High Physiological Prolactin Induced by Pituitary Transplantation Decreases BMD and BMC in the Femoral Metaphysis, but Not in the Diaphysis of Adult Female Rats

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Abstract: High physiological prolactin (PRL) stimulated intestinal calcium absorption and renal calcium uptake in mammals. Previous histomorphometric study revealed a significant increase in bone turnover in the trabecular part of the PRL-exposed long (cortical) bone; however, whole-bone densitometric analysis was unable to demonstrate such effect. We therefore studied differential changes in bone mineral density (BMD) and contents (BMC) of the femoral diaphysis and metaphysis in adult female rats exposed to high PRL induced by anterior pituitary (AP) transplantation. The estrogen-dependent effects of PRL on the femur were also investigated. We found that chronic exposure to PRL had no effect on BMD or BMC of the femoral diaphysis, which represented the cortical part of the long bone. It is interesting that 7 weeks after an AP transplantation, BMD and BMC of the femoral metaphysis were significantly decreased by 8% and 14%, respectively. Ovariectomy (Ovx) for 2, 5, and 7 weeks also decreased BMD and BMC in the femoral metaphysis, but not in the diaphysis. However, the AP transplantation plus Ovx (AP+Ovx) produced no additive effects. Nevertheless, 2.5 µg/kg 17β-estradiol (E2) supplementation abolished the osteopenic effects of both Ovx and AP+Ovx on the femur. As for the L5–6 vertebrae, BMD and BMC were not affected by PRL exposure, but were significantly decreased by Ovx and AP+Ovx, and such decreases were completely prevented by E2 supplementation. It could be concluded that high physiological PRL induced a significant osteopenia in the trabecular part, i.e., the metaphysis, of the femora of adult female rats in an estrogen-dependent manner. Since PRL had no detectable effect on the vertebrae, the effects of PRL on bone appeared to be site-specific.

Key words: diaphysis, femur, metaphysis, prolactin, vertebrae.

Prolactin (PRL), as a calcium-regulating hormone, has been reported to stimulate intestinal calcium absorption, enhance renal calcium reabsorption, and induce high bone turnover in nonmated female rats [1–3]. By regulating calcium mobilization in these target organs during pregnancy and lactation, the high physiological PRL of 75–300 ng/ml increases calcium availability for fetal growth and milk production [4]. Moreover, the basal PRL level of 7–10 ng/ml was required for the maintenance of normal bone turnover and duodenal calcium absorption in rats [5, 6].

It has been shown that PRL acted directly on the intestinal absorptive cells to enhance calcium absorption [3]. In bone, osteoblasts but not osteoclasts strongly expressed functional PRL receptors (PRLR), indicating that bone was also a target of PRL [7]. However, the direct effect of PRL on osteoblasts to enhance bone turnover was complicated by its indirect actions through hyperprolactinemia-induced hypogonadism, which, in turn, induced chronic estrogen deficiency [8, 9]. Moreover, the PRL-enhanced calcium absorption may indirectly contribute to the increase in bone calcium deposition, as previously suggested by the 45Ca kinetic and histomorphometric techniques [10–12]. Nevertheless, the effects of PRL on bone were mostly confined to the trabecular sites [13], e.g., sternum, vertebrae, and trochanter, because a 2-week exposure to high physiological PRL altered calcium deposition or the total calcium content in these bones, but not in the tibia and femur of adult rats [6, 14]. By using bone histomorphometry, we found that the trabecular microarchitecture of long bone was indeed markedly changed by long-term exposure to PRL, i.e., increased trabecular separation and decreased trabecular number [12]. Such changes were not detectable by the whole-bone densitometric study [12, 14]. We therefore hypothesized that in vivo exposure to high physiological PRL, despite showing no effect on the whole femur, may, in fact, alter bone mineral density.
week-old donors were then decapitated, enabling us to remove the renal capsule of the recipient rat. Two anesthetized 10- to 1.0 cm paracostal incisions were made to expose the left kidney and a gentle touch of the renal fascia with forceps. The visual examination of a well-vascularized hypophyseal graft was performed at the end of the experiments to assure successful AP transplantation.

To confirm that PRL was synthesized in the transplanted glands, we dissected the hypophyseal grafts from the periurethral tissues for an immunohistochemical analysis of PRL production, as previously described [14]. In brief, formalin-fixed, paraffin-embedded 4.0 µm sections of grafts were incubated for 60 min with 1:300 PRL polyclonal primary antibody (Dako, Carpinteria, CA, USA), and they were later incubated for 10 min with 1:3,000 biotin-conjugated antirabbit secondary antibody and peroxidase-conjugated streptavidin (Dako). The chromogenic reaction was carried out with 3,3′-diaminobenzidine to produce a brownish product. Digital images were acquired from a light microscope (model BX51 TRF, Olympus, Tokyo, Japan). All AP grafts were positive for PRL immunoreactivity (Fig. 1). This technique has also been known to raise plasma PRL to 90–100 ng/ml (comparable to the levels during pregnancy) [16] and to suppress plasma estrogen below 50 pg/ml [17].

**Bilateral ovariectomy.** Bilateral ovariectomy (Ovx) has been a widely accepted surgical procedure to induce chronic estrogen deficiency [17]. In brief, the rat was anesthetized with diethylether before two 1.5-cm paralumbar incisions were made. The distal parts of both fallopian tubes were ligated prior to the removal of the ovaries. The

![Fig. 1. Representative image of a hypophyseal allograft excised from a 7-week AP rat; magnification ×40 (n = 6). The slide was stained with anti-PRL antibody. The anterior pituitary gland (AP) is strongly labeled with brownish products of peroxidase, whereas the surrounding renal capsule (RC) is negative.](image)
skin was finally sutured with sterile silk 3/0 and cleaned with 70% ethanol and povidone-iodine. Vital signs were carefully monitored until the rat recovered from anesthesia. The sham operation was similar to the bilateral ovariec-
tomy, except that both ovaries were gently touched with forceps and left in place. Uterine weight and vaginal
cytology confirmed the success of the surgery.

**BMD and BMC measurements.** BMD and BMC were
determined by the modified method of Binkley et al. [19].
Under 50 mg/kg sodium pentobarbitone i.p. (Abbott Labor-
atories, North Chicago, IL, USA) anesthesia, BMD and
BMC of the femora were assessed by dual-energy X-ray
absorptiometry (DXA; model Lunar PIXImus2, GE Medical
Systems, Madison, WI, USA), operated with a soft-
ware version 2.10. The dual-energy supply was 80/35 kVp
at 500 µA. The animals were laid prone on a supporting
board with reproducible positioning. Both femora were
placed parallel to the scan direction with knees flexed at
90°. The regions of interest (ROI) for femoral metaphysis
included the distal 8 mm of the femur, whereas the ROI of
femoral diaphysis included the middle part of femur be-
tween its 8-mm ends.

As for the vertebral BMD and BMC, L5–6 vertebrae
were dissected and cleaned of adhering tissues. Fat-con-
taining tissues were eluted by a 1:1 mixture of 100% etha-
nol and diethyl ether. Thereafter the bones were dried at
80°C for 48 h to obtain a constant dry weight. Ex vivo
BMD and BMC were then determined. The ROI of verte-
brae included the L5–6 segments.

**Experimental design.** To study the effect of high physi-
ological PRL on the cortical and trabecular sites in the
presence and absence of chronic estrogen deficiency, we
divided the rats into 6 groups, i.e., sham-operated (Sham),
ovariectomized (Ovx), Ovx supplemented with 2.5 µg/kg
17β-estradiol s.c. (Sigma) 3 times a week (Ovx+E2), AP-
grafted (AP), AP+Ovx, and AP+Ovx+E2. Both AP trans-
plantation and ovariecotomy were performed on the same
day (week 0). Intact BMD and BMC of the femoral dia-
phys and metaphysis were measured at 2, 5, and 7 weeks
postsurgery. At week 7, all rats were sacrificed for ex vivo
BMD and BMC measurements in the L5–6 vertebrae.

**Statistical analysis.** The results are expressed as mean ±
SE. Multiple comparisons were performed by one-way
analysis of variance (ANOVA) with a Newman-Keuls
posttest. The level of significance for all statistical tests
was P < 0.05. The data were analyzed by GraphPad Prism
4.0 for Mac OS X (GraphPad Software, San Diego, CA,
USA).

**RESULTS**

**High PRL did not affect body weight of AP rats**

At 7 weeks postsurgery, the body weights of Ovx (n =
10, P < 0.001) and AP+Ovx rats (n = 6, P < 0.05) were sig-
nificantly increased (Fig. 2) and could be completely nor-
malized by 17β-estradiol (E2) supplementation. The body
weights of AP rats were comparable to those of Sham rats.

**PRL decreased BMD and BMC in the femoral
metaphysis in an estrogen-dependent manner**

It has been known that exposure to high physiological
PRL for 4–7 weeks did not change the BMD and BMC of
the whole cortical bones, including the femur [12]. In
the present study, we found that AP transplantation for 2, 5,
and 7 weeks had no effect on BMD and BMC of the fem-
oral diaphysis (Fig. 3). We found it interesting that a 7-
week AP transplantation significantly decreased the meta-
physeal BMC (Fig. 4) by 8%, i.e., from 0.229 ± 0.002 (n =
6) to 0.210 ± 0.003 g/cm² (n = 10, P < 0.01), and the meta-
physeal BMC by 14%, i.e., from 0.169 ± 0.003 (n = 6) to
0.146 ± 0.002 g (n = 10, P < 0.01).

Similar to AP rats, the Ovx rats manifested a decrease
in BMD in the metaphysis, but not in the diaphysis (Figs.
3 and 4), i.e., from 0.209 ± 0.002 (n = 7) to 0.186 ± 0.001
g/cm² (n = 10, P < 0.001) at 2 weeks, from 0.225 ± 0.003
(n = 6) to 0.205 ± 0.003 g/cm² (n = 10, P < 0.01) at 5
weeks, and from 0.229 ± 0.003 (n = 6) to 0.210 ± 0.004 g/
cm² (n = 10, P < 0.05) at 7 weeks, post-Ovx. The meta-
physeal BMC in Ovx rats was also consistently decreased
with BMD, i.e., from 0.143 ± 0.004 (n = 7) to 0.125 ±
0.003 g (n = 10, P < 0.05) at 2 weeks, from 0.160 ± 0.004
(n = 6) to 0.139 ± 0.002 g (n = 10, P < 0.01) at 5 weeks,
and from 0.169 ± 0.003 (n = 6) to 0.144 ± 0.003 g (n = 10,
P < 0.01) at 7 weeks, post-Ovx. In rats with AP transplan-
tation plus Ovx (AP+Ovx), their decreased metaphyseal
BMD and BMC at 2, 5, and 7 weeks postsurgery were
comparable to those in Ovx rats, suggesting that high
physiological PRL had no additional osteopenic effect on
the femoral metaphysis in Ovx rats.

**Fig. 2.** Body weight of Sham, Ovx, Ovx+E2, AP, AP+Ovx, and
AP+Ovx+E2 rats at 7 weeks postsurgery. *P < 0.05, "P <
0.001 compared with Sham. †††P < 0.001 compared with
Ovx+E2. #P < 0.05 compared with AP. Numbers in paren-
theses are the numbers of experimental animals.
The E2 supplementation of 2.5 µg/kg s.c. 3 times a week completely abolished the osteopenic effect of Ovx on the femoral metaphysis (Figs. 3 and 4), indicating that the supplement regimen was sufficient to provide circulating estrogen for the maintenance of normal bone mass. We noticed with interest that E2 supplementation also re-

Fig. 3. In situ BMD and BMC of the femoral diaphysis of Sham, Ovx, Ovx+E2, AP, AP+Ovx, and AP+Ovx+E2 rats at 0, 2, 5, and 7 weeks postsurgery. The ROI of the femoral diaphysis was the area between the 8-mm ends of the left femur. Numbers in parentheses are the numbers of experimental animals.

Fig. 4. In situ BMD and BMC of the femoral metaphysis of Sham, Ovx, Ovx+E2, AP, AP+Ovx, and AP+Ovx+E2 rats at 0, 2, 5, and 7 weeks postsurgery. The ROI of the femoral metaphysis was the area in the 8-mm distal end of the left femur. *P < 0.05, **P < 0.01, ***P < 0.001 compared with Sham. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with Ovx+E2. §P < 0.05, §§P < 0.01 compared with AP. ¶P < 0.05, §§§P < 0.01 compared with AP+Ovx. Numbers in parentheses are the numbers of experimental animals.
versed BMD and BMC in AP+Ovx rats to the normal levels. Since osteopenia in AP+Ovx rats could be restored by E2 supplement, our results suggested that high physiological PRL induced a significant bone loss in the femoral metaphysis in an estrogen-dependent manner.

**High physiological PRL did not change vertebral BMD and BMC**

To demonstrate a site-specific action of PRL, we investigated whether high PRL induced by AP transplantation affected BMD and BMC of the L5–6 vertebrae in normal and Ovx rats (Fig. 5). Seven weeks after an Ovx, the vertebral BMD and BMC were decreased by 7% and 14%, i.e., from 0.178 ± 0.002 (n = 10) to 0.166 ± 0.001 g/cm² (n = 10, P < 0.001), and from 0.318 ± 0.007 (n = 10) to 0.275 ± 0.014 g (n = 10, P < 0.05), respectively. AP transplantation did not affect BMD and BMC of the vertebrae, and both parameters in AP+Ovx rats were comparable to those in the Ovx rats. E2 supplementation restored BMD and BMC in the Ovx and AP+Ovx rats to the Sham levels. The results indicated that in contrast to the trabecular part of the femur, the L5–6 vertebrae showed no decreases in the densitometric parameters after chronic exposure to high physiological PRL, implying a site-specific action of PRL on bone.

**DISCUSSION**

In the present study, we provide evidence that high physiological PRL of 90–100 ng/ml (comparable to the levels during pregnancy) could induce bone loss in the femoral metaphysis as demonstrated by DXA. In contrast, BMD and BMC of the L5–6 vertebrae, which were primarily trabecular bones, were not changed by PRL, suggesting a site-specific action of PRL. Our findings could be physiologically relevant, since chronic exposure to high physiological PRL for a few months, similar to that occurring during pregnancy, does not usually induce overt osteopenia in rats and humans [12, 20, 21]. On the other hand, long-term exposure to pathological hyperprolactinemia (up to ~1,000 ng/ml), such as in prolactinoma or prolonged antipsychotic drug uses, leads to massive bone loss and increased risk of osteoporosis [22–24].

High plasma PRL level, especially under pathological conditions, has been known to be associated with gonadal dysfunction [8]. In schizophrenic patients treated with antipsychotic drugs that induced hyperprolactinemia, peak serum E2 in periovulatory phase was suppressed below 100 pg/ml by PRL [25]. AP rats, similar to Ovx rats, also manifested a decrease in the plasma estrogen concentration to less than 50 pg/ml [17]. PRL was therefore thought to indirectly cause bone loss by inducing estrogen deficiency. However, our recent histomorphometric study in the tibial metaphysis of AP rats showed that besides the Ovx-like effects on bone, such as decreases in bone volume and trabecular number, high PRL also exerted an estrogen-independent action by increasing bone formation rate [12]. The presence of PRLR in osteoblasts confirmed a direct action of PRL on bone [7]. By using RT-PCR and Southern blot technique, Bataille-Simoneau and co-workers [26] demonstrated the expression of PRLR in two human osteoblast-like cell lines, MG-63 and Saos-2. Neonatal rat osteoblasts and primary osteoblasts derived from the tibia of adult rats also expressed functional PRLR [7, 12], implicating rat osteoblasts as targets of PRL. Recently, we reported that MG-63 osteoblast-like cells, when directly exposed to recombinant human PRL for 48 h, exhibited significant decreases in osteocalcin mRNA expression, osteoprotegerin protein expression, and alkaline phosphatase activity [12]. Thus PRL was able to exert both direct and indirect actions on bone, though their relative contributions were not known. In the present study with PRL-stimulated bone loss in the femoral metaphysis, because the AP+Ovx rats produced no more severe osteopenia than that seen in Ovx or AP alone suggested that the estrogen-dependent (indirect) action of PRL was predominant. This was confirmed by the finding that E2 supplementation completely restored BMD and BMC in AP+Ovx rats to the Sham levels. Although high-dose E2 supplementation of 50 µg/week may increase serum PRL concentration by enhancing pituitary PRL secretion [17], a lower dose of ~2.2 µg/week used in the present study was unlikely to induce significant PRL secretion, either from the intracranial gland or implanted glands. An indirect action of PRL on bone through other calcitropic hormones, e.g., parathyroid hormone, has never been reported.

Besides the estrogen-dependent action, the effect of PRL on bone was also dependent on the circulating levels...
of PRL and duration of exposure [6, 16]. We previously showed that the basal levels of PRL of ~7–10 ng/ml were necessary for the maintenance of the normal rate of bone turnover in nonmated adult female rats [6]. This was consistent with reports of less developed and poorly ossified calvariae of 18.5-day PRL-Knockout (PRL−/−) murine fetuses and a 60% decrease in the bone formation rate of 8-week-old adult PRL−/− mice [27]. During pregnancy with higher physiological PRL concentrations in the range of 75–100 ng/ml, PRL was found to increase both the rates of bone formation and the resorption, i.e., inducing a state of high bone turnover, thus resulting in minimal or undetectable changes in the densitometric parameters [13, 21]. In long-term lactation with an increased loss of calcium in milk, higher PRL levels in the range of 200–300 ng/ml could induce significant bone loss, which was totally reversible after the cessation of lactation [20, 28, 29]. Prolonged pathological hyperprolactinemia, on the other hand, was accompanied by persistent bone loss with low BMD and BMC and increased risks of osteoporosis and fracture [22–24]. The present AP rats with the plasma PRL levels in the same range as that of pregnant rats could provide a model of PRL-induced bone loss during pregnancy with regional trabecular osteopenia.

Apparently the site-specific trabecular bone loss was also observed during lactation [13]. Generally, the effects of high physiological PRL are not substantial enough to be detectable by the macroscopic techniques, such as the total body or whole-bone DXA. Only when the more sensitive 45Ca kinetic technique and bone histomorphometry were used were the effects demonstrated of high physiological PRL, which accelerated bone turnover to mobilize calcium to satisfy the increased calcium demand [4, 20]. However, when the trabecular region of cortical bone was examined separately as femoral metaphysis, significant decreases in BMD and BMC after exposure to high PRL could be detected by DXA, implying that the trabecular part of cortical bones was more responsive or susceptible to PRL than the primarily trabecular bone, i.e., vertebrae. Meanwhile, the unaffected BMD and BMC of the femoral diaphysis confirmed the absence of the effects of high PRL and estrogen deficiency on the cortical sites [12, 14].

At the histological level, PRL-enhanced bone turnover in the trabecular microarchitecture was associated with concurrent increases in the eroded surface, osteoblast surface, and osteoclast surface [12]. Moreover, bone mineral apposition rate was also elevated together with an increase in trabecular bone calcium deposition [6], suggesting that new calcium was supplied to bone to overcome bone loss, possibly through the parallel stimulation of the intestinal calcium absorption by PRL [2, 30]. Nevertheless, these compensatory mechanisms were inadequate for the maintenance of the femoral metaphysis, whereas they may successfully prevent an overt bone loss in the vertebrae. Although other mechanisms, such as different regional distribution of PRLR, might also explain the absence of PRL effect on the vertebrae, the present results clearly demonstrated the heterogeneity of bones in their responses to high physiological PRL. Since the metaphyseal bone loss weakens the whole bone, further investigations are required to demonstrate whether the biomechanical properties of the femoral metaphysis and fracture risk are also changed by long-term exposure to high physiological PRL.

In conclusion, we provided evidence that high physiological PRL induced bone loss in the trabecular part of the cortical bones, i.e., the femoral metaphysis, in estrogen-dependent and site-specific manners. The effects of high PRL and estrogen deficiency on the femoral diaphysis were undetectable by the DXA technique. Since decreases in BMD and BMC were found in the femoral metaphysis but not in the vertebrae, these changes in the femur may have some physiological significance or clinical relevance that needs to be further investigated.

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