Abstract Since hyperprolactinemia was found to induce osteopenia in the metaphysis of long bone in non-mated female rats, pregnant and lactating rats with sustainedly high plasma prolactin (PRL) levels might also exhibit some changes in their long bones. We performed a longitudinal study in pregnant, lactating and post-weaning rats, using dual-energy X-ray absorptiometry to demonstrate site-specific changes (i.e., metaphysis vs. diaphysis) in femoral bone mineral density (BMD) and content (BMC). The results showed that femoral metaphyseal BMD and BMC were higher when compared to their age-matched controls during pregnancy, before decreasing in late lactation and post-weaning. On the other hand, femoral diaphyseal BMC increased during pregnancy, early lactating and mid-lactating periods without change during late lactation and post-weaning. After 7 days of bromocriptine administration, which inhibited endogenous PRL secretion, the lactation-induced increases in BMC during early and mid-lactating periods were abolished. Moreover, a decrease in metaphyseal BMD during late lactation was restored to the control levels by bromocriptine. However, bromocriptine did not antagonize the pregnancy-induced increases in BMD and BMC. It could be concluded that the effect of PRL on bone was variable during the reproductive periods. While having no effect on femoral BMD and BMC during pregnancy, PRL was responsible for bone gain in early and mid-lactating periods, but induced bone loss during late lactating period.

Keywords Bone density · Dual-energy X-ray absorptiometry (DXA) · Femur · Lactation · Pregnancy

Introduction

Previous investigations in non-mated female rats demonstrated that short-term exposure to high plasma prolactin (PRL) levels induced by 4 weeks of anterior pituitary transplantation (AP) led to net bone gain, while prolonged PRL exposure longer than 4 weeks gradually led to net bone loss [1, 2]. Such hyperprolactinemic effects appeared to be observed primarily at the trabecular sites, e.g., vertebrae and sternum, but not the cortical sites, e.g., tibiae and femora [3, 4]. However, by using dual-energy X-ray absorptiometric (DXA) analysis on specific regions of rat femur, we found that prolactin could decrease bone mineral density (BMD) and content (BMC) of the femoral metaphysis, which was predominantly trabecular bone, but not diaphysis which was predominantly cortical bone [5]. Thus, it was possible that elevated plasma PRL levels during pregnancy (75–100 ng/mL) and lactation (100–300 ng/mL), i.e., physiological hyperprolactinemia, could alter BMD and BMC of the long bone.

Reports on site-specific changes in BMD and BMC (metaphysis vs. diaphysis) of long bone during pregnancy and lactation in rats were controversial. Zeni and co-workers
[6] reported that in situ BMDs of the proximal and distal tibiae were not changed on the first day postpartum, but were decreased at the end of lactation. Similarly, single-photon absorptiometric analysis revealed greater lactation-induced bone loss in the femoral metaphysis than in the diaphysis [7]. However, densitometric changes at mid-pregnancy and mid-lactation, which have high rates of maternal calcium loss, were presently not known. Nishiwaki and co-workers [8] demonstrated decreases in BMDs in the distal femur, lumbar spine and caudal spine (i.e., trabecular sites) at week 2–3 of lactation, but changes in cortical BMDs were not studied. Thus, a complete longitudinal densitometric study was first performed to demonstrate changes in site-specific femoral BMD and BMC during these reproductive periods.

To investigate the effects of endogenous PRL on BMD and BMC during pregnancy and lactation, an inhibitor of pituitary PRL secretion, bromocriptine, was administered to the animals. Bromocriptine is a dopaminergic D2 receptor agonist which mimics the hypothalamic PRL-inhibitory factor dopamine, thereby suppressing PRL release from the pituitary lactotrophs [9, 10]. Since osteoblasts were found to directly respond to several monoamines, e.g., serotonergic and adrenergic agonists [11], expression of D2 receptors, which have two isoforms known as long and short isoforms, was also determined to exclude possible direct actions of bromocriptine on osteoblasts.

Materials and methods

Animals

Non-mated (nulliparous) and pregnant (primiparous) Sprague–Dawley rats (8-week-old, weighing 210–220 g), were obtained from the Animal Centre of Thailand, Salaya, Thailand. They were placed in hanging stainless steel cages, fed standard chow containing 1% w/w calcium and 4,000 IU/kg vitamin D (Perfect Companion, Bangkok, Thailand), and distilled water ad libitum under 12:12 h light:dark cycle. Room temperature was controlled at 23–25°C, and the relative humidity was about 50–60%. Body weight and food intake were recorded daily. After delivery, the litter size was adjusted to eight pups per dam. Animals were cared for in accordance with the “Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences”. This study has been approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Science, Mahidol University.

Experimental design

Rats were divided into 6 groups, i.e., 14-day pregnancy (P14; mid-pregnancy), 21-day pregnancy (P21; late pregnancy), 8-day lactation (L8; early lactation), 14-day lactation (L14; mid-lactation), 21-day lactation (L21; late lactation), and 15-day post-weaning (PW), with ages of 10, 11, 12, 13, 14, and 16 weeks, respectively. In some experiments, prior to the removal of femora, pregnant and lactating rats were administered for 7 days with 4 mg/kg per day bromocriptine s.c., which have been known to diminish plasma PRL in pregnant and lactating rats [1, 9]. Thus, each group consisted of vehicle-treated mated (pregnant or lactating) rats, bromocriptine-treated mated rats, and vehicle-treated age-matched non-mated control rats. Bromocriptine (Sigma, St. Louis, MO, USA) was first dissolved in a mixture (vehicle) of tartaric acid, absolute ethanol, and normal saline, as described previously [12]. After the rats were killed, BMD and BMC of proximal metaphysis, distal metaphysis (trabecular sites), and diaphysis (cortical site) of the femur were determined.

Primary osteoblast culture

As described previously [13], tibiae were dissected from a 9-week-old non-mated female rat by sterile surgical technique. After removing the connective tissues and marrow cells, the bones were cut into small dice, and incubated on a shaker (60 cycles/min) for 2 h at 37°C in a 25-cm² T-flask (Corning, NY, USA) containing DMEM supplemented with 100 U/mL penicillin/streptomycin and 2 mg/mL collagenase (all purchased from Sigma). Thereafter, bone dice were washed with DMEM, and then cultured in DMEM supplemented with 15% FBS, 100 U/mL penicillin/streptomycin, 100 µg/mL ascorbate-2-phosphate, and 0.5 mM sodium pyruvate (Sigma) at 37°C with 5% CO₂. Osteoblasts proliferated and migrated from bone dice into the media within 3 days. Media were changed daily until day 20 for expression study. Rat osteoblast-like UMR-106 cells [American Type Culture Collection (ATCC) no. CRL-1661] were cultured as described by the ATCC’s instruction.

Polymerase chain reaction (PCR) and sequencing

Total RNA was prepared from primary rat osteoblasts, UMR-106 cells, pituitary glands, gastrocnemius muscle (negative control), and brain (positive control) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and purified with RNeasy Mini kit (Qiagen, Valencia, CA, USA). 1 µg of the total RNA was reverse-transcribed with oligo-dT₂₀ primer and iScript kit (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as a control gene to check the consistency of reverse transcription. Conventional PCR was performed for 36 cycles with the GoTaq Green Master Mix (Promega, Madison, WI, USA) and Bio-rad.
MyCycler. Oligonucleotide sequences used as primers were 5'-CAGTCGAGCTTTCAGAGC CAA-3' and 5'-TCC ATTCTCCGCTTCA-3' for long isoform of rat D2 receptor (amplicon 129 bp), 5'-TATGGCTTGAAGACCGTG-3' and 5'-TGCTGGCTTCTTCTTGA-3' for short isoform of rat D2 receptor (amplicon 134 bp), and 5'-AGTCTACTGCGCTTAC-3' and 5'-TCATATTTC TCCTTCTGA-3' for GAPDH (amplicon 133 bp). PCR products were visualized on a 1.5% agarose gel stained with 1.0 l g/mL ethidium bromide under a UV transilluminator (Alpha Innotech, San Leandro, CA, USA). After electrophoresis, PCR products were purified by the HiYield Gel/PCR DNA Extraction kit (Real Biotech Corporation, Taipei, Taiwan), and were sequenced by the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

**BMD and BMC measurements**

Site-specific BMD and BMC were determined by the modified methods of Binkley et al. [14] and Thongchote et al. [5]. Femora of pregnant and lactating rats were removed and cleaned of adhesive tissue. Femoral BMD and BMC were assessed by a dual-energy X-ray absorptiometer (model Lunar PIXImus2; GE Medical Systems, Madison, WI, USA), operated with software version 2.10. The dual-energy supply was 80/35 kVp at 500 mA. The regions of interest (ROI) for femoral metaphysis included the proximal and distal 8 mm of the femur, whereas the ROI of femoral diaphysis included the middle part of the femur between its 8-mm ends. The machine was calibrated daily using a phantom provided by the manufacturer. The interassay coefficient of variation was less than 0.3%.

**Statistical analysis**

Results are expressed as mean ± SE. Two-group and multiple comparisons were performed by Student’s t test and one-way ANOVA with Newman–Keuls post-test, respectively. The level of significance for all statistical tests was P < 0.05. Data were analyzed by GraphPad Prism 4.0 for Mac OS X (GraphPad Software, San Diego, CA, USA).

**Results**

Prior to investigating the effects of PRL on site-specific BMD and BMC of femora of pregnant and lactating rats, expression of dopaminergic D2 receptor was determined. As shown in Fig. 1, long isoform D2 receptor expression was observed only in pituitary gland and brain (positive control), but not in primary rat osteoblasts, osteoblast-like UMR-106 cells, or gastrocnemius muscle (negative control). Short isoform of D2 receptor mRNAs were not expressed in osteoblasts. Thus, the results indicated that bromocriptine had no direct action on osteoblasts.

Densitometric analysis in non-mated control rats showed that femoral BMD and BMC of both trabecular sites (proximal and distal metaphyses) and cortical site (diaphysis) increased with age (Figs. 2, 3). When compared to non-mated control, BMD of proximal femoral metaphysis in the P14, P21, and L8 groups were significantly higher, while that of the L21 and PW groups were lower (Fig. 2a). BMC of the proximal metaphysis was also higher during pregnancy (P14 and P21), early lactation (L8), and mid-lactation (L14), without any difference from control thereafter (Fig. 2b). Similarly, BMD of distal femoral metaphysis in the P14 and P21 groups were markedly elevated when compared to non-mated control, and became lower in the L21 and PW groups (Fig. 2c). BMC of distal femoral metaphysis was higher in the P14 and P21, and L14 groups, and later became lower in the PW group (Fig. 2d). Neither BMD nor BMC of distal metaphysis in the L8 group was different from the control values. As for the cortical site, when compared to control, significantly higher BMD was found in the P14, P21, and L14 groups (Fig. 3a), while higher diaphyseal BMC was found in the P14, P21, L8, and L14 groups (Fig. 3b).

To demonstrate the effects of PRL on femoral BMD and BMC in pregnant and lactating rats, bromocriptine was administered for 7 days prior to removal of the femora. As shown in Fig. 4, suppression of PRL secretion had no effect on the pregnancy-induced increases in BMD and BMC in the P14 and P21 groups. On the other hand, during lactation, an absence of PRL abolished increases in metaphyseal and diaphyseal BMC, but not BMD, in the L8 and L14 groups (Fig. 5a–d). In the L21 group, absence of PRL secretion also prevented a decrease in BMD and restored it to the control levels (Fig. 5e), without effect on BMC (Fig. 5f).

![Fig. 1 Expression of long and short isoforms of subtype 2 dopamine (D2) receptor mRNAs in primary rat osteoblasts (OB), osteoblast-like UMR-106 cells, and pituitary gland. Gastrocnemius muscle and brain were used as negative and positive controls, respectively. Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) is a housekeeping gene. This result was a representative of three independent repeats](image-url)
Discussion

Little is currently known regarding the effects of hyperprolactinemia during pregnancy and lactation on the long bone. In the present study, we demonstrated that BMD and BMC of both trabecular and cortical parts of the rat femur were significantly increased during pregnancy until mid-lactation (L14). During late lactation (L21) and 15-day post-weaning, BMD of trabecular bone, but not cortical bone, was decreased. Furthermore, effects of PRL on bone during these reproductive periods were evaluated by administration of a selective D2 receptor agonist bromocriptine. The results suggested that PRL secreted from the pituitary gland was responsible for BMD and BMC changes during lactation, but not pregnancy. Daily administration of 4 mg/kg per day bromocriptine for 7 days was recently shown to decrease serum PRL of L7 rats by greater than 50% [15]. Moreover, in L7 rats with suckling, a single dose of bromocriptine injection markedly decrease serum PRL level from \*430 to \*100 ng/mL [15]. Since direct effect of bromocriptine on bone has never been reported, and osteoblasts did not express D2 receptor (Fig. 1), the bromocriptine effects observed in the present study

Fig. 2 BMD and BMC of a–b proximal and c–d distal femoral metaphyses in mated (pregnant, lactating, and post-weaning) and non-mated age-matched control rats (n = 7–9 per group). *P < 0.05, **P < 0.01, ***P < 0.001 compared with its respective age-matched control group. \(^{†}P < 0.01\) compared with age-matched control of P14 group. \(^{\#}P < 0.05\) compared with P14 group. P14 and P21 designate 14- and 21-day pregnant rats, while L8, L14, and L21 designate 8-, 14-, and 21-day lactating rats, respectively. PW indicates 15-day post-weaning rats.

Fig. 3 a BMD and b BMC of femoral diaphyses in mated (pregnant, lactating, and post-weaning) and non-mated age-matched control rats (n = 7–9 per group). *P < 0.05, ***P < 0.001 compared with its respective age-matched control group. \(^{†}P < 0.01\) compared with age-matched control of P14 group. \(^{\#}P < 0.01\) compared with P14 group. PW indicates 15-day post-weaning rats.
should occur through suppression of pituitary PRL release.

In general, fetal development during pregnancy leads to a massive drain on maternal calcium pool [16]. In humans, ~200–300 mg/day calcium was lost during the second and third trimester to maintain bone calcium accretion in the fetus [17]. However, changes in bone density during pregnancy remained controversial. Several investigators showed that there was no change in BMD in either pregnant women or rats [6, 16, 18]. In contrast, Gonen and co-workers [19] reported increased BMD of lumbar vertebrae, femur and tibia in pregnant rats, whereas others reported pregnancy-induced osteoporosis in humans [20, 21]. Such controversy could partly be due to the fact that an in situ densitometric measurement in living subjects is not sensitive enough to detect a slight change in bone density. Herein, by using ex vivo densitometric measurement, BMD and BMC of pregnant rats were found to increase at both femoral metaphysis and diaphysis. Despite the lack of evidence for the underlying mechanism, we speculated that the enhanced intestinal calcium absorption, in part, due to elevated serum PRL, placental lactogen and/or 1,25(OH)2D3 levels, was responsible for supplying calcium both for fetuses, and for maternal trabecular and cortical bone calcium accretion [15, 22, 23], thereby leading to increases in ash weight, calcium content, and cross-sectional area of femur [1, 24]. Changes in the size and cross-sectional area of femur without proportional change in the total calcium content partially explained why BMC changes occurred in an absence of BMD change. Increased bone calcium content during pregnancy, as indicated by BMC (Figs. 2, 3), may also provide a reserved calcium pool for later use in milk production.

During lactation, an increase in BMC persisted until mid-lactation, presumably due to the lactation-induced increase in intestinal calcium absorption and renal calcium reabsorption [15, 16, 25]. PRL surge during suckling (~400–650 ng/mL) may further contribute to the elevated BMC, since short-term hyperprolactinemia of 2–4 weeks could increase bone calcium content in the rat femur, and further augment calcium absorption in the small intestine [1, 15, 26]. Besides metaphysis, PRL also had a positive effect on the femoral diaphysis of L8 and L14 rats, despite having no effect on the cortical sites in non-mated rats [4]. Although diaphyseal bone gain could be due to periosteal or endosteal bone formation, little is known regarding the direct effect of PRL on both processes in early lactation. We speculated that PRL did affect cortical bone but only in rats fed high-calcium diet [4], and perhaps in rats with enhanced intestinal calcium absorption. Bone histomorpohometric study in hyperprolactinemic rats as well as in early lactating rats should reveal the effect of PRL on endosteal and periosteal calcium accretion.

When breastfeeding was prolonged, ongoing calcium loss in milk production eventually culminated in osteopenia in late lactation (L21), which was still detected at day 15 post-weaning. In exclusively breastfeeding women, calcium loss during this period can be as high as
400 mg/day [17], while lactating rats lose 20–30% of their skeletal mass over 21 days of lactation [27, 28]. Interestingly, the observed bone loss only at trabecular site (metaphysis) was similar to that previously reported in rat vertebrae and distal femur [8]. Predominant bone loss in metaphyses may be due to the fact that the trabecular sites have more surface areas for bone resorption than the cortical sites. Several weeks after weaning, bone mass appeared to be completely restored to the normal level [16], presumably due to an increase in osteoclast apoptosis and decreased expression of osteoblast-derived bone resorption mediator, receptor activator of nuclear factor κB ligand (RANKL) [29].

A number of hormones act in concert to regulate bone resorption process during lactation. For example, parathyroid hormone-related protein (PTHrP) produced by the mammary gland is able to stimulate bone resorption and mobilize bone calcium for lactogenesis, whereas calcitonin helps to limit bone loss during lactation [16, 30]. In the present study, a decrease in metaphyseal BMD during late lactation was likely to be due to a lactogenic hormone PRL. Although PRL could induce a net bone gain in young growing rats or after short-term exposure in adults, chronic hyperprolactinemia longer than 5 weeks may lead to progressive trabecular osteopenia [2]. In non-mated female rats, chronic hyperprolactinemia markedly decreased trabecular bone volume and trabecular number, while enhancing bone turnover [2].

Since high plasma PRL levels suppressed ovarian estrogen synthesis [31–33], hyperprolactinemia-induced osteopenia was previously thought to be due to estrogen depletion. However, the presence of PRL receptors in primary rat osteoblasts and osteoblast-like MG-63 cells indicated direct PRL action on bone [2, 13].
molecular level, PRL upregulated RANKL and downregulated osteoprotegerin (OPG) in osteoblasts, thereby enhancing osteoclastogenesis and osteoclast-mediated bone resorption [2]. PRL also suppressed alkaline phosphatase activity and osteocalcin expression, both of which were important for the osteoblast-mediated bone formation [2]. Although the aforementioned mechanism is able to explain metaphyseal bone loss in rat femur during late lactation, the molecular mechanism by which PRL increased bone mass during early and mid-lactation is currently unknown. In addition to the PRL-enhanced intestinal calcium absorption to supply more calcium for bone formation [23], it was possible that, during these reproductive periods, PRL may upregulate OPG rather than RANKL in osteoblasts of lactating rats, similar to that observed in human fetal osteoblasts [34], thus in turn reducing osteoclast proliferation.

In conclusion, from pregnancy to mid-lactation, BMD and BMC of both femoral metaphysis and diaphysis were increased. On the other hand, net bone loss was observed only in the femoral metaphysis during late lactation and day 15 post-weaning. Such bone changes during lactation, but not pregnancy, were found to be under the regulation of PRL. Interestingly, in contrast to non-mated rats in which PRL predominantly affected trabecular sites [3, 4], PRL induced net bone gain in both trabecular and cortical parts of femur of L8 and L14 rats, presumably to expand the calcium storage pool. Therefore, the present findings, together with the facts that PRL is able to stimulate intestinal calcium absorption and renal calcium reabsorption [1, 15, 23], suggested that PRL orchestrated total body calcium metabolism during lactation to guarantee an adequate calcium supply for milk production.

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Conflict of interest statement The authors declare no conflicts of interest.

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