily PTH injections. Also, it was desirable to compare these effects with the effect of a combination of sCT infusion and daily PTH injections. Alzet osmotic minipumps (Alza Corporation, Palo Alto, CA) were used to administer all the infusions.

MATERIALS AND METHODS

Materials

Salmon calcitonin and PTH (1-34) were purchased from Bachem, Inc (Torrance, CA). Alzet osmotic pumps were purchased from Alza Corporation (Palo Alto, CA). Female Sprague-Dawley rats that weighed approximately 225 g and were 90 days old were purchased from Harlan Labs (Indianapolis, IN), and the studies were conducted at the University of Kentucky College of Pharmacy Animal Research Facility in accordance with the institutional guidelines.

In vivo evaluation of Alzet osmotic pumps

The Alzet osmotic pumps were evaluated in vivo for their ability to maintain elevated serum PTH and sCT levels. Five female Sprague-Dawley rats that weighed approximately 225 g and were 90 days old were lightly anesthetized using ether. The rats were then implanted with Alzet osmotic pumps subcutaneously at the back of the neck. The pumps were filled with a PTH and sCT stock solution in 0.1 M acetate buffer, pH 4.0. The pumps had been precalibrated to infuse at a rate of 0.25 μ L/h for 28 days. All implantations were accomplished within 2 minutes in each rat. Blood samples were collected from the tail vein under

light ether anesthesia at 0, 2, 6, 9, 14, 20, and 28 days after treatment. The samples were centrifuged in Microtainer tubes (Becton Dickinson, Franklin Lakes, NJ), and serum was collected. Serum samples were frozen and stored at -20°C until analysis.

Serum analyses

Serum sCT and PTH levels were measured by ¹²⁵I radioimmunoassay with commercially available kits (Peninsula Laboratories, San Carlos, CA). The sCT antiserum supplied by Peninsula has a 100% cross-reactivity with sCT, and the PTH (1-34) antiserum has a 100% cross-reactivity with PTH (1-34). The sensitivity of the assay for both sCT and PTH was 10 pg/mL. In general, serum samples were incubated separately with rabbit peptide (sCT and PTH) antiserum for 24 hours at 4°C, followed by addition of ¹²⁵I-labeled peptide. After an additional 24-hour incubation, a second antibody, goat antirabbit immunoglobulin G, was added, followed by 90-minute incubation at room temperature to separate the antibody-bound and free peptide. The antibody-bound radioactivity was then measured by a MINAXI γ -counter (Packard, Downers Grove, IL).

Skeletal effects of PTH with or without sCT

Female Sprague-Dawley rats (n = 43) were anesthetized with an intraperitoneal injection of ketamine hydrochloride and xylazine at doses of 50 and 10 mg/kg body weight (BW), respectively. Ketamine hydrochloride was supplied as Ketaset (Fort Dodge Animal Health, Fort Dodge, IA), and xylazine hydrochloride was purchased from Sigma Chemical Co (St Louis, MO). Bilateral ovariectomies were performed in 36 rats from a dorsal approach. The remaining 7 rats were subjected to sham surgeries in which the ovaries were exteriorized but not removed. Beginning the day after surgery, the OVX and sham-operated (control) rats were treated for 4 weeks according to the regimens described in **Table 1**. The investigator was not blinded to the treatments. PTH and sCT were infused using Alzet osmotic pumps.

Table 1. Treatment Regimens of PTH Injection or Infusion to OVX and Sham-Operated Rats With or Without Concurrent Infusion of sCT

Group	n	Description	Dose (μ g/kg/day)
1	7	OVX + PTH infusion	40
2	8	OVX + PTH infusion + sCT infusion	PTH 40, sCT 10
3	6	OVX + PTH injection daily + sCT infusion	PTH 80, sCT 10
4	7	OVX + PTH injection daily	80
5	8	OVX + vehicle daily	0.0
6	7	Sham + vehicle daily	0.0

PTH indicates parathyroid hormone; OVX₂ ovariectomized; sCT, salmon calcitonin.

The pumps were filled with PTH or sCT stock solutions prepared in 0.1 M acetate buffer, pH 4.0, and implanted subcutaneously at the back of the neck. The pumps were precalibrated to infuse at a rate of 0.25 μ L/h for 28 days. PTH was dissolved in 0.9% saline and was administered subcutaneously every day. Groups 5 (OVX, untreated rats) and 6 (sham-operated controls) received subcutaneous vehicle injections of 0.9% saline daily. Food was available ad libitum to all the rats. The BW of rats was measured at 0, 4, 7, 21, and 28 days after starting the treatments. The rats were sacrificed after 28 days of treatment by exsanguination under carbon dioxide.

Figure 1. Serum PTH levels after 4 weeks of infusion of 40 μ g/kg/day of PTH in female Sprague-Dawley rats using Alzet osmotic pumps ($n = 5$). The zero time average is the average serum PTH level before the treatments were started.

Figure 2. Serum sCT levels after 4 weeks of infusion of 10 μ g/kg/day of sCT in female Sprague-Dawley rats using Alzet osmotic pumps ($n = 5$). The zero time average is the average serum sCT level before the treatments were started.

The success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns. Estrogen levels were not measured because previous studies had shown that the success of ovariectomy can be confirmed at necropsy by failure to detect the ovarian tissue and by the observation of marked atrophy of the uterine horns^{25,26}. After necropsy, the tibiae were isolated by cutting through the knee joint and the ankle joint with a scalpel. The tibiae were stripped of musculature and cut in cross-section midway between their proximal and distal ends to expose the marrow cavity with a hand-held saw. Both sections were placed in glass scintillation vials (25 mL) containing 10% phosphate buffered formalin (pH 7.0-7.4) for fixation. After 24 hours of incubation at 4°C, the bone samples were rinsed with and stored in 70% ethanol.

Bone analyses

The bone samples were dehydrated in ethanol and embedded undecalcified in methyl methacrylate. Longitudinal sections (4 μ m) were stained according to the Von Kossa method with a tetrachrome counterstain (Polysciences Inc, Warrington, PA). Bone measurements were performed in the cancellous bone tissue of the proximal tibial metaphysis at distances greater than 1 mm from the growth plate-metaphyseal junction to exclude the primary spongiosa. In general, 2 sections of the proximal tibia with 40 to 50 mm cancellous bone perimeter were sampled in each animal (ie, there were 2 measurements per animal).

Bone measurements were performed with the Bioquant Bone Morphometry System (R & M Biometrics Corp, Nashville, TN). Cancellous bone volume as a percentage of bone tissue area was measured.

Statistical analyses

Results for body weights and percent cancellous bone volumes were first analyzed for statistical significance using one-way analysis of variance. When significant differences were observed ($p < .05$), Scheffe's test was used for multiple comparisons.

RESULTS

Figures 1 and 2 show serum PTH and sCT levels after infusion of 40 and 10 μ g/kg/day of each, respectively, for 28 days. Elevated serum PTH and

sCT levels were maintained during the course of infusion. The average serum PTH and sCT levels during 28 days of infusion were 135 ± 31 and 321 ± 54 pg/mL, respectively. The zero time averages for PTH and sCT were 25.3 and 17.7 pg/mL, respectively. These values are comparable to the zero time values ranging between 10 and 30 pg/mL that have been previously reported^{6, 25}.

The BW for the 6 groups of rats is shown in **Figure 3**. The BW of sham-operated control rats remained fairly constant over the 28 days, gaining only 6% weight during the course of the study (260.5 ± 6.0 g at day 0 vs. 275.7 ± 6.8 g at day 28). Vehicle-treated OVX rats gained 22% weight (270.8 ± 6.9 g at day 0 vs. 329.5 ± 9.4 g at day 28) and weighed significantly more (19%) than control rats after 28 days of treatment. Rats that underwent sCT infusion with either concurrent PTH infusion or daily PTH injection exhibited decreased BW during the first 4 days. However, after the initial decrease, BW increased steadily over the course of the study. Nevertheless, the average values after 28 days of sCT infusion with either concurrent PTH infusion (289.2 ± 12.7 g) or daily PTH injection (298 ± 13.2 g) were significantly lower (12% and 10%, respectively) than the average values for the vehicle-treated OVX group. The BWs of groups treated with daily injection and continuous infusion of PTH increased similarly (17% and 23%, respectively) to those of the vehicle-treated OVX group (22%) and were significantly higher than those of control rats after 28 days of treatment.

Figure 3. Effect of administration of PTH either continuously or intermittently (daily injections) with or without continuous infusion of sCT on body weights of ovariectomized rats. 1. PTH infusion alone. 2. PTH + sCT infusion. 3. sCT infusion + daily PTH injection. 4. Daily PTH injection. 5. Vehicle. 6. Vehicle + sham-operated rats. The number of rats in each group is reported in Table 1.

Bone histomorphometric data are shown in **Figure 4**. Cancellous bone volume was 35% lower in vehicle-treated OVX rats, although it was not statistically different from that of vehicle-treated control rats ($14.0 \pm 1.8\%$ vs. $21.5 \pm 5.4\%$). Cancellous bone volume of rats injected with 80μ g/kg of PTH alone daily ($46.3 \pm 8.1\%$) was significantly higher than that of vehicle-treated OVX and control rats (231% and 115% higher, respectively). Infusing PTH alone at a dose of 40μ g/kg also increased the cancellous bone volume ($49.8 \pm 7.6\%$) significantly compared to vehicle-treated OVX and control rats (256% and 132% more, respectively). However, this increase was not significantly different from that with daily injection of PTH alone. A treatment regimen of sCT infusion at a dose of 10μ g/kg/day along with daily injections of PTH at a dose of 80μ g/kg caused the highest (statistically significant) increase in cancellous bone volume ($51.8 \pm 7.8\%$) over that seen in vehicle-treated OVX and control rats (270% and 141% more, respectively). This increase was not significantly different from the increase after PTH treatments alone either as an infusion or a daily injection. Combining PTH and sCT as an infusion at 40 and 10μ g/kg/day doses, respectively, caused the smallest increase in cancellous bone volume ($35.0 \pm 9.8\%$) compared with that in vehicle-treated OVX and control rats (150% and 63% more, respectively). This increase in cancellous bone volume, though significantly higher than that in the vehicle-treated OVX and control groups, was significantly lower than that seen after the other 3 nonvehicle treatments.

Figure 4. Effect of administration of PTH either continuously or intermittently (daily injections) with or without continuous infusion of sCT on cancellous bone volume of ovariectomized rats. a indicates results significantly different from those of group 5; b, significantly different from group 6; c, significantly different from group 2. The number of rats in each group is reported in Table 1.

DISCUSSION

Elevated serum PTH and sCT levels were maintained for 28 days after subcutaneous implantation of Alzet osmotic pumps. These observations provided strong evidence for the successful delivery of PTH and sCT over 4 weeks. There was a significant increase in body weight associated with ovariectomy, which was consistent with previous work²⁷. Continuous sCT infusion either with concurrent PTH infusion or daily PTH injection significantly lessened the increase in body weight when compared to the BW increase in vehicle-treated OVX rats. The anorexic effect of sCT in rats is already well known^{28,29}. PTH as an infusion or as a daily injection resulted in a significant increase in BW over that of sham-operated controls and a similar increase in BW over vehicle treated OVX rats. Thus, unlike sCT administration, PTH administration as an infusion or daily injection did not have an anorexic effect on rats. Although the vehicle-treated OVX rats lost 35% more bone than control rats did, this difference was not statistically significant. The vehicle-treated OVX rats gained 16% more weight than did the control rats during the course of the study. In a previous study, Wronski and colleagues²⁷ showed that bone loss is compensated for through weight gain in OVX rats. In their study, these researchers found that all OVX rats lost bone significantly as compared to control rats 14 weeks after ovariectomy ($p < .001$). However, OVX rats that weighed a similar amount as the control rats lost more bone than did obese OVX rats that weighed much more than control rats (statistically significant at $p < .02$). In our study, the statistically insignificant decrease in cancellous bone volume in OVX rats treated with vehicle as compared to control rats could be due to this effect of body weight on osteopenia in OVX rats. Statistically significant bone loss 14 weeks after ovariectomy and significant cancellous bone loss as early as 6 weeks after ovariectomy have been reported in OVX rats treated with vehicle as compared to vehicle-treated controls^{25,26}. Nevertheless, in our study, cancellous bone volume was 35% lower in vehicle-treated OVX rats than in control rats.

As expected, daily PTH injections at a dose of 80 μ g/kg/day increased cancellous bone volume significantly over that of vehicle-treated OVX and control rats. However, similar to daily PTH injection,

continuous infusion of PTH alone at a dose of 40 μ g/kg/day also significantly increased cancellous bone volume over that of vehicle-treated OVX and control rats. A daily PTH dose of 80 μ g/kg is known to be anabolic to the rat skeleton without resulting in hypercalcemia⁶. However, this dose has been shown to be too high when administered as a continuous infusion, resulting in severe hypercalcemia and mortality in rats⁶. Hence, a lower dose (40 μ g/kg/day) was administered as a continuous infusion because even though this dose results in increased calcium levels, it seems to reduce the risk of life-threatening hypercalcemia in rats⁶. The significant increase in cancellous bone volume after PTH infusion alone is interesting because a previous study in OVX rats had shown that cancellous bone volume did not increase when PTH was infused alone at doses of 7.2 and 14.8 μ g/kg/day²². However, Zhou and colleagues²⁴ also reported an increase in cancellous bone volume in OVX rats after infusion of PTH alone at a dose of 30 μ g/kg/day similar to the increase seen in vehicle-treated OVX rats. In our study, however, the cancellous bone volume after infusion of PTH alone in OVX rats was significantly higher than even that of vehicle-treated control rats, unlike as observed by Zhou. The higher dose of PTH infused in our study (40 μ g/kg/day), as opposed to 30 μ g/kg/day infused by Zhou, might account for this difference. Also, these researchers reported no evidence of any deleterious effect on cortical width resulting from continuous PTH treatment alone at a dose comparable to that administered in our study. However, it is prudent to emphasize that, because a loss of bone mineral density and increased cortical porosity has been reported by Shen²³ and Zhou²⁴ after infusion of PTH alone, further studies are necessary to determine whether the bone resulting from such treatment is of normal architecture and strength. The studies of Shen and Zhou also indicate that there is a significant increase in serum calcium level well beyond the normal physiological range at such doses. However, the possible changes in calcium regulation were not evaluated in our study.

Continuously infusing sCT along with daily PTH injections also increased cancellous bone volume significantly over that of vehicle-treated OVX and control rats. However, the effect was not different from the effect of daily injections of PTH alone. This indicates that combining a 4-week sustained release

sCT formulation (such as sCT microspheres) with daily PTH injections would not result in more cancellous bone volume than would daily injections of PTH alone.

A concurrent infusion of PTH and sCT produced the least increase in cancellous bone volume as compared to the other 3 nonvehicle treatments. Although cancellous bone volume after concurrent PTH and sCT infusion was significantly lower than that seen with the other 3 nonvehicle treatments, it was still significantly higher than that of the vehicle-treated OVX and control groups. This is significant because combining PTH infusion with another antiresorptive agent such as estrogen has been shown to form bone, with bone mineral density and mechanical strength comparable to that of bone in normal rats²³.

CONCLUSION

In conclusion, the data presented here suggest that it might be possible to enhance bone formation by continuous administration of PTH either alone or in combination with an antiresorptive agent such as sCT. However, at the doses tested, these effects were not significantly better than those that can be achieved with PTH injection alone. Additional studies are warranted to determine whether the bone strength and architecture are compromised after such treatment.

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