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journal or publication title	Journal of Veterinary Medical Science
volume	80
number	1
page range	63-67
year	2017-12
URL	http://id.nii.ac.jp/1588/00004149/

doi: info:doi/10.1292/jvms.17-0465





NOTE

Wildlife Science

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80(1): 63–67, 2018

doi: 10.1292/jvms.17-0465

Received: 19 August 2017

Accepted: 7 November 2017

Published online in J-STAGE:
17 November 2017

ABSTRACT. The blood biochemical analysis of bone markers could have a role in the early diagnosis of metabolic bone disease in animals; however, there is limited information on bone markers in captive Asian elephants (*Elephas maximus*). Serum samples from ten captive Asian elephants were obtained to clarify the relationship between age and the blood bone markers tartrate-resistant acid phosphatase isoform 5b (TRAP5b) and bone specific alkaline phosphatase (BALP). Serum TRAP5b and BALP activities were negatively correlated with age. A positive correlation was observed between TRAP5b activity and BALP activity. These results may contribute to the health management of captive Asian elephants.

KEY WORDS: Asian elephant (*Elephas maximus*), bone marker, bone specific alkaline phosphatase (BALP), tartrate-resistant acid phosphatase isoform 5b (TRAP5b)

Asian elephants (*Elephas maximus*), one of the largest land mammals, are popular at zoological parks, safari parks, and national wildlife sanctuaries. According to recent estimates, about 50,000 Asian elephants live in Thailand, Sri Lanka, India, Laos, Vietnam, Malaysia, Indonesia, and other Southeast Asian countries. Almost 14,000 captive Asian elephants are kept in their countries of origin, and 1,000 are kept in zoos and circuses in western countries. The number of Asian elephants has declined in recent years because of decreased habitat caused by the expansion of agricultural areas and disafforestation or excess hunting for ivory. The Asian elephant is currently listed as “endangered” on the IUCN Red List of threatened species.

Ninety-one captive Asian elephants were kept in Japanese zoological parks in 2016. The first report of the captive breeding of Asian elephants was in 2004; since then, eleven successful breeding cases of captive Asian elephants have been reported. Zoological experts predict that captive Asian elephants are destined to become extinct in the next 50 years in Japan. The worldwide mortality rate is high among captive Asian elephants <5 years old [13]. Therefore, the success of breeding and rearing is important for maintaining the number of captive Asian elephants in Japan and other countries.

To date, three elephant calves that were bred in Japan suffered from astasia and suspected bone disease. Therefore, the status of bone metabolism is important for the success of raising elephant calves. Blood biochemical analysis of bone markers is a diagnostic tool to evaluate bone metabolism. Blood bone markers are used for monitoring age-related trends in bone formation and resorption, the effects of exercise, fracture healing, skeletal neoplasia, and subchondral bone changes associated with osteoarthritis in canine, equine, and bovine medicine [2, 7]. Several reports in Asian elephants have considered the serum activities of select bone markers such as bone specific alkaline phosphatase (BALP) and osteocalcin as bone formation markers, and cross-linked telopeptide domain of type I collagen as a bone resorption marker [3, 12, 22].

Tartrate-resistant acid phosphatase (TRAP) belongs to the family of acid phosphatase isoenzymes found in bone, erythrocytes, platelets, the spleen, and the prostate [6]. TRAP isoform 5b (TRAP5b) is a lysosomal enzyme secreted by activated osteoclasts [6]; circulating levels of this isoform are reportedly well correlated with the number of osteoclasts in bone [18]. Few studies have evaluated blood TRAP5b activity in Asian elephants, but we have reported a fluorometric method to measure circulating TRAP5b activity in cattle [14, 25]. Here, we clarified the relationship between serum TRAP5b and BALP activities in clinically healthy captive Asian elephants at different ages.

Ten healthy Asian elephants (three males aged 3 months to 25 years and seven females aged 3 to 39 years) at Ichihara Elephant Kingdom Zoological Park were studied (Table 1). They were kept in an elephant-keeping zoo with indoor and outdoor enclosures and fed grass hay, commercial grain, and some vegetables and fruits, and given water *ad libitum*. One elephant calf (male, 3

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Table 1. Data from the ten captive Asian elephants (*Elephas maximus*) in this study

No.	Age (years)	Sex	TRAP5b ^a (U/l)	BALP ^b (U/l)	B/T index ^c
1	39	Female	0.8	71.7	9.3
2	30	Female	2.4	160.3	6.7
3	26	Female	1.2	126.3	10.8
4	25	Male	1.8	342.3	19.2
5	25	Female	3.2	243.8	7.6
6	18	Female	3.7	302.0	8.2
7	9	Female	5.9	398.0	6.7
8	3	Female	8.9	551.0	6.2
9	2	Male	8.8	531.0	6.0
10	0.25	Male	4.5	1,076.8	24.1

a) TRAP5b: tartrate-resistant acid phosphatase isoform 5b, b) BALP: bone-specific alkaline phosphatase, c) B/T index: serum BALP activity (U/l)/serum TRAP5b activity (U/l) × 10.

months old) was raised by human caretakers and fed a mixture of goat's milk (Kimura goat farm, Oita, Japan) and artificial elephant's milk (Morinyu Sunworld, Tokyo, Japan) once per hour. All elephants went to a playground with mahouts to exercise twice daily and performed for park visitors. The animals were declared healthy by clinical examinations.

Blood was withdrawn from the auricular vein using a 5-ml disposable syringe (Nipro Medical, Osaka, Japan) with a 23-G butterfly needle (Top Medical, Tokyo, Japan) and transferred into a blood collection tube (GP-SP1029, Nipro Neo-tube; Nipro Medical). After the blood clotted and was centrifuged, the serum samples were frozen at -20°C until analysis.

Serum TRAP5b activity was measured fluorometrically [14, 25]. Briefly, 10 µl of serum were added to 50 µl of a substrate solution consisting of 0.25 mM N-ASBI-P (Wako Pure Chemical Industries, Osaka, Japan) in 100 mM sodium acetate and 50 mM sodium tartrate (Wako Pure Chemical Industries) buffer. The solution contained 2% Nonidet P-40 (NP-40; BioVision, Mountain View, CA, U.S.A.), 1% ethylene glycol monomethyl ether (Wako Pure Chemical Industries) and heparin (23 U/ml) and 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 NaOH containing 0.05% NP-40 (stop solution). Reagent blanks were prepared for each serum sample by adding 125 µl of stop solution. A standard calibration curve was constructed using acid phosphatase solutions of known concentrations (Wako Pure Chemical Industries). Fluorescence was measured at an excitation wavelength of 405 nm and a peak emission wavelength of 535 nm.

Serum BALP activity was measured spectrophotometrically using the wheat germ lectin technique [4]. Briefly, 30 µl of serum sample was incubated with 30 µl of wheat germ lectin (lectin from *Triticum vulgare*; Sigma-Aldrich, St. Louis, MO, U.S.A.) solution (5 mg/ml) and 3 µl of diluted Triton-X (Wako Pure Chemical Industries) at 37°C for 30 min. The mixtures were cooled on ice and centrifuged at 2,000 × g for 10 min; then, duplicate 20 µl aliquots were removed to measure lectin-insensitive alkaline phosphatase (ALP) isoenzyme levels. Serum activities of total ALP and the lectin-insensitive ALP isoenzyme were determined using a LabAssay ALP kit (Wako Pure Chemical Industries). BALP was calculated by subtracting the activity of the lectin-insensitive aliquot from the total activity of the unheated sample.

The ratio of BALP to TRAP5b (B/T index) was calculated in the serum of each elephant as shown in the following equation because the ratio and index applying two bone markers has been speculated to be more informative than individual markers [24]:

$$B/T \text{ index} = \text{serum BALP activity} / (\text{serum TRAP5b activity} \times 10).$$

All statistical analyses were performed using Prism ver. 6 for Windows (GraphPad Software Inc., La Jolla, CA, U.S.A.). The threshold for statistical significance was $P < 0.05$. Spearman's rank correlation analysis was performed with the data for all ten elephants to evaluate the significance of the correlation between the serum activities of each serum parameter (TRAP5b, BALP, or B/T index) and age or between the two bone markers (TRAP5b and BALP).

Table 1 shows the serum TRAP5b and BALP activities and the B/T index in the ten elephants. Serum TRAP5b activity was 4.5 U/l in a 3-month-old elephant calf and ranged from 0.8 to 8.9 U/l in elephants aged 2–39 years old. Serum BALP activity was highest in a 3-month-old elephant calf (1,076.8 U/l), whereas the levels seemed to fall with increasing age in the other elephants aged 2–39 years old (551.0–71.7 U/l). The B/T index, which shows the balance of bone condition that is too small wave to define in individual markers, was high in two elephants aged 3 months and 25 years old (24.1 and 19.2, respectively) and ranged between 6.0 and 10.8 in the other eight elephants.

Spearman's rank correlation analysis revealed that serum TRAP5b and BALP activities (Fig. 1A and 1B) were negatively correlated with age ($\rho = -0.87$, $P = 0.002$ and $\rho = -0.95$, $P < 0.0001$, respectively). Serum TRAP5b activity was positively correlated with serum BALP activity ($\rho = 0.86$, $P = 0.003$; Fig. 1C). The B/T index did not correlate with the age of any of the elephants ($\rho = -0.40$, $P = 0.09$; Fig. 1D).

Serum and/or plasma TRAP5b activities have been measured in cattle [14, 25], sheep [19], and dogs [20]. TRAP5b is a good indicator of bone resorption because serum TRAP5b activity has low diurnal variability without any effects of non-bony collagen metabolism, feeding, and renal or hepatic failure and reflects the number of osteoclasts [18]. The present study detected a negative correlation between serum TRAP5b activity and age, suggesting that the number of osteoclasts decreased in accordance with increasing elephant age. This result coincides with previous data on other blood bone resorption markers reported previously in

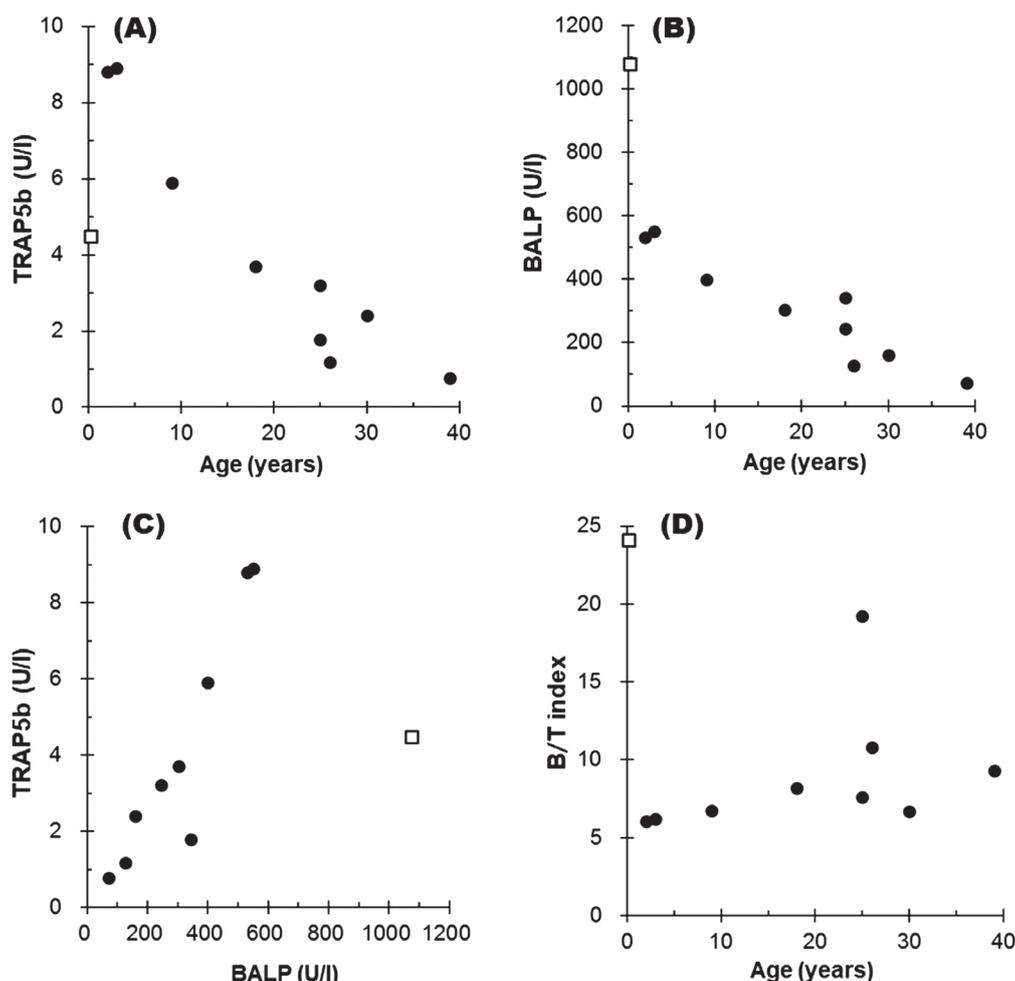


Fig. 1. Scatter diagrams showing the relationship between each bone marker and age in the ten elephants. (A) Correlation between serum tartrate-resistant acid phosphatase isoform 5b (TRAP5b) activity with age. (B) Correlation between serum bone-specific alkaline phosphatase (BALP) activity with age. (C) Correlation between serum TRAP5b with BALP activities in the ten elephants. (D) Correlation between the B/T index with age in the ten elephants. White square: 3-month-old elephant calf. Black circles: values in nine elephants aged >2 years. B/T index: BALP to TRAP5b ratio.

Asian elephants and other mammals [3, 16, 17, 25].

BALP, a non-collagenous protein secreted by osteoblasts, is essential for bone mineralization and is a highly specific marker of osteoblast function [6]. The present study showed that serum BALP activity was negatively correlated with age. This result corresponds with previous serum BALP data in Asian elephants [3, 12], suggesting that bone mineralization and the function of the osteoblasts were inactivated with increasing age of the elephants.

The present study revealed a significant positive correlation between TRAP5b and BALP activities in the sera of the Asian elephants, suggesting that osteoclast-mediated bone resorption and osteoblast-mediated bone formation were well balanced during bone turnover. It has been reported that bone formation markers are closely correlated with bone resorption markers in clinically healthy humans [17], horses [16], and cattle [25], whereas this balance of formation and resorption may be dissociated under certain disease conditions or physiological situations during changing calcium homeostasis [7, 21].

The proportions of BALP and TRAP5b activities in human serum reveal the balance between the two markers [24]. In the present study, the BALP to TRAP5b ratio in the sera of the elephants was calculated as the B/T index, which was not correlated with age. The B/T index in eight elephants (nos. 1–3 and 5–9) was 6.0–10.8, whereas the values in the other two elephants (nos. 4 and 10) were twice as high (19.2 and 24.1) than those in the other eight elephants.

Elephant no. 4 was a 25-year-old adult male and the only bull in the present study. Previous reports have described that the blood levels of bone markers may be influenced by sex in humans [17] and other animals [5, 9]. Androgens in males have suppressive effects on osteoclastogenesis, supporting osteoblast activity [11]. Male adult Asian elephants have a period of elevated androgen levels and heightened sexual activity, which is called musth [8]. Elephants in musth show a significant elevation in fecal testosterone levels [8]. Therefore, further study is needed to evaluate serum TRAP5b and BALP activities and the B/T index in bull elephants during musth and non-musth periods by measuring testosterone levels.

Elephant no. 10 was a 3-month-old male calf, which was fed milk by human caretakers. This calf seemed to show outlier levels of TRAP5b and BALP compared with the other calves aged 2 to 9 years (nos. 7–9), although serum TRAP5b and BALP activities were negatively correlated with the age of all ten elephants (Fig. 1A and 1B). Namely, the serum TRAP5b and BALP activities in this 3-month-old elephant appeared to be lower and higher, respectively, than their predicted levels. It has been reported that passive exercise with gentle joint compression reduces bone resorption and increases bone formation in human infants [23]. Moreover, lactoferrin in bovine milk is a promoter of osteoblast growth and an inhibitor of osteoclastogenesis [10, 15]. Limited information is available on lactoferrin and the other components in the natural elephant milk [1] and artificial milk used in this study. Therefore, further study is necessary to clarify the factors affecting the balance of osteoclastic and osteoblastic bone markers during the neonatal and nursing periods in Asian elephants.

In summary, serum TRAP5b activity in conjunction with BALP levels were negatively correlated with age in ten captive Asian elephants. The age-related changes in serum TRAP5b and BALP activities appeared to indicate a decrease in the number of osteoclasts and inactivity of osteoblasts with increasing age in the elephants. A positive correlation was observed between TRAP5b and BALP activities in the sera of the elephants. The B/T index ranged from 6.0 to 10.8 in eight of the ten elephants. These data suggest that osteoclast-mediated bone resorption was well balanced with osteoblast-mediated bone formation in most of the elephants. Therefore, measuring TRAP5b and BALP in sera may contribute to the health management of captive Asian elephants. To establish clinical application in the future, we need additional study about bone metabolism in Asian elephants because this study of sample was low and there was a few information about this field.

ACKNOWLEDGMENTS. We would like to thank all staff and students of Obihiro University of agriculture and veterinary medicine who assisted us. We express our gratitude to Dr. Preecha P. (Thai Elephant Hospital), Dr. K. Hanaki (Kobe Oji zoo), Dr. N. Imada (Toyohashi Zoo & Botanical Park), Dr. Y. Hirano (Ueno Zoological Gardens) who gave advices and cooperated. We thank Mr. R. Kunitachi, Mr. Loy S., Mr. Nattapong S., Mr. Prateep S., Mr. Suriya S., Mr. Arthit A., Mr. Thanawut S. and all Japanese staff and Thai mahouts of Ichihara Elephant Kingdom zoological park who helped collecting samples. Finally, we are grateful to Ms. S. Sakamoto, the curator of Ichihara Elephant Kingdom zoological park.

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