NOTE *Internal Medicine*

Diurnal Variation of Plasma Bone Markers in Japanese Black Calves

Asako MATSUO[1)], Akira TOGASHI[1)], Kouya SASAKI[2)], Bhuminand DEVKOTA[1,3)], Toh-ichi HIRATA[4)] and Norio YAMAGISHI[1,2)]*

1)Cooperative Department of Veterinary Medicine, Iwate University, Morioka, Iwate, 020–8550, Japan
2)United Graduate School of Veterinary Sciences, Gifu University, Gifu, Gifu, 501–1193, Japan
3)Agriculture and Forestry University, Rampur, Chitwan, 44209, Nepal
4)Field Science Center, Faculty of Agriculture, Iwate University, Shizukuishi, Iwate 020–0581, Japan

(Received 9 January 2014/Accepted 4 March 2014/Published online in J-STAGE 9 March 2014)

ABSTRACT. To evaluate diurnal variation of plasma bone markers, blood samples were collected from five calves at 2-hr intervals throughout a 24-hr period. Tartrate-resistant acid phosphatase isoform 5b (TRAP5b), carboxy-terminal collagen crosslinks of type-I collagen (CTX), hydroxyproline, bone specific alkaline phosphatase (BALP) and osteocalcin were measured. Cosinor analysis showed a significant rhythm in all bone markers. The acrophase of each bone marker appeared from the early to late morning. The percentage ratio of the amplitude to mesor and the within-subject variability for CTx and osteocalcin were significantly larger than those for TRAP5b and BALP. This marked diurnal variation in five bone markers suggested that the time of blood sampling should be fixed when studying bone marker concentrations in bovine plasma.

KEY WORDS: blood sample, bone marker, cattle, diurnal variation.

doi: 10.1292/jvms.14-0021; *J. Vet. Med. Sci.* 76(7): 1029–1032, 2014

Bone remodelling is the main process of bone metabolism in humans and animals. Information on bone metabolism can be obtained by measuring biochemical bone markers in blood or urine derived from the activity of the cells involved [20]. In canine, feline and equine medicine, bone markers are used as non-invasive diagnostic tools for monitoring age-related trends in bone metabolism and the skeletal effects of exercise or several diseases [1, 5, 13]. The clinical application of bone markers to bovine medicine has not yet been established, although they may aid diagnosis of skeletal diseases in young cattle. Generally, bone formation markers are detectable in the blood (serum or plasma), whereas many bone resorption markers are detectable in both blood and urine [1]. Because it is difficult to collect frequent whole urine samples from cattle, the assessment of blood bone markers seems to be feasible as a routine laboratory procedure in bovine medicine. In humans and several animal species, the concentration of biochemical bone markers is higher during growth than at maturity and exhibits diurnal rhythms [1, 17, 22]. However, to our knowledge, few studies have focused on diurnal variation of plasma bone markers in cattle. Here, we examined the diurnal variations of several bone markers in plasma of healthy young cattle.

The study protocol and experimental design were approved by the Iwate University Laboratory Animal Care and Use Committee. Five castrated Japanese Black male calves (age 7.2 to 9.0 months, weight 210 to 300 kg) at the Iwate University Farm were used. The calves were castrated at least 2 months before the experiment. They were kept in two adjoining pens and fed grass hay and commercial grains and given water *ad libitum*. The day-light hour on the day of experiment was from 4:42 to 18:39 hr. All animals were declared healthy by clinical examinations. One intravenous catheter (UK catheter kit UA-1608-S, 16G, 20 cm, Unitika Medical, Osaka, Japan) was inserted into one jugular vein in each calf. The next day, 13 heparinized blood samples were collected at 2-hr intervals from 10:00 to the next 10:00 (10:00b) hr. The blood was placed on ice immediately and centrifuged, and the separated plasma was frozen at −60°C until analysis.

Plasma TRAP5b activity was measured fluorometrically [22]. Briefly, 10 μl of the plasma samples were added to 50 μl of substrate consisting of 0.25 mM naphthol-ASBI-phosphate (N-ASBI-P) in 100 mM sodium acetate buffer containing 50 mM sodium tartrate, 2% Nonidet P-40 (NP-40), 1% ethylene glycol monomethyl ether (EGME) and heparin (23 U/m*l*) adjusted to pH 6.6. The reaction was carried out for 30 min at 37°C and stopped by adding 125 μl of 0.1 M sodium hydroxide (NaOH) containing 0.05% NP-40. Reagent blanks were prepared for each plasma sample by adding 125 μl of 0.1 M NaOH containing 0.05% NP-40. A standard calibration curve was constructed using acid phosphatase solutions of known concentration (Wako Pure Chemical Industries, Osaka, Japan). Fluorescence was measured with an excitation wavelength of 405 nm and a peak emission wavelength of 535 nm. Plasma CTx concentration was assayed using an ELISA kit (Serum CrossLaps ELISA, Nordic Bioscience Diagnostics, Herlev, Denmark; minimum detection limit=0.02 *n*g/m*l*). The plasma HYP concentration was determined spectrophotometrically [4]. Plasma BALP

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

Table 1. Rhythm parameters and variability of five bone markers over 24 hr in five Japanese Black calves

	Mesor [1)]	Amplitude [2)]	Acrophase [3)]	Amp/Mes [4)]	iCV	*P* value [5)]
TRAP5b	2.18 ± 0.64 U/*l*	0.21 ± 0.08 U/*l*	11:40 ± 02:37 hr	10.1 ± 3.0% [a)]	6.1 ± 1.7% [a)]	0.00004
CTx	0.58 ± 0.14 *n*g/m*l*	0.10 ± 0.03 *n*g/ m*l*	2:00 ± 5:28 hr	17.7 ± 4.4% [b)]	11.0 ± 2.6% [b)]	0.003
HYP	2.91 ± 0.20 μg/ m*l*	0.32 ± 0.08 μg/ m*l*	9:00 ± 3:05 hr	11.1 ± 2.4% [a)]	8.0 ± 2.3%	0.0003
BALP	27.4 ± 8.0 U/*l*	2.67 ± 0.77 U/*l*	8:55 ± 7:03 hr	10.1 ± 2.6% [a)]	5.9 ± 2.0% [a)]	0.0003
OC	43.2 ± 10.7 *n*g/m*l*	6.8 ± 3.9 *n*g/m*l*	9:57 ± 2:35 hr	15.6 ± 7.0%	10.8 ± 5.8% [b)]	0.015

1) Mesor: midline estimating statistics of the rhythm of the fitted cosine curve. 2) Amplitude: one half of the difference between the highest and lowest point of the fitted cosine curve. 3) Acrophase: occurrence of maximum in hours, which may not take place at the time during which maximum concentration was observed. 4) Amp/Mes: estimated amplitude of mesor expressed as a percentage. 5) *P* values were derived from cosinor periodicity. a, b) Values with different superscripts within Amp/Mes or iCV (within-subject variavility) indicate significant differences ($P<0.05$).

activity was measured spectrophotometrically using the heat-inactivation method [15]. Briefly, 200 μl of plasma samples were incubated at 56°C for 15 min. The samples were then cooled on ice, and duplicate 25 μl aliquots were removed for the measurement of heat-insensitive ALP isoenzyme. Plasma activities of total ALP and heat-insensitive ALP isoenzyme were determined using a LabAssay ALP kit (Wako Pure Chemical Industries). BALP was calculated by subtracting the activity of the heated sample from the total activity of the unheated sample. The plasma OC concentration was determined immunoradiometrically using a BGP IRMA Mitsubishi kit (Mitsubishi Chemical Medience, Tokyo, Japan; minimum detection limit=1 *n*g/m*l*). The intra- and inter-assay coefficients of variation (CVs) were as follows: TRAP5b: 5.6 and 7.8%; CTx: 6.3 and 7.2%; HYP: 8.7 and 8.1%; BALP: 7.9 and 10.0%; OC: 7.4 and 5.8%, respectively.

All numerical data were expressed as means ± standard deviations (SD). Diurnal rhythmicity was analyzed using a cosinor procedure using the Cosinor Periodicity software (www.circadian.org/softwar.html). The Acro software (http://www.circadian.org/softwar.html) was used to compute the mesor (midline estimating statistics of the rhythm of the fitted cosine curve), the amplitude (one half of the difference between the highest and lowest point of the fitted cosine curve) and the acrophase (occurrence of maximum in hours, which may not take place at the time during at which the maximum concentration was observed) of each bone marker in each animal, using the best-fitting cosine wave. The ratio of the amplitude to the mesor was expressed as a percentage (Amp/Mes) and was calculated as a quantitative measure of variability [19]. Within-subject variability (iCV) was also calculated to evaluate the daily variation over 24 hr using the formula for CV [14]. The differences in the Amp/Mes and CVs among five bone markers were checked for normal distribution using Kolmogorov–Smirnov normality tests and assessed by repeated measures ANOVA and Tukey's multiple comparison test or by Friedman test and Dunn's multiple comparison test. To evaluate changes in plasma bone marker levels, the values were checked for normal distribution using Kolmogorov–Smirnov normality tests, and assessed by repeated measures ANOVA and Dunnett's test or by Friedman test and Dunn's multiple comparison test, using the maximal value of each parameter. These statistical analyses were performed using Prism version 6 for Windows (GraphPad Software Inc., La Jolla, CA, U.S.A.). The level of significance was set at $P<0.05$.

Table 1 shows the results of rhythm parameters analyzed by the cosinor procedure and the variability of five bone markers in calves. The mean mesors of plasma TRAP5b, CTx, HYP, BALP and OC were 2.18 U/*l*, 0.58 *n*g/m*l*, 2.91 μg/m*l*, 27.4 U/*l* and 43.2 *n*g/m*l*, respectively. The acrophase of each bone marker was from 02:00 to 11:40 hr. The Amp/Mes values of TRAP5b, HYP and BALP were significantly lower than that of CTx ($P<0.05$). The iCVs of TRAP5b and BALP were significantly lower than those of CTx and OC ($P<0.05$). Significant rhythm was observed in all bone markers ($P=0.015$ to 0.00004).

Figure 1 shows the changes in plasma concentrations of five bone markers during 24 hr in five calves. The maximum plasma TRAP5b activity was shown at 12:00 (2.4 ± 0.7 U/*l*) and was significantly higher than the levels from 16:00 to 10:00 hr ($P<0.05$ or 0.01), except at 4:00 hr. Plasma CTx levels peaked at 10:00 hr (0.62 ± 0.14 *n*g/m*l*), but did not change significantly during the 24-hr period. The maximum plasma HYP concentration was shown at 10:00b hr (3.4 ± 0.5 μg/m*l*) and was significantly higher than that at 18:00 hr ($P<0.05$). Plasma BALP activity was highest at 10:00 hr (29.4 ± 8.6 U/*l*) and was significantly higher than at 20:00, 4:00, 6:00 and 8:00 hr ($P<0.05$ or 0.01). Plasma OC peaked at 10:00b hr (49.2 ± 11.0 *n*g/m*l*) and was significantly higher than at 20:00 and 22:00 hr ($P<0.05$).

Bone metabolic parameters are influenced by factors, such as age, nutrition, exercise [20] and the time of blood sampling [11]. To exclude all factors other than the timing of blood collection, the present study involved 5 cattle on one farm with similar age, rearing environment and castration status; these cattle underwent simultaneous blood samplings within the same 24-hr period. In the present study, significant rhythm was observed with the cosinor procedure in all measured plasma bone markers.

A pattern of diurnal variation has been identified in several bone markers in humans and several other animal species. Among the resorption markers, peak serum TRAP5b activity has been reported to occur at ~9:00, with a diurnal rhythm of small amplitude, in humans [9]. Plasma concentrations of CTx have been shown to peak during the late night to early morning in humans [17, 21], canines [11] and equines [3].

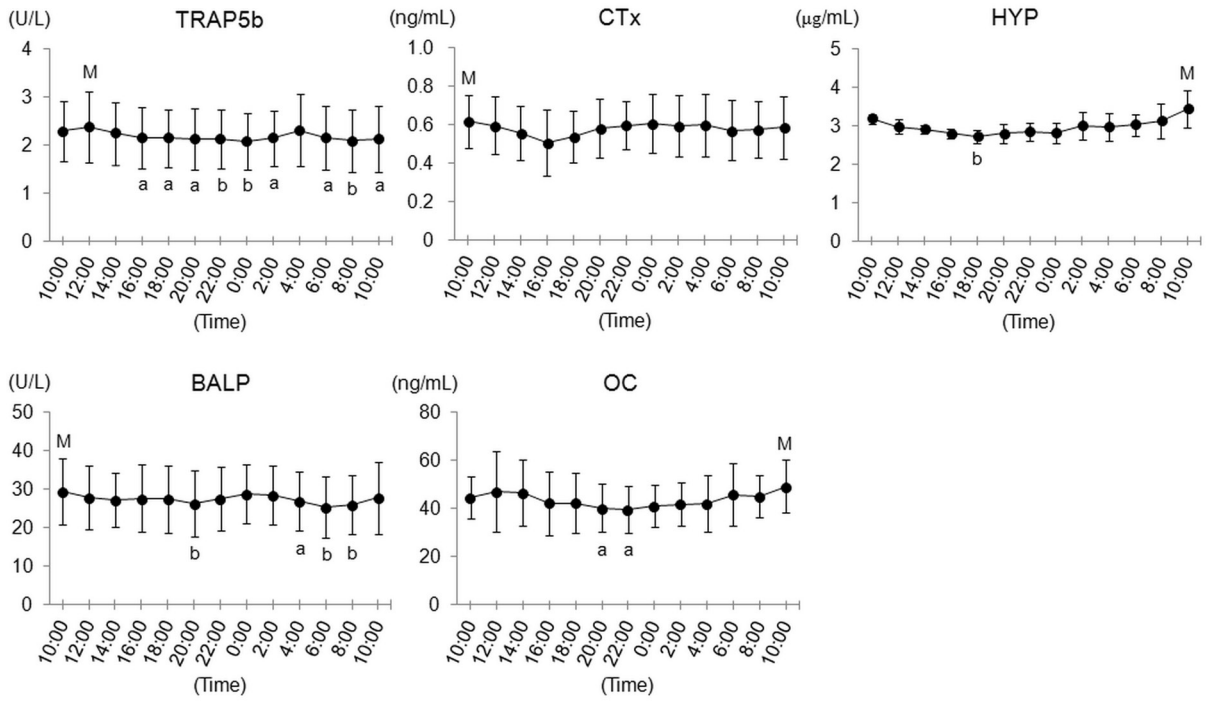

Fig. 1. Changes in plasma concentrations of tartrate-resistant acid phosphatase isoform 5b (TRAP5**)**, carboxy-terminal collagen crosslinks of type-I collagen (CTx), hydroxyproline (HYP), bone specific alkaline phosphatase (BALP) and osteocalcin (OC) in Japanese Black calves (n=5) over 24 hr. Data are expressed as means ± SD. Significant differences are shown compared with the maximal value (M) for each bone marker (a: $P<0.05$, b: $P<0.01$).

Beagle dogs on dietary HYP restriction revealed no obvious diurnal variations in serum HYP concentration [18]. Among the bone formation markers, serial measurements of plasma OC revealed increased levels during the late night to early morning in humans [9], equines [3] and canines [11]. Plasma BALP activities in humans and canines peak between late morning and early afternoon [7, 11], whereas one report on plasma BALP showed no apparent diurnal pattern in humans [6]. In the present study, the acrophase in all bone markers occurred from the early to late morning, suggesting that formation and resorption are higher during the night to morning than during the day in cattle.

In the present study, both the Amp/Mes and iCV of plasma CTx concentrations were significantly greater than those of plasma TRAP5b and BALP activities. Similarly, the iCV of plasma OC concentration was higher than those of plasma TRAP and BALP. These observations suggest that the amplitude and variability of CTx and OC are larger than those of other bone markers, in accordance with previous reports of a smaller amplitude and a diurnal rhythm for TRAP5b than for CTx [10, 21]. The reason for this difference is unknown, but the mechanism of clearance of these resorption markers differs substantially. TRAP5b is not cleared directly by the kidney, whereas CTx is cleared largely through glomerular filtration [10]. The daily fluctuation in the serum OC level was greater than that in the serum BALP level in camels [2]. These bone formation markers have different half-lives; that of OC is ~5 min [2, 12], while BALP has a longer half-life of 1–2 days [11].

The etiology of the daily rhythms in the various biochemical parameters of bone metabolism is not completely understood. The timing and magnitude of these rhythms appear to be constrained by the light:dark cycle, feeding schedule and endocrine relationship [16]. Several published observations suggest that parathyroid hormone and glucocorticoids might play a role in the synchronization of the bone metabolism periodicity [16]. In addition, age and/or gender differences in blood bone markers have been reported in humans [8]. Therefore, further studies are necessary to examine the etiological factors involved in the bone metabolism periodicity of cattle.

In conclusion, marked diurnal variations in the five measured bone markers were observed in calves. Therefore, it is important to fix the time of blood sampling when assessing concentrations of these bone markers in bovine plasma.

REFERENCES

1. Allen, M. J. 2003. Biochemical markers of bone metabolism in animals: uses and limitations. *Vet. Clin. Pathol.* **32**: 101–113. [Medline] [CrossRef]
2. Al-Sobayil, F. A. 2010. Circadian rhythm of bone formation biomarkers in serum of dromedary camels. *Res. Vet. Sci.* **89**: 455–459. [Medline] [CrossRef]

3. Black, A., Schoknecht, P. A., Ralston, S. L. and Shapses, S. A. 1999. Diurnal variation and age difference in the biochemical markers of born turnover in horses. *J. Anim. Sci.* **77**: 75–83. [Medline]
4. Dabev, D. and Struck, H. 1971. Microliter determination of free hydroxyproline in blood serum. *Biochem. Med.* **5**: 17–21. [Medline] [CrossRef]
5. DeLaurier, A., Jackson, B., Ingham, K., Pfeiffer, D., Horton, M. and Price, J. 2002. Biochemical markers of bone turnover in the domestic cat: relationships with age and feline osteoclastic resorptive lesions. *J. Nutr.* **132**: 1742S–1744S. [Medline]
6. Generali, D., Berruti, A., Tampellini, M., Dovio, A., Tedoldi, S., Bonardi, S., Tucci, M., Allevi, G., Aguggini, S., Milani, M., Bottini, A., Dogliotti, L. and Angeli, A. 2007. The circadian rhythm of biochemical markers of bone resorption is normally synchronized in breast cancer patients with bone lytic metastases independently of tumor load. *Bone* **40**: 182–188. [Medline] [CrossRef]
7. Greenspan, S. L., Dresner-Pollak, R., Parker, R. A., London, D. and Ferguson, L. 1997. Diurnal variation of bone mineral turnover in elderly men and women. *Calcif. Tissue Int.* **60**: 419–423. [Medline] [CrossRef]
8. Gundberg, C. M., Looker, A. C., Nieman, S. D. and Calvo, M. S. 2002. Patterns of osteocalcin and bone specific alkaline phosphatase by age, gender, and race or ethnicity. *Bone* **31**: 703–708. [Medline] [CrossRef]
9. Hannon, R. and Eastell, R. 2000. Preanalytical variability of biochemical markers of bone turnover. *Osteoporos. Int.* **11** Suppl 6.: S30–S44. [Medline] [CrossRef]
10. Hannon, R. A., Clowes, J. A., Eagleton, A. C., Hadari, A. A., Eastell, R. and Blumsohn, A. 2004. Clinical performance of immunoreactive tartrate-resistant acid phosphatase isoform 5b as a marker of bone resorption. *Bone* **34**: 187–194. [Medline] [CrossRef]
11. Ladlow, J. F., Hoffmann, W. E., Breur, G. J., Richardson, D. C. and Allen, M. J. 2002. Biological variability in serum and urinary indices of bone formation and resorption in dogs. *Calcif. Tissue Int.* **70**: 186–193. [Medline] [CrossRef]
12. Lee, A. J., Hodges, S. and Eastell, R. 2000. Measurement of osteocalcin. *Ann. Clin. Biochem.* **37**: 432–446. [Medline] [CrossRef]
13. Lepage, O. M., Carstanjen, B. and Uebelhart, D. 2001. Non-invasive assessment of equine bone: An update. *Vet. J.* **161**: 10–22. [Medline] [CrossRef]
14. Lovie, P. 2005. Coefficient of variation, pp. 317–318. *In*: Encyclopedia of Statistics in Behavioral Science, vol. 1 (Everitt, B. S. and Howell, D. C. eds.), John Wiley & Sons Ltd, Chichester.
15. Mohebbi, A., Khaghani, A. and Mohammadnia, A. 2010. Bone-specific alkaline phosphatase activity in dairy cows. *Comp. Clin. Pathol.* **19**: 33–36. [CrossRef]
16. Ostrowska, Z., Kos-Kudla, B., Marek, B., Kajdanjuk, D. and Ciesielska-Kopacz, N. 2002. The relationship between the dairy profile of chosen biochemical markers of bone metabolism and melatonin and other hormone secretion in rats under physiological condition. *Neuroendocrinol. Lett.* **23**: 417–425. [Medline]
17. Seibel, M. J. 2005. Biochemical markers of bone turnover. Part I: biochemistry and variability. *Clin. Biochem. Rev.* **26**: 97–122. [Medline]
18. Svanberg, G. K. 1988. Biological variations in serum total hydroxyproline concentration in the beagle dog. *Lab. Anim.* **22**: 157–161. [Medline] [CrossRef]
19. Veldhuis, J. D., Iranmanesh, A., Johnson, M. L. and Lizarralde, G. 1990. Twenty-four-hour rhythms in plasma concentrations of adenohypophyseal hormones are generated by distinct amplitude and/or frequency modulation of underlying pituitary secretory bursts. *J. Clin. Endocrinol. Metab.* **71**: 1616–1623. [Medline] [CrossRef]
20. Watts, N. B. 1999. Clinical utility of biochemical markers of bone remodeling. *Clin. Chem.* **45**: 1359–1368. [Medline]
21. Wichers, M., Schmidt, E., Bidlingmaier, F. and Klingmüller, D. 1999. Diurnal rhythm of crosslaps in human serum. *Clin. Chem.* **45**: 1858–1860. [Medline]
22. Yamagishi, N., Takehana, K., Kim, D., Miura, M., Hirata, T., Devkota, B., Sato, S. and Furuhama, 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]