

## Plasma fluctuation in estradiol-17 $\beta$ and bone resorption markers around parturition in dairy cows

Bhuminad DEVKOTA<sup>1,2</sup>, Masahiro TAKAHASHI<sup>1,3</sup>, Saori SATO<sup>1</sup>, Kouya SASAKI<sup>4,5</sup>, Atsushi UEKI<sup>6</sup>, Takeshi OSAWA<sup>1,7</sup>, Masahiro TAKAHASHI<sup>1,5,8</sup> and Norio YAMAGISHI<sup>1,5</sup>\*

<sup>1</sup>Cooperative Department of Veterinary Medicine, Iwate University, Morioka, Iwate 020–8550, Japan

<sup>2</sup>Center for Biotechnology, Agriculture and Forestry University, Rampur, Chitwan, Nepal

<sup>3</sup>Atsumi Dairy Herds Management Service, Sendai, Miyagi 989–3205, Japan

<sup>4</sup>Iwate Veterinary Hospital, Iwate-machi, Iwate 028–4307, Japan

<sup>5</sup>United Graduate School of Veterinary Sciences, Gifu University, Gifu 501–1193, Japan

<sup>6</sup>Ueki Veterinary Hospital, Morioka, Iwate 020–0887, Japan

<sup>7</sup>Department of Veterinary Sciences, Faculty of Agriculture, University of Miyazaki, Miyazaki 889–2192, Japan

<sup>8</sup>Department of Advanced Pathobiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Izumisano, Osaka 598–8531, Japan

(Received 8 January 2015/Accepted 26 February 2015/Published online in J-STAGE 9 March 2015)

**ABSTRACT.** Blood samples were obtained sequentially from 10 dairy cows around the time of parturition to assess plasma fluctuations in estradiol-17 $\beta$  (E<sub>2</sub>) levels in association with those of several bone resorption markers. Plasma E<sub>2</sub> concentration increased sharply a few days prepartum and decreased quickly after parturition. In terms of bone resorption markers, the plasma level of tartrate-resistant acid phosphatase isoform 5b (TRAP5b) rose significantly, commencing 1 week prepartum, and was maintained at this level to a few days postpartum. The plasma concentration of carboxyterminal collagen cross-links of type-I collagen (CTX) increased significantly after parturition. These observations suggest that osteoclast-mediated bone resorption was activated after parturition when plasma E<sub>2</sub> concentrations decreased.

**KEY WORDS:** dairy cow, osteoclast-mediated bone resorption, osteocytic remodeling, parturition, tartrate-resistant acid phosphatase isoform 5b (TRAP5b)

doi: 10.1292/jvms.15-0018; *J. Vet. Med. Sci.* 77(7): 875–878, 2015

Nearly all dairy cows experience some degree of hypocalcemia around the time of parturition, and milk fever (parturient paresis) is a hypocalcemic disorder associated with the commencement of lactation [6]. A reduction in plasma calcium (Ca) concentration occurs, because Ca in the extracellular fluid pool enters the mammary gland faster than it can be replaced by intestinal Ca or via the bone resorption of Ca [6]. Estrogen, estradiol-17 $\beta$  (E<sub>2</sub>) and estrone, known to play important roles in skeletal homeostasis [2], are often considered to be potent inhibitors of bone resorption [4]. In humans, estrogen deficiency caused by menopause triggers increased bone resorption exceeding bone formation, and this imbalance causes the bone loss predisposing one to osteoporosis [2]. In dairy cows, some earlier studies suggested that cows with higher plasma concentrations of estrogen at the time of parturition were at a greater risk of developing milk fever [7, 16], whereas several other studies failed to make such findings [5, 20]. Thus, further work is required to clarify the relationship between estrogen level and bone resorption in parturient cows. The present study was designed

to assess plasma fluctuations in E<sub>2</sub> levels together with those of several bone resorption markers around the time of parturition in dairy cows.

The study protocol was approved by the Iwate University Laboratory Animal Care and Use Committee (#A201331). Ten normally parturated Holstein cows from 2 commercial farms (A and B) in Iwate, Japan, were studied. The dry-up period commenced 2 months prepartum, and the close-up period began 3 weeks prepartum in all cows. On farm A, the cows (4.1–7.0 years old; n=4) were housed in free-stall barns. The cows were fed grass hay *ad libitum*, with 4 kg/day of concentrate, together with 30 g Ca sulfate as an additive, during the close-up period [0.59% Ca, 0.47% phosphorus (P) and 0.24% magnesium (Mg) in dry matter (DM), all w/w]. On farm B, the cows (3.3–6.3 years old; n=6) were housed in a free-stall barn before parturition and in a tie-stall barn after calving. The cows were fed grass hay *ad libitum* with 3 kg/day of concentrate containing 80 g Mg oxide and 50 g dicalcium phosphate as additives during the close-up period (0.50% Ca, 0.40% P and 0.47% Mg in DM, all w/w). The close-up dietary cation–anion difference (DCAD) calculated from the dietary mineral levels was 0.1 and 26.7 mEq/100 g of dietary DM on farms A and B, respectively. During lactation, the cows on each farm were fed a total mixed ration of grass silage and concentrates, with plenty of grass hay. The milk yield (mean  $\pm$  standard deviation) of cows on farms A and B was 35.8  $\pm$  8.2 and 35.5  $\pm$  4.9 kg/day, respectively, in the first month after parturition.

\*CORRESPONDANCE TO: YAMAGISHI, N., Cooperative Department of Veterinary Medicine, Iwate University, 3–18–8 Ueda, Morioka 020–8550, Japan. e-mail: yamagisi@iwate-u.ac.jp

©2015 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <http://creativecommons.org/licenses/by-nc-nd/3.0/>.

Heparinized blood samples were obtained via coccygeal venipuncture at 3 weeks (18–24 days prepartum; W-3), 1 week (5–7 days prepartum; W-1) and 1–2 days (5–43 hr prepartum; D-2) before parturition, and at 1 or 2 days (18–43 hr) postpartum (D+2) and 3 weeks (20–24 days) postpartum (W+3). Placental expulsion occurred within 12 hr of calf delivery in all cows. Plasma was separated within 3 hr of collection via centrifugation at  $1,780 \times g$  for 20 min and stored at  $-50^{\circ}\text{C}$  prior to blood biochemical analysis.

The plasma  $\text{E}_2$  concentration was measured using time-resolved fluorescence immunoassay kits (DELFIA Estradiol Reagents; Perkin Elmer Life and Analytical Sciences, Akron, OH, U.S.A.) as described by Togashi *et al.* [21], while the plasma Ca and Mg concentrations were determined via an *o*-cresolphthalein complexone and xylydyl blue methods, respectively, using an automated clinical chemistry analyzer (ACCUTE TBA-40FR autoanalyzer; Toshiba Medical Systems Co., Otawara, Japan). The plasma intact parathyroid hormone (iPTH) concentration was measured via an electrochemiluminescence immunoassay (Elecsys PTH Kit; Roche Diagnostics, Mannheim, Germany). As a check of the assay validity, plasma samples from cows with experimentally induced hypocalcemia ( $n=4$ ) [19] were preliminary subjected to measuring iPTH concentrations. Plasma iPTH concentration (median, minimum–maximum) increased to the maximum level during experimental hypocalcemia (1,228 pg/ml, 755–1,587 pg/ml) compared to the level before the experiment (60.8 pg/ml, 19.7–128.3 pg/ml). Plasma iPTH concentrations in clinical healthy parturient cows and spontaneous milk fever cases using this assay have been described in our previous study [19]. Plasma tartrate-resistant acid phosphatase isoform 5b (TRAP5b) activity was measured fluorometrically as described previously [13, 22]. The plasma carboxy-terminal collagen cross-links of type-I collagen (CTx) concentration was assayed using an ELISA kit validated for cattle [9] (Serum CrossLaps ELISA; Nordic Bioscience Diagnostics, Copenhagen, Denmark), while the plasma hydroxyproline (HYP) concentration was determined spectrophotometrically as described previously [3].

Blood biochemical data are presented as medians with interquartile ranges (IQRs). To evaluate fluctuation in each parameter, the values were checked for normal distribution using D'Agostino-Pearson omnibus normality test and assessed by repeated measures ANOVA and Holm-Sidak's multiple comparisons test or by Friedman test and Dunn's multiple comparison test, using the prepartum W-3 levels as controls. The Mann-Whitney *U*-test was used to compare differences between levels in the cows of each farm at each time point. All statistical analyses were performed with the aid of Prism version 6 for Windows (GraphPad Software Inc., La Jolla, CA, U.S.A.). The level of significance was set at  $P < 0.05$ .

Figure 1 shows the plasma  $\text{E}_2$ , Ca, iPTH, TRAP5b, CTx and HYP levels in 10 dairy cows from prepartum W-3 to postpartum W+3. Plasma  $\text{E}_2$  concentrations significantly increased at prepartum D-2 (median, IQRs: 168.0 pg/ml, 147.0–231.2 pg/ml) and significantly decreased at postpartum W+3 (0.4 pg/ml, 0.1–1.3 pg/ml) compared to the level

at prepartum W-3 (7.7 pg/ml, 6.4–11.3 pg/ml). Plasma Ca concentration decreased significantly at postpartum D+2 (8.8 mg/dl, 8.5–9.1 mg/dl) compared to the level at prepartum W-3 (9.6 mg/dl, 9.4–9.9 mg/dl). The plasma iPTH concentration was significantly elevated at postpartum D+2 (41.7 pg/dl, 33.7–56.8 pg/ml) compared to that at prepartum W-3 (15.6 pg/ml, 7.5–23.0 pg/ml). The plasma TRAP5b level was significantly lower at prepartum W-1 (0.73 U/l, 0.51–0.84 U/l) compared to postpartum D+2 (1.04 U/l, 0.78–1.33 U/l) and prepartum W-3 (0.64 U/l, 0.48–0.76 U/l). The plasma CTx concentration was significantly higher at postpartum D+2 (0.75 ng/ml, 0.54–1.64 ng/ml) and W+3 (1.43 ng/ml, 0.95–2.14 ng/ml) than at prepartum W-3 (0.44 ng/ml, 0.24–0.58 ng/ml). The plasma HYP and Mg concentrations did not fluctuate significantly around the time of parturition. When the two farms were compared, the plasma iPTH concentration at prepartum W-1 on farm A (19.0 ng/ml, 13.5–24.1 ng/ml) was significantly higher than that on farm B (5.9 pg/ml, 5.7–6.2 pg/ml). There were no significant differences in other plasma parameters between 2 farms at each time point.

Estrogen is the principal hormonal regulator of bone metabolism in humans and animals, and recent studies have shown that osteoclasts are directly targeted by the hormone [10]. Osteoclast-specific deletion of the estrogen receptor decreased trabecular bone mass attributable to an increase in the osteoclast life span caused by a reduction in apoptosis [10]. Estrogen blocks the receptor activator of NF $\kappa$ B ligand (RANKL)/macrophage-colony stimulating factor (M-CSF)-induced activator protein-1-dependent transcription and suppresses RANKL-induced osteoclast differentiation [10]. In addition to these direct effects on osteoclasts, estrogen indirectly regulates osteoclast formation and activity. Recent studies have shown that estrogen suppresses RANKL production by osteoblasts, and T- and B-cells, and also stimulates the production of osteoprotegerin (OPG), which is the decoy receptor for RANKL [10]. Estrogen deficiency has been reported to increase production of several of bone-resorbing cytokines (including interleukin-1, tumor necrosis factor- $\alpha$  and M-CSF), resulting in bone loss in both humans and animals [10]. In the present study, the plasma levels of  $\text{E}_2$ , Ca, iPTH and several bone resorption markers were analyzed in dairy cows around the time of parturition to explore the suppressive effect of estrogen on bone resorption.

The fluctuations in plasma  $\text{E}_2$  and Ca concentrations described here appear to be typical in parturient dairy cows and are consistent with previously reported findings. The plasma  $\text{E}_2$  concentration has been reported to increase sharply a few days prepartum and to fall swiftly after parturition [7, 14], whereas the plasma Ca level declines soon after calving [6]. The parathyroid gland produces iPTH and secretes the hormone into the bloodstream whenever the blood Ca level declines [6]. We found that the plasma iPTH concentration increased significantly when the plasma Ca attained its minimum level (postpartum D+2). We also found a significant difference in plasma iPTH concentrations between the 2 farms at prepartum D-2, possibly due to differences in the concentrations of Mg and DCADs in the close-up diets on

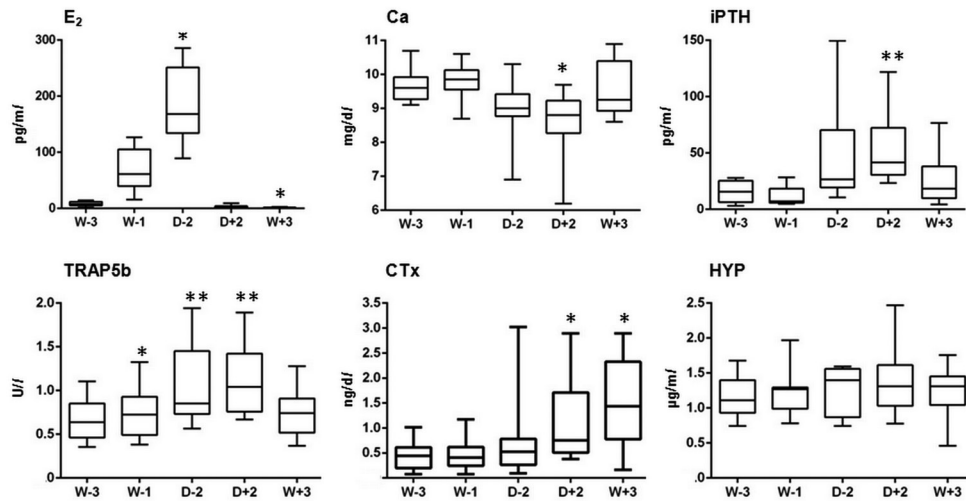


Fig. 1. Box-and-whisker plots (medians, interquartile ranges, maxima and minima) showing the plasma estradiol-17 $\beta$  ( $E_2$ ), calcium (Ca), intact-parathyroid hormone (iPTH), tartrate-resistant acid phosphatase isoform 5b (TRAP5b), carboxy-terminal collagen cross-links of type-I collagen (CTx) and hydroxyproline (HYP) levels around the time of parturition in Holstein dairy cows ( $n=10$ ). Significant differences are shown [compared with levels 3 weeks prepartum (W-3)] for each parameter (\*:  $P<0.05$ , \*\*:  $P<0.01$ ).

the 2 farms, because such factors affect Ca metabolism by changing tissue sensitivity to PTH [6]. However, there were no significant differences in plasma Ca and Mg concentrations between 2 farms at that time point (plasma Ca concentration: 9.9 mg/dl, 8.9–10.5 mg/dl in farm A and 9.9 mg/dl, 9.6–10.1 mg/dl in farm B; plasma Mg concentration: 2.1 mg/dl, 1.7–2.4 mg/dl in farm A and 1.9 mg/dl, 1.8–2.1 mg/dl in farm B).

Turning to bone resorption markers, the levels of TRAP5b, a lysosomal enzyme secreted by activated osteoclasts, are reportedly well correlated with osteoclast numbers [17]. CTx is a fragment of the peptide-bound metabolite of type I collagen, an important biochemical marker of bone resorption [1, 18]. This fragment is generated via breakdown of collagen type I mediated by osteoclast-derived acid proteases [1]. In the present study, plasma TRAP5b activity became significantly elevated commencing 1 week prepartum (W-1), and these levels were maintained up to a few days postpartum (D+2). Elevation of plasma TRAP5b activity in cows soon after parturition has been reported previously [9]. However, to the best of our knowledge, this is the first report to describe a rise in plasma TRAP5b activity commencing in the final gestational week. The plasma CTx concentrations in our cows increased significantly after parturition (postpartum D+2 to W+3), similar to findings of previous studies measuring the plasma levels of CTx [8, 12] or other breakdown fragments of collagen type I [11, 12] during early lactation in cows, goats and sheep. These observations on 2 bone markers appear to indicate that osteoclast numbers were elevated prepartum despite any effect of plasma  $E_2$ ; however, bone resorption mediated by osteoclasts was suppressed prepartum when plasma  $E_2$  levels were high and activated postpartum when plasma  $E_2$  levels decreased.

To date, osteoclasts have been thought to be solely responsible for removal of the bone matrix. Recent studies have shown that osteocytes can also remove the bone matrix by reversibly remodeling their perilacunar/canalicular matrix [10, 15]. Qing *et al.* [15] reported that osteocytes from lactating mice exhibited elevated expression of genes and proteins known to be utilized by osteoclasts, including TRAP5b and cathepsin K; these returned to virgin levels upon weaning, suggesting that the increased Ca demand caused by milk production induced osteocytic remodeling (osteocytic osteolysis) to remove mineralized matrix. Therefore, we suggest that the elevated plasma TRAP5b activity also reflects the number of TRAP5b-positive osteocytes involved in osteocytic remodeling in response to the increased Ca demand commencing 1 week prepartum in parturient dairy cows. However, a further study is needed to clarify the relationship between estrogen and osteocytic remodeling in parturient cows.

The plasma HYP concentrations of the present study did not fluctuate significantly in the time around parturition. HYP is an amino acid contributing to collagen orientation within the bone matrix and serves as a marker of bone resorption in cattle [11, 22]. However, the utility of HYP measurements is controversial, because plasma HYP is influenced by the diet and the metabolism of non-bony collagen, such as that of muscle, skin and liver [11, 22].

In summary, our present study suggests that osteoclast-mediated bone resorption is activated after parturition when the plasma  $E_2$  concentration decreases, following the suppression of osteoclast activity prepartum when the plasma  $E_2$  level was high. In addition, the fluctuation in plasma TRAP5b activity suggests that the number of osteocytes involved in osteocytic remodeling increases commencing

1 week prepartum in dairy cows. Further work is necessary to verify the utilities of these bone resorption markers in the monitoring of osteoclastic and/or osteocytic bone resorption around the time of parturition in dairy cows.

#### REFERENCES

- Allen, M. J. 2003. Biochemical markers of bone metabolism in animals: uses and limitations. *Vet. Clin. Pathol.* **32**: 101–113. [Medline] [CrossRef]
- Clarke, B. L. and Khosla, S. 2010. Physiology of bone loss. *Radiol. Clin. North Am.* **48**: 483–495. [Medline] [CrossRef]
- Dabev, D. and Struck, H. 1971. Microliter determination of free hydroxyproline in blood serum. *Biochem. Med.* **5**: 17–21. [Medline] [CrossRef]
- Devkota, B., Itagaki, K., Kim, D., Sasaki, K., Osawa, T., Furuhashi, K. and Yamagishi, N. 2012. Relationship between sex hormone fluctuations and biomarkers of bone resorption in bovine plasma during the oestrous cycle. *Vet. J.* **194**: 256–258. [Medline] [CrossRef]
- Edqvist, L. E., Ekman, L., Gustafsson, B. and Lindell, J. O. 1974. Estrone and progesterone plasma levels of normal cows and cows with parturient paresis. *Acta Vet. Scand.* **15**: 587–596. [Medline]
- Goff, J. P. 2014. Calcium and magnesium disorders. *Vet. Clin. North Am. Food Anim. Pract.* **30**: 359–381. [Medline] [CrossRef]
- Hollis, B. W., Draper, H. H., Burton, J. H. and Etches, R. J. 1981. A hormonal assessment of bovine parturient paresis: evidence for a role of oestrogen. *J. Endocrinol.* **88**: 161–171. [Medline] [CrossRef]
- Holtenius, K. and Ekelund, A. 2005. Biochemical markers of bone turnover in the dairy cow during lactation and the dry period. *Res. Vet. Sci.* **78**: 17–19. [Medline] [CrossRef]
- Kim, D., Yamagishi, N., Ueki, A., Miura, M., Saito, F., Sato, S. and Furuhashi, K. 2010. Changes in plasma bone metabolic markers in periparturient dairy cows. *J. Vet. Med. Sci.* **72**: 773–776. [Medline] [CrossRef]
- Khosla, S., Oursler, M. J. and Monroe, D. G. 2012. Estrogen and the Skeleton. *Trends Endocrinol. Metab.* **23**: 576–581. [Medline] [CrossRef]
- Liesegang, A., Eicher, R., Sassi, M. L., Risteli, J., Kraenzlin, M., Riond, J. L. and Wanner, M. 2000. Biochemical markers of bone formation and resorption around parturition and during lactation in dairy cows with high and low standard milk yields. *J. Dairy Sci.* **83**: 1773–1781. [Medline] [CrossRef]
- Liesegang, A., Risteli, J. and Wanner, M. 2006. The effects of first gestation and lactation on bone metabolism in dairy goats and milk sheep. *Bone* **38**: 794–802. [Medline] [CrossRef]
- Matsuo, A., Togashi, A., Sasaki, S., Devkota, B., Hirata, T. and Yamagishi, N. 2014. Diurnal variation of plasma bone markers in Japanese Black calves. *J. Vet. Med. Sci.* **76**: 1029–1032. [Medline] [CrossRef]
- Patel, O. V., Hirako, M., Takahashi, T., Sasaki, N. and Domeki, I. 1995. Sex steroid levels throughout gestation in cows carrying normal and malformed fetuses. *J. Vet. Med. Sci.* **57**: 659–663. [Medline] [CrossRef]
- Qing, H., Ardeshirpour, L., Pajevic, P. D., Dusevich, V., Jahn, K., Kato, S., Wysolmerski, J. and Bonewald, L. F. 2012. Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation. *J. Bone Miner. Res.* **27**: 1018–1029. [Medline] [CrossRef]
- Pyörälä, E., Pyörälä, S., Mero, M. and Adlercreutz, H. 1992. Oestrogens and milk fever—is there a link? *Acta Vet. Scand.* **33**: 335–339. [Medline]
- Rissanen, J. P., Suominen, M. I., Peng, Z. and Halleen, J. M. 2008. Secreted tartrate-resistant acid phosphatase 5b is a Marker of osteoclast number in human osteoclast cultures and the rat ovariectomy model. *Calcif. Tissue Int.* **82**: 108–115. [Medline] [CrossRef]
- Rosen, H. N., Moses, A. C., Garber, J., Iloputaife, I. D., Ross, D. S., Lee, S. L. and Greenspan, S. L. 2000. Serum CTX: a new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. *Calcif. Tissue Int.* **66**: 100–103. [Medline] [CrossRef]
- Sasaki, K., Yamagishi, N., Kizaki, K., Sasaki, K., Devkota, B. and Hashizume, K. 2014. Microarray-based gene expression profiling of peripheral blood mononuclear cells in dairy cows with experimental hypocalcemia and milk fever. *J. Dairy Sci.* **97**: 247–258. [Medline] [CrossRef]
- Sasser, R. G., Falk, D. E. and Ross, R. H. 1979. Estrogen in plasma of parturient paretic and normal cows. *J. Dairy Sci.* **62**: 551–556. [Medline] [CrossRef]
- Togashi, M., Tsujimoto, T., Yamauchi, K., Deguchi, Y., Hashizume, K., Kizaki, K., Honjou, S., Izaike, Y. and Osawa, T. 2009. Plasma and fecal sex steroid hormone profile during estrous cycle in a Japanese Serow (*Capricornis crispus*). *J. Reprod. Dev.* **55**: 412–417. [Medline] [CrossRef]
- Yamagishi, N., Takehana, K., Kim, D., Miura, M., Hirata, T., Devkota, B., Sato, S. and Furuhashi, K. 2009. Fluorometric method for measuring plasma tartrate-resistant acid phosphatase isoform 5b and its application in cattle. *J. Vet. Med. Sci.* **71**: 1637–1642. [Medline] [CrossRef]