

The effects of recombinant human parathyroid hormone, rhPTH(1–84), on bone mass in undernourished rats

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Abstract

Energy intake restriction reduces bone formation both in protein–energy malnourished children and in undernourished rats, and such conditions might cause partial or irreversible bone loss. Because the use of anti-resorptive agents in this situation is seemingly limited, we examined the effect of the anabolic agent, recombinant human parathyroid hormone (rhPTH(1–84)), on bones in undernourished conditions. First, the osteopenic changes of rat bones with 40% restricted diet for 4 weeks were confirmed. Subsequently, another set of the rats were randomized into four groups and studied for 8 weeks: the freely fed group (control group); the restricted diet, then freely fed group (restriction–*ad libitum* group); the restricted diet–vehicle-treated group (restriction–vehicle group); and the restricted diet–PTH-treated group (restriction–PTH group). In the restriction–vehicle group, total femoral bone mineral density (BMD) was lower and femoral length was shorter than the control group

by 15.4% and 8.1% respectively ($P < 0.05$). In the restriction–*ad libitum* group, these parameters recovered fully to those of the control group. In the case of intermittent PTH treatment in the persistent undernourished state, the BMD of total femur caught up with those of the control or the restriction–*ad libitum* group. However, the femoral length remained shorter than those of the other groups. Serum osteocalcin was significantly reduced in continuously undernourished rats, whereas it was elevated in the restriction–PTH group. In conclusion, BMD of total femur was low in undernourished rats. However, it increased after re-feeding *ad libitum* or intermittent PTH treatment. We suggest that rhPTH(1–84) may be a possible therapeutic agent for ongoing bone loss, especially in patients in a chronically undernourished condition.

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Introduction

Nowadays, simple undernourishment is not as common as it was in the past, though conditions inducing cachexia such as anorexia nervosa and cancer are hard to overlook. Moreover, the restriction of energy intake is known to reduce bone formation both in malnourished children and undernourished rats (Ndiaye *et al.* 1995a,b). The prevalence of low bone mass in anorexia nervosa is about 50%, and occurs relatively early. In untreated anorexia nervosa patients, approximately 4–10% of bone loss occurs each year (Maugars *et al.* 1996). Furthermore, the affected age of anorexia nervosa patients overlaps with the period of peak bone formation. The risk of lifetime fracture in anorexia nervosa patients is about 2.9 times the norm (Lucas *et al.* 1999).

The characteristic bone biochemical marker profiles in protein–energy malnourished children and in restricted-fed rats show a reduced level of bone formation marker

(Ndiaye *et al.* 1995a,b). Osteocalcin levels are also reduced in anorexia nervosa patients, and an elevation in the bone resorption markers, deoxypyridinoline (Dpd) or N-telopeptide (NTX) has been observed in anorexia nervosa (Grinspoon *et al.* 1996). Even after the administration of estrogen or the recovery of near normal body weight and menstruation, bone loss was only partially reversed or sometimes bone density is not recovered at all (Rigotti *et al.* 1991, Klibanski *et al.* 1995, Baker *et al.* 2000). Anti-resorptive agents such as estrogen and bisphosphonate, etc. have limited effects on bone changes related to anorexia nervosa (Klibanski *et al.* 1995). The effects of anabolic agents such as parathyroid hormone (PTH) have not been studied in anorexia nervosa. The aim in the present study was to determine whether the intermittent administration of PTH would be effective on bones in persistently undernourished state.

When PTH is administered as a constant infusion to normal animals or those in a disease state such as

hyperparathyroidism, where its level is constantly elevated, bone is lost, especially at cortical sites (Dobnig & Turner 1997). In contrast, when the hormone is treated in an intermittent fashion, it has been reported to strongly increase lumbar bone mineral density (BMD) both in the normal and the ovariectomized rats (Gunnesh-Hey & Hock 1984, Hock *et al.* 1988, Hori *et al.* 1988, Liu *et al.* 1991, Kimmel *et al.* 1993, Li *et al.* 1995).

Therefore, it is hypothesized that recombinant human parathyroid hormone, rhPTH(1–84), a strong anabolic agent, would restore bone loss related to malnourishment. To test the effects of rhPTH(1–84) on the bone, an undernourished osteopenic rat model was established and the resulting rats were treated with rhPTH(1–84) intermittently.

Materials and Methods

Animals and food intake

Ten-week-old virgin female Sprague–Dawley rats were purchased from the Department of Laboratory Animal Medicine at Yonsei Medical Research Center (Seoul, Korea). They were housed in a room maintained at $22 \pm 2^\circ\text{C}$ and on 12 h light : 12 h darkness cycles. The animals were fed in two different ways: a freely fed group and a group that was given 40% of the total amount of the food intake by the freely fed group. Purina laboratory rodent chow (Hagribland Purina Korea Co., Kunsan, Korea) that contained 1.17% calcium, 0.77% phosphorous, and 2.6 IU vitamin D per gram was used. The total daily food intake of the control group was 16.8 ± 0.3 g during the first 4 weeks and 17.1 ± 0.6 g during the last 4 weeks. Water was provided and animals allowed to drink *ad libitum*. All animals were treated in accordance with the guidelines and regulations for the use and care of animals of Yonsei University, Seoul, Korea.

Experimental protocols

The animals were weight-matched and divided into six groups when they were 11 weeks old. There were five to seven rats per group, and housed two rats per cage. The six groups were as follows: the 4-week control group, freely fed for 4 weeks and then sacrificed; the restriction group, a 40% restricted diet for 4 weeks prior to sacrifice; the 8-week control group where rats were freely fed for 8 weeks then sacrificed; the restriction–*ad libitum* group, fed on a 40% restricted diet for the first 4 weeks and then freely fed for another 4 weeks, then sacrificed; the restriction–vehicle group, a 40% restricted diet for the whole 8 weeks and treated with a vehicle during the last 4 weeks, then sacrificed; and the restriction–PTH group, a 40% restricted diet for 8 weeks and treated with rhPTH(1–84) during the last 4 weeks, then sacrificed.

Table 1 Experimental protocols. Words shown on the lines designate the time period where vehicle or PTH was given to the assigned groups. PTH, rhPTH(1–84) (150 µg/kg per day s.c.). *Ad libitum* diet is expressed with a bold line and 40% restricted diet with a dotted line

Groups	Time (weeks)		
	0	4	8
4 weeks			
<i>Ad libitum</i>	—————		
Restriction		
8 weeks			
<i>Ad libitum</i>	—————		
Restriction– <i>ad libitum</i>	—————
Restriction–vehicle		
Restriction–PTH		
		← Vehicle →	
		← PTH →	

rhPTH(1–84) was a gift from the Mogam biotechnology research institute (Kyungi, Korea), dissolved in 0.9% normal saline. The restriction–PTH group was treated daily for 5 days per week with intermittent subcutaneous injections of 150 µg/kg rhPTH(1–84) per day. The restriction–vehicle group was injected subcutaneously with an equivalent volume of 0.9% normal saline at the same frequency. The detailed protocol used is shown in Table 1. At the end of the fourth and eighth weeks of experimental period, the rats were sacrificed by exsanguinations from the abdominal aorta under ketamine/xylazine anesthesia. Urine was collected via metabolic cages over 24 h at timed intervals. Rat blood samples were taken at timed intervals. Both the collected urine and serum were stored at -20°C .

Measurement of bone length and density

The right femur lengths were measured using a Mitutoyo digital caliper (Cole Parmer, Vernon Hills, IL, USA). The femoral length was measured from the greater trochanter to intercondylar fossa. The BMD of the excised femurs was measured by dual energy X-ray absorptiometry (QDR–4500A, Hologic, Waltham, MA, USA). Triplicate determinations of the five different femurs with repositioning showed a coefficient of variation of 0.59%.

Osteocalcin and N-telopeptide assays

The osteocalcin concentration of the rat serum was measured using a rat osteocalcin IRMA assay kit (Immutopics Inc., San Clemente, CA, USA). The urinary cross-linked N-telopeptide of type 1 collagen (NTX) was measured by ELISA (Osteomark, Ostex International, Seattle, WA, USA). Intra-assay coefficients of variation

(CV) for rat osteocalcin were 2.0% at 1.5 ng/ml and 2.3% at 4.3 ng/ml, and the inter-assay CV was 4.0% at 4.0 ng/ml. The intra-assay CVs for NTX were 18% at 26 nM of bone collagen equivalents (BCE), 10% at 439 nM of BCE and 8% at 690 nM of BCE, and the inter-assay CV for NTX was 6% at 80 nM of BCE. Urine creatinine was measured by Jaffe's reaction using a Hitachi 747 autoanalyzer (Boehringer Mannheim, Mannheim, Germany). The intra-assay CV was less than 3.0% for the urine tests. The groups that were experimented on after 4 weeks were excluded in the measurement of the biochemical markers.

Cortisol and estradiol assays

The cortisol and estradiol concentration of rat serum were measured using a rat cortisol RIA assay kit (Beckman Coulter Inc., Marseille, France) and estradiol RIA assay kit (Beckman Coulter Inc.). Intra- and inter-assay CVs for rat cortisol were 2.2% at 407 nmol/l and 4.6% at 416.5 nmol/l respectively, and intra- and inter-assay CVs for estradiol were 7.2% at 64 pg/ml and 7.9% at 57 pg/ml. These parameters were only measured in rats killed after 4 weeks.

Statistical analysis

SAS software (6.12 for Windows; SAS, Cary, NC, USA) was used. The data were expressed as the means \pm s.d. The differences in the BMD and femoral length between the groups were compared using ANOVA with a Bonferroni's multiple group comparison procedure. The data on body weight, serum osteocalcin and NTX at the timed intervals were analyzed by repeated measures of the ANOVA method. The difference between the osteocalcin and NTX concentration were analyzed by the Kruskal–Wallis test and compared with the results of the Mann–Whitney *U* test. A *P* value of less than 0.05 was accepted as statistically significant.

Results

Changes of body weight

Even after 4 weeks of a restricted diet, body weight reduction was obvious. After 8 weeks of dietary control, the animals fed a continuously restricted diet showed 51% lower body weight than the freely fed control group ($P < 0.001$, Fig. 1). The initial reduction in body weight after the restricted diet was recovered to the levels of those of the control group after 4 weeks of free feeding.

Rat cortisol and estradiol concentrations

Rat cortisol concentrations were significantly reduced after 4 weeks of restricted diet ($P < 0.05$), but there were no

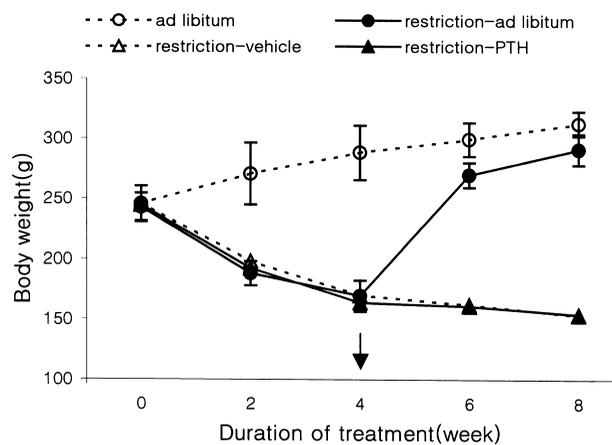


Figure 1 Effects of dietary control and PTH treatment on body weight. The rats were 11 weeks old when the study started. Values are presented as means \pm s.d. Two groups with restricted diet were injected with vehicle and rhPTH(1–84) respectively from the 4th week (arrow) to the 8th week. $P < 0.001$, by repeated measures ANOVA.

significant differences in the estradiol levels of freely fed and undernourished rats (data not shown).

Femoral length

The femoral lengths after 8 weeks in the groups that had been provided with a 40% restricted diet were significantly shorter than in the control group, and also shorter than those of the restriction–*ad libitum* group (Fig. 2 and Table 2). Four weeks of restricted diet was found to result in a reduced femoral length. The femoral length reduction was reversed after feeding *ad libitum* for a period of 4 weeks to a similar length as that of the control group. However, the administration of rhPTH(1–84) had no effect on the femoral length.

BMD of right femur

BMD decreased after 4 weeks of a restricted diet, but this was without statistical significance (data not shown), however, after 8 weeks the BMD was significantly lower than in the freely fed group ($P < 0.05$, Fig. 3 and Table 2). The BMD was significantly increased to the level of the control group in the restriction–PTH group, and also in the restriction–*ad libitum* group ($P < 0.05$, Fig. 3 and Table 2). The body weight was positively correlated with the BMD ($r = 0.678$; $P < 0.0001$).

Serum osteocalcin

Serum osteocalcin concentrations tend to decrease with increasing age in all groups except the restriction–PTH group (Table 3). In the restriction–PTH group, the serum

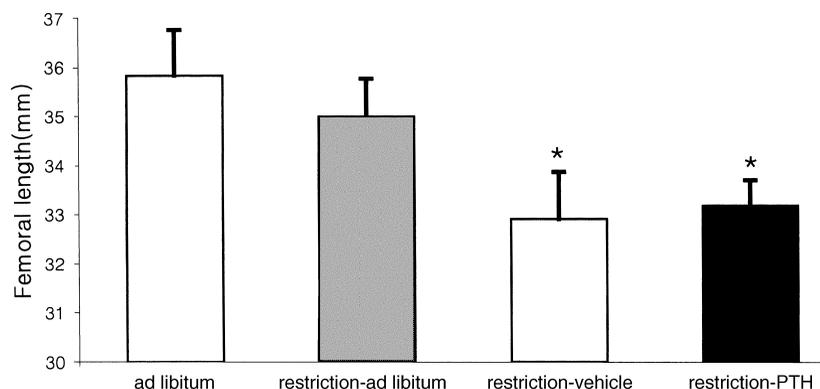


Figure 2 Effects of dietary control and PTH treatment on right femoral length at the 8th week. Each bar represents the mean \pm s.d. * $P < 0.01$ vs *ad libitum* group or *restriction-ad libitum* group.

osteocalcin concentration at the eighth week was increased significantly compared with those of the restriction-vehicle group ($P < 0.05$, Table 3). In the restriction-*ad libitum* group, the serum osteocalcin concentration showed slight increasing tendency without statistical significance.

NTX levels in 24-h urine

No differences in the NTX levels in the 24-h urine were found among the groups (Table 4).

Discussion

In this study, it was found that a restricted diet in growing rats significantly reduced the body weight, linear growth and bone formation, and led to low BMD. PTH treatment significantly improved the BMD and markedly increased the serum osteocalcin levels even in the group that was maintained on a restricted diet. In the group with nutritional recovery, both the BMD and the body weight recovered significantly.

Undernourishment affects the bone metabolism detrimentally. Data on energy-protein malnourished children or undernourished rats has shown that the bone formation marker osteocalcin is present at reduced levels (Ndiaye *et al.* 1995a,b). The calcium content of rat bones that have suffered food restriction of 60% was significantly decreased (Kalu *et al.* 1988). The analysis of histomorphometric data has also shown decreased mineral apposition rate in the bones of rats fed on energy-restricted diets (Ndiaye *et al.* 1995a). More astonishingly, anorexia nervosa leads to partially reversible or sometimes irreversible bone loss, even after recovering the body weight to a normal level and the resumption of menstruation, or even after hormonal replacement therapy (Rigotti *et al.* 1991, Klibanski *et al.* 1995, Baker *et al.* 2000).

The exact mechanism of bone loss in malnourishment is not clear. However, several plausible factors have been proposed. The synthesis of insulin-like growth factor-I (IGF-I) in the bone as well as in the liver has been found to be significantly reduced in patients with anorexia nervosa (Canalis *et al.* 1989, Counts *et al.* 1992, Soyka *et al.* 1999). Especially in anorexia nervosa, estrogen deficiency

Table 2 Effects of dietary control and PTH treatment on femoral length and density. Values are means \pm s.d.

Parameters	8 weeks			
	<i>Ad libitum</i>	Restriction- <i>ad libitum</i>	Restriction-vehicle	Restriction-PTH
<i>n</i>	6	6	6	6
Femoral length (mm)	35.83 \pm 0.93	34.99 \pm 0.77	32.90 \pm 0.96 ^a	33.17 \pm 0.52 ^a
BMC (g)	0.479 \pm 0.004	0.440 \pm 0.004	0.347 \pm 0.004	0.397 \pm 0.002
Area (cm ²)	1.821 \pm 0.120	1.737 \pm 0.006	1.555 \pm 0.009 ^b	1.612 \pm 0.038 ^c
BMD (g/cm ²)	0.262 \pm 0.009	0.254 \pm 0.013	0.221 \pm 0.016 ^d	0.247 \pm 0.007

^a $P < 0.001$ vs *ad libitum* and restricted-*ad libitum* diet groups; ^b $P < 0.05$ vs *ad libitum* and restricted-*ad libitum* diet groups; ^c $P < 0.05$ vs *ad libitum*; ^d $P < 0.05$ vs *ad libitum*, restricted-*ad libitum* diet and restricted diet-PTH-treated groups. BMC, bone mineral content.

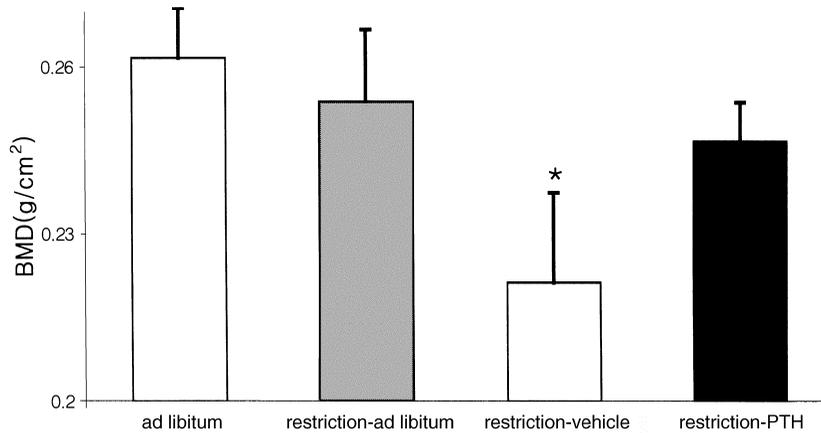


Figure 3 Effects of dietary control and PTH treatment on BMD of right total femur at the 8th week. Each bar represents mean \pm s.d. * $P < 0.01$ vs *ad libitum* group and $P < 0.05$ vs restriction-PTH group.

is one of the contributing factors, because the degree of bone loss is directly correlated with the duration of amenorrhea (Biller *et al.* 1989). Hypercortisolism, present in about 22% of patients, can also accelerate bone loss (Lucas *et al.* 1999). Another worrisome problem might be an increase in the PTH concentration during food restriction. However, Kalu *et al.* showed that there was no significant change or difference in PTH between freely fed and restricted-diet rat groups (Kalu *et al.* 1988).

In undernourished children, it was shown that reduced osteocalcin levels are fully normalized during nutritional recovery (Prudhon *et al.* 1991). However, in conditions such as anorexia nervosa, several trials aimed at preventing bone loss in these patients have been conducted in vain. Neither daily calcium supplements for 23 months nor estrogen replacement for 1 year increased the BMD in osteopenic patients with anorexia nervosa (Rigotti *et al.* 1991, Klibanski *et al.* 1995). A 6-day trial of low dose rhIGF-I in women with anorexia nervosa increased the carboxyl-terminal propeptide of the type 1 procollagen (PICP) by up to 21.6%, without any evidence of enhanced bone resorption (Lucas *et al.* 1999). However, the clinical application of IGF-I has several limitations, such as short plasma half-life and some side effects, including hypoglycemia and parotid swelling (Guler *et al.* 1989, Kovacs

et al. 1999). Therefore, another intervention trial to prevent or treat bone loss associated with this disorder is warranted.

Intermittent administration of PTH stimulates both bone formation and resorption and has a greater net impact on bone formation, resulting in an increase in bone mass (Gunness-Hey & Hock 1984, Hock *et al.* 1988, Hori *et al.* 1988, Liu *et al.* 1991, Kimmel *et al.* 1993). The intermittent administration of PTH doubled the expression of the IGF-I mRNA in the osteoblasts and stimulated collagen synthesis (Watson *et al.* 1995, Urena *et al.* 1993). Recently, Jilka *et al.* reported that PTH suppressed osteoblasts apoptosis, resulting in prolonged survival of the osteoblasts (Jilka *et al.* 1999). Assuming that bone loss in undernourishment may have a deleterious effect on bone due to the chronic propensity for fracture, the aim of this study was to determine whether or not intermittent PTH treatment could enhance BMD in a malnourished state. The calorie intake was restricted to about 40% of the quantity normally consumed by the control rat group in this experiment. The rationales for reducing the calorie intake to 40% in this experiment were as follows. Patients with anorexia nervosa are known to consume approximately 60% of the daily intake of the controls, without any significant differences in the composition of

Table 3 Changes of serum osteocalcin. Values are means \pm s.d. (ng/ml)

	Initial	Fourth week	Eighth week
<i>Ad libitum</i>	53.0 \pm 15.9	48.3 \pm 29.9	34.2 \pm 3.0
Restriction- <i>ad libitum</i>	49.5 \pm 13.9	26.0 \pm 15.1	36.1 \pm 11.9
Restriction-vehicle	55.1 \pm 20.0	39.6 \pm 12.7	30.6 \pm 4.2
Restriction-PTH	50.8 \pm 5.3	35.7 \pm 9.3	59.4 \pm 16.8 ^a

^a $P < 0.05$ vs restriction-vehicle group by Mann-Whitney *U* test.

Table 4 Changes of 24-h urine NTX. Values are means \pm s.d. (nM BCE/mM creatinine)

	Initial	Fourth week	Eighth week
<i>Ad libitum</i>	24.8 \pm 7.4	32.0 \pm 14.4	21.0 \pm 7.0
Restriction- <i>ad libitum</i>	32.8 \pm 23.4	32.3 \pm 19.5	25.4 \pm 7.7
Restriction-vehicle	52.0 \pm 44.6	86.7 \pm 159.9	32.3 \pm 16.4
Restriction-PTH	21.7 \pm 6.8	29.8 \pm 12.8	30.1 \pm 8.3

protein, fat and carbohydrate (Gwirtsman *et al.* 1989). However, in a previous animal study, only 0.9% bone loss with a concomitant 16.5% decrease in body weight was observed after restricting the calorie intake to 60% of that normally consumed by rats for 18 weeks (Banu *et al.* 1999).

As expected, intermittent rhPTH(1–84) administration increased the BMD to the level of the control group (10.5% vs restriction–vehicle group; $P < 0.05$). Interestingly, nutrition enrichment alone after 4 weeks of dietary restriction also improved the BMD in rats. Our observation of an impressive increment in BMD after intermittent rhPTH(1–84) treatment in a persistently malnourished state is meaningful, especially with respect to potential therapies against bone loss.

While the serum osteocalcin concentration was decreased in accordance with the duration of the restricted diet and the degree of weight loss, it significantly increased after intermittent rhPTH(1–84) treatment. Nutritional enrichment also tends to increase the osteoblastic activity. However, no significant changes in the urinary bone resorption marker, NTX, were observed in any group. The wide distribution of the data and the high coefficient of variation in the urinary NTX concentration undoubtedly contributed to this result.

Recently, Kronenberg *et al.*, after observing that mice were missing either the PTH-related peptide (PTHrP) or the PTH/PTHrP receptor gene, proposed that PTHrP controls the pace of differentiation in the growth plate, and that the Indian hedgehog (*Ihh*)-PTHrP feedback system regulates the rate of chondrocyte differentiation, thereby balancing the growth and ossification of the long bones (Kronenberg *et al.* 1996). Our previous study also showed that intermittent PTH treatment promoted linear growth in ovariectomized juvenile rats (Lim *et al.* 1999). However, in contrast to the BMD increment, linear growth did not recover to that of the control group despite rhPTH(1–84) treatment in the persistent malnourished state. This suggests that the process of linear growth is a more complex process that is regulated by many systemic and local factors under optimal nutritional conditions. However, more investigation is needed in order to determine the relationship between linear growth impairment and nutritional deprivation.

In conclusion, the intermittent administration of rhPTH(1–84) stimulated bone formation and increased the BMD in malnourished rats. It is suggested that rhPTH(1–84) may be a possible therapeutic and preventive agent for ongoing bone loss, especially for those in a chronically undernourished condition.

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