ORIGINAL ARTICLE

Circadian variations in biochemical markers of bone metabolism in horse of different age

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Summary

The author studied in thirty Thoroughbred horses the influence of age and gender on daily rhythms of serum osteocalcin (OC). The animals were divided into two groups. Group A: six male and six female 2 years old; Group B: six male, six female and six geldings 6 years old. They were housed individually in box-like stalls under natural photoperiod and environmental conditions. Blood samples were collected every 3 hours over a 48 hour period. Statistical differences of serum OC concentration due to different gender were observed in both Groups A and B. Daily rhythms of serum OC concentration were observed only in Group B, with nocturnal acrophase. In females the acrophase was statistically postponed for 1 hour compared to male and gelding. Male and female showed a more robust daily rhythm than the geldings. The results showed that blood sampling for determination of serum OC should be strictly standardized with regard to the time of the day.

Key words: circadian rhythms; osteocalcin; age; gender; horses

INTRODUCTION

Bone turnover is characterized by two opposing, but complementary, metabolic activities: the resorption of old bone by osteoclastic cells and the deposition of newly formed bone by osteoblasts. Age and rate of growth at weaning may have an effect on the rate of bone formation. The rate of bone turnover can be

assessed *in vivo* by measuring the serum, plasma or urine concentrations of specific biochemical markers, which, although mostly developed for studies of human bone metabolism, have in some cases been tested also in the horse (McIlwraith 2005).

The high incidence of orthopaedic diseases in young and adult athletic horses raises questions concerning the factors that contribute to these problems, as well as a need to detect and monitor them. Various techniques are described to non-invasively evaluate and monitor equine bone (Jeffcott et al. 1988, Lepage et al. 2001). Most of them, however, are expensive, time consuming or require specialized staff. Interestingly, osteocalcin (OC) has been extensively used to assess bone metabolism in this species. OC is a small abundant non-collagenous calcium binding protein, indigenous to the organic matrix of bone dentin and possibly other mineralized tissues which circulate in the blood.

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This protein is synthesised by osteoblasts, incorporated in new bone matrix and released into the circulation during bone resorption. As a result, its serum level reflects bone turnover (Khosla and Kleerekoper 2003). Several studies have demonstrated that serum OC as a marker of bone metabolism provides useful information in metabolic bone diseases and in the management of their treatment (Lepage et al. 1992). However, before markers of bone turnover can be applied to study orthopaedic diseases, sources of variation, such as age differences and diurnal rhythms, must be described to allow appropriate sample collection. Studies conducted on 2 and 3 year old Thoroughbreds indicated that bone biomarkers have a better predictive value of bone diseases in older horses or when measured serially in the same animals (Jackson et al. 2009).

In Thoroughbred and Standardbred horses, age-related changes in circulating levels of bone markers have been observed, and changes reflect the decrease in bone turnover with increasing skeletal maturity (Lepage et al. 1990, Price et al. 1995). In juvenile horses consistent age related patterns in biomarker serum concentrations were found, indicating a markedly higher metabolism before age 20 weeks. In these horses OC concentrations were not affected by feeding level (Donabédian et al. 2008). An age-related decrease in concentrations of bone markers was also seen in Hanoverian foals during the first 200 days of life. These changes were correlated to the date of birth indicating that there are differences in skeletal development between early- and late-born foals (Vervuert et al. 2007). OC levels were also observed to change in relation to the season with higher concentrations during winter (Pastoret et al. 2007).

In humans, it has been well-documented that biochemical markers of bone metabolism show a circadian rhythm (Nielsen et al. 1990, Hassager et al. 1992, Gertz et al. 1998). Circadian rhythmicity of bone cell metabolic activity has been documented also in rats and mice (Aardal and Laerum 1983, Ohtsuka et al. 1998). In horses the results are not clear: Black et al. (1999) investigated serum OC diurnal variation in six Standardbred, one Thoroughbred and one Quarter horse. They found a rise in OC concentration during the night with the lowest values during midday. This pattern was similar to that reported by Lepage et al. (1991) in nine adult Standardbreds, in which serum OC levels reached their highest point during the night, but were not significantly different during the day from 07:00 and 20:00. Jackson et al. (2003) found a circadian rhythm of serum OC in six 2 years old Thoroughbred.

But in all these studies the procedures used in circadian physiology for the analysis of full time series were not applied (Refinetti 2006).

The purpose of this study was to determine whether there is daily rhythm in serum OC concentration in Thoroughbred horses and how this may be affected by age and gender. It is of key importance to establish the appropriate sampling time for optimal use of biochemical markers to reflect bone turnover change in clinical investigations of metabolic disease and to value the effects of training on skeletal adaptation.

MATERIALS AND METHODS

Animals

Our study was conducted on thirty Thoroughbred horses regularly trained from the same horse training centre. Before the start of the study, all subjects underwent a heart examination, respiratory auscultations, and routine haematology and plasma biochemistry at rest. Only clinically healthy animals were used. Animals were divided into two groups: Group A consisted of twelve horses (six male and six female), 2 years old and with a mean body weight of 380 ± 30 kg; Group B consisted of eighteen horses (six male, six female and six geldings), 6 years old and with a mean body weight of 560 ± 40 kg. They were housed individually in box-like stalls (4.5×4.5 m) under natural photoperiod (sunrise at 06:30; sunset at 18:30), at indoor temperature and humidity ($18\text{--}21$ °C; $50\text{--}60$ Rh%). Thermo-hygrometric recordings were carried out inside the box throughout the entire study by means of a data logger (Gemini, Chichester, West Sussex, UK). The horses were fed traditionally with hay and a mix of cereals (oats and barley), three times a day (08:00, 12:00 and 19:00) and received water *ad libitum*. General animal care was carried out by professional staff not associated with the research team. All housing and care conformed to the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 86/609 CEE.

Blood samples

Blood samples were collected at 3 hour intervals over a 48 hour period (starting at 08:00 on day 1 and finishing at 08:00 on day 3) via a jugular intravenous catheter into Vacutainer tubes (Terumo Corporation, Japan) without anticoagulant and stored at 4 °C for a maximum of 30 min before centrifugation (3000 rpm for 15 min) and freezing (-20 °C). Samples were collected at 3 hour intervals in order not to

excessively disturb the animals. A dim red light (<3lux, 15 W Safelight lamp filter 1A, Kodak Spa, Milano, Italy) was used to sample horses during the dark phase. Serum OC concentrations were quantified by the use of an equine-specific RIA test (Diasorin S.P.A., Saluggia, Italy). The validity of this kit for horse OC was reported by Inoue et al. (2008) and Donabédian et al. (2008). The serum OC concentration was expressed as nanogram (ng) of OC per millilitre (ml). The sensitivity of the RIA was 0.2 ng/ml.

Statistical analysis

All the results were expressed as mean $\pm$ SD. Data were normally distributed ($p < 0.05$, Kolmogorov-Smirnov test). Two-way repeated measure ANOVA was used to determine a statistical significant effect of time of the day and age on the serum OC concentration. An unpaired Student t-test and an ANOVA were used to evaluate statistical differences of serum OC concentration due to different genders within Group A and B, respectively. Data were evaluated at the significance level $2\alpha = 0.05$. The data was analyzed using STATISTICA 7 software (StatSoft Inc., Tulsa, USA).

In addition, we applied a trigonometric statistical model to the average values of each time series, so as to describe the periodic phenomenon analytically, by characterizing the main rhythmic parameters according to the single cosinor procedure (Nelson et al. 1979). Four rhythmic parameters were determined: mean level, amplitude (the difference between the peak, or trough, and the mean value of a wave), acrophase (the time at which the peak of a rhythm occurs), and robustness (strength of rhythmicity). For each parameter, the mean level of each rhythm was computed as the arithmetic mean of all values in the data set (9 data points), the amplitude of a rhythm was calculated as half the range of oscillation, which in its turn was computed as the difference between peak and trough. Rhythm robustness was computed as a percentage of the maximal score attained by the chi-square periodogram statistic for ideal data sets of comparable size and 24 h periodicity (Refinetti 2004). Robustness greater than 40% is above noise level and indicates statistically significant rhythmicity.

RESULTS

The application of two-way repeated measure ANOVA showed a statistical significant effect of time of the day ($F_{(7,524)} = 2.94$, statistically significant) and of age ($F_{(7,524)} = 1050.26$, statistically significant) on

the serum OC concentration. In Group A male and female horses showed different trends of serum OC concentration (Fig. 1); a higher serum OC concentration in male (19.70 ± 0.36 ng/ml) than in female was observed (18.37 ± 0.73 ng/ml) ($t_{34} = 6.86$, statistically significant, unpaired Student t-test). In Group B all horses showed the same trend of serum OC concentration (Fig. 2); statistically significant differences in serum OC concentration were observed ($F_{(2,53)} = 45.31$, statistically significant, one-way ANOVA) between the genders (Figs 2–3). The higher concentration was observed in males (13.96 ± 1.03 ng/ml) followed by geldings (12.30 ± 1.01 ng/ml) and females (10.63 ± 1.10 ng/ml).

The application of the periodic model and the statistical analysis of cosinor enabled us to define the periodic parameters and their acrophases during the 48 h of monitoring. Group A did not show a daily rhythm of serum OC concentration in both genders. Group B showed a daily rhythm of serum OC concentration in all genders. Statistical significant differences of acrophases and robustness of rhythm between the genders were observed. The mean level for the four rhythmic parameters of serum OC levels recorded during the 48 h of monitoring, with their statistically differences, are reported in Fig. 3.

DISCUSSION

Our results showed a different bone metabolism in horses of different ages and genders. A higher serum OC concentration was observed in Group A than in Group B. Serum OC is synthesized by osteoblasts and reflects new bone formation. Therefore, its decrease suggests a decrease in bone formation and resorption. The immature growing skeleton of a 2 year old Thoroughbred has neo-formative activity, and maximal mineral content of the third metacarpal bone is not reached until six years of age (Lawrence et al. 1994). More recent studies indicated a markedly higher metabolism before 20 weeks of life (Donabédian et al. 2008), the decrease in bone metabolism persists during the first 200 days of life, and seems to be related to the date of birth (Vervuert et al. 2007).

As previously reported by Lepage et al. (1991), in Standardbred these data indicate a significant slowdown in the rate of bone formation in adults compared to foals. An age-related decrease of serum OC had previously been described in female Standardbred horses (Lepage et al. 1990) and in humans (Kruse and Kracht 1986) in which the normal range of serum OC is high in children and declines to

Fig. 1. Mean pattern of serum osteocalcin concentration in male and female Thoroughbred horses of Group A (2 year olds). Each point represents the mean of six horses. The grey bar indicates the dark phase of the natural photoperiod.

Fig. 2. Daily rhythms of serum osteocalcin concentration in male, female and gelding Thoroughbred horses of Group B (6 year olds). Each point represents the mean of six horses. The grey bar indicates the dark phase of the natural photoperiod.

adult levels with completion of puberty. In humans the changes observed in serum OC at puberty showed gender-related differences (Cole et al. 1985). Influences of gender on serum OC concentrations were also observed in Quarter horses (Fletcher et al. 2000) in contrast to the observations of Lepage et al. (1992) which concluded that serum OC concentrations of Standardbred horses less than 5 years of age were not affected by gender. The exact hormonal mechanism that influence the serum OC concentration in male and female horses during their maturation period is not completely understood. Fletcher et al. (2000) attributed gender differences to

the different levels of peripheral steroid hormone concentration that act on the steroid receptors located on the osteoblastic cells to a higher degree in females than males and geldings.

The high rates of skeletal modelling and remodelling during growth and the resulting greater variability in bone marker concentrations were likely to explain the lack of a circadian rhythm in young Standardbred horses compared to adults (Black et al. 1999). In agreement with this, Fletcher et al. (2000) also failed to observe any detectable change in serum OC over a 24 hours sampling period in Quarter foals. Hope et al. (1993) reported no significant changes in

Fig. 3. Analysis of four rhythmic parameters of 48 h records of serum osteocalcin concentration in Group B (6 year olds). Each bar corresponds to the mean ($\pm$ SD) of six horses of each gender in the 48 h of monitoring; * significant versus all.

24 hours serum OC concentrations in a study that used animals covering a wide range of ages, whereas Geor et al. (1995) observed no circadian rhythm in a study of 3 to 5 years old Thoroughbreds. On the contrary Jackson et al. (2003) reported in 2 years old Thoroughbred a circadian rhythm of serum OC concentration with an estimated peak time at 09:00.

Lepage et al. (1991) observed significant 24 hour variation in serum OC concentrations in adult Standardbred, and Black et al. (1999) also found serum OC concentrations in adult geldings to exhibit a significant circadian pattern. Studies in human and animals have suggested that endogenous factors, including hormones, may play an important role in regulating daily rhythms in bone metabolism (Nielsen et al. 1990, Ostrowska et al. 2002). It was noted by Lepage et al. (1991) that circadian changes in serum OC appeared to be related to photoperiod. They suggest that physical activity does not explain the differences between light and dark periods because the activity of the horses was restricted to movements in the box stall during the experiment. Also they excluded an influence of meals on serum OC concentration because after food it was not possible to detect variations. In humans, it has been shown that

part of the circadian variation of serum OC may be influenced by food intake (Schlemmer and Hassager, 1999, Bjarnason et al. 2002). On the basis of our knowledge, in horses, the pattern of diurnal variation was found to be affected by a number of endogenous and exogenous factors, such as age, season, feeding times and fasting, change in light intensity and environmental temperature (Piccione et al. 2005, 2008a, Fazio et al. 2006, Bertolucci et al. 2008). To find the exact zeitgeber it is necessary to test the parameter under constant environmental conditions, in which the circadian rhythms free-run with periods slightly different from 24 hours, and to subject the animals to different light/dark, temperature and feeding schedules (Piccione et al. 2008b, Bertolucci et al. 2009). Since circulating levels of serum OC reflect bone turnover it has been proposed that variations in the circulating pool may be linked to an intrinsic rhythmic activity occurring in bone (Gunderberg et al. 1985).

In summary, bone metabolism is different in horses of different ages. It has a higher rate in 2 year old than 6 year old Thoroughbreds. The different rate of bone turnover influences also the circadian rhythm of serum OC concentration, that is evident only in

mature Thoroughbreds. Within the same age-group the influence of gender on serum OC concentration has been observed. These differences were probably due to the reproductive hormones, but no exact data are present in the literature about their mechanism.

In conclusion, our results are indicative of the existence of daily rhythms in osteoblastic activity. Their activity peaks during the night and decreases in the morning both in males and females, as well as in geldings. The results, however, support the interaction between sex hormones and bone metabolism. Single samples from individuals are of little value for monitoring marker activity; a series of measurements should be taken over a period of time, or samples should be collected at precise times for results to be meaningful. Blood sampling for determination of serum OC should be strictly standardized with regard to the time of the day and the time since last medication or intervention with factors that may affect osteoblastic activity. Lack of daily rhythm in serum OC of young horses suggests that regulating the time of sampling for serum OC determinations in metabolic studies may not be necessary in skeletally immature horses.

REFERENCES

- Aardal NP, Laerum OD: Circadian variation in mouse bone marrow. *Exp Hematol* 11:792–801, 1983.
- Bertolucci C, Giannetto C, Fazio F, Piccione G: Seasonal variations in daily rhythms of activity in athletic horses. *Animal* 2:1055–1060, 2008.
- Bertolucci C, Giudice E, Fazio F, Piccione G: Circadian intraocular pressure rhythms in athletic horses under different lighting regime. *Chronobiol Int* 26:348–358, 2009.
- Bjarnason NH, Henriksen EE, Alexandersen P, Christgau S, Henriksen DB, Christiansen C: Mechanism of circadian variation in bone resorption. *Bone* 30:307–313, 2002.
- Black A, Schoknecht PA, Ralston SL, Shapses SA: Diurnal variation and age differences in the biochemical markers of bone turnover in horses. *J Anim Sci* 77:75–83, 1999.
- Cole DEC, Carpenter TO, Gundberg CM: Serum osteocalcin levels in children with metabolic bone disease. *J Pediatr* 106:770–776, 1985.
- Donabédian M, Van Weeren PR, Perona G, Fluerance G, Robert C, Léger S, Bergero D, Lapage O, Martin-Rosset W: Early changes in biomarkers of skeletal metabolism and their association to the occurrence of osteochondrosis (OC) in the horse. *Equine Vet J* 40:1–7, 2008.
- Fazio F, Assenza A, Crisafulli G, Piccione G, Caola G: The influence of exercise on daily rhythm of serum homocysteine in horses. *J Physiol Sci* 55:455–458, 2006.
- Fletcher KL, Topliff DR, Cooper SR, Freeman DW, Geisert RD: Influence of age and sex on serum osteocalcin concentrations in horses at weaning and during physical conditioning. *J Equine Vet Sci* 20:124–126, 2000.
- Geor R, Hope L, Lauper S, Piela S, Klassen J, King V, Murphy M: Effect of glucocorticoids on serum osteocalcin concentration in horses. *Am J Vet Res* 56:1201–1205, 1995.
- Gertz BJ, Clemens DJ, Holland SD, Yuan W, Greenspan S: Application of a new serum assay for type-I collagen cross-linked N-telopeptide: Assessment of diurnal changes in bone turnover with and without Alendronate treatment. *Calcif Tissue Int* 63:102–106, 1998.
- Gunderberg CM, Markowitz ME, Mizruchi M, Rosen JF: Osteocalcin in human serum: a circadian rhythm. *J Clin Endocrinol Metab* 60:736–736, 1985.
- Hassager C, Resteli J, Resteli L, Jensen SB, Christiansen C: Diurnal variation in serum markers of type I collagen synthesis and degradation in healthy premenopausal women. *J Bone Miner Res* 7:1307–1311, 1992.
- Hope E, Johnston SD, Hegstad RL, Geor RJ, Murphy MJ: Effects of sample collection and handling on concentration of osteocalcin in equine serum. *Am J Vet Res* 54:1017–1020, 1993.
- Inoue Y, Matsui A, Asai Y, Aoki F, Yoshimoto K, Matsui T, Yano H: Response of biochemical markers of bone metabolism to exercise intensity in Thoroughbred horses. *J Equine Sci* 19:83–89, 2008.
- Jackson BF, Blumsohn A, Goodship AE, Wilson AM, Price JS: Circadian variation in biochemical markers of bone cell activity and insulin-like growth factor-I in two year old horses. *J Anim Sci* 81:2804–2810, 2003.
- Jackson BF, Dyson PK, Lonnel C, Verheyen KLP, Pfeiffer DU, Price JS: Bone biomarkers and risk of fracture in two- and three-year-old Thoroughbreds. *Equine Vet J* 41:410–413, 2009.
- Jeffcott LB, Buckingham SH, McCarthy RN, Cleeland JC, Scotti E, McCartney RN: Non-invasive measurement of bone: a review of clinical and research applications in the horse. *Equine Vet J* 6:0S71–79, 1988.
- Khosla S, Kleerekoper M: Biochemical markers of bone turnover. In Favus MJ (ed): *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*, 5th edition, American

- Society for Bone and Mineral Research, Washington 2003, pp 166–172.
- Kruse K, Kracht U: Evaluation of serum osteocalcin as an index of altered bone metabolism. *Eur J Pediatr* 145:27–33, 1986.
- Lawrence LA, Ott EA, Miller GJ, Poulos PW, Piotrowski G, Asquith RL: The mechanical properties of equine third metacarpals as affected by age. *J Anim Sci* 72:2671–2623, 1994.
- Lepage OM, Marcoux M, Tremblay A: Serum osteocalcin of bone Gla-protein, a biochemical marker for bone metabolism in horses: Differences in serum levels with age. *Can J Vet Res* 54:223–226, 1990.
- Lepage OM, Des Coteaux L, Marcoux M, Tremblay A: Circadian rhythms of osteocalcin in equine serum. Correlation with alkaline phosphatase, calcium, phosphate and total protein levels. *Can J Vet Res* 55:5–10, 1991.
- Lepage OM, Marcoux M, Tremblay A, Dumas G: Sex does not influence serum osteocalcin levels in Standardbred horses of different ages. *Can J Vet Res* 56:379–381, 1992.
- Lepage OM, Carstanjen B, Uebelhart D: Non-invasive assessment of equine bone: an update. *Vet J* 161:10–22, 2001.
- McIlwraith CW: Use of synovial fluid and serum biomarkers in equine bone and joint disease: a review. *Equine Vet J* 37:473–482, 2005.
- Nelson W, Tong U, Lee J, Halberg F: Methods for cosinor rhythmometry. *Chronobiologia* 6:305–323, 1979.
- Nielsen HK, Brixen K, Mosekilde L: Diurnal rhythm in serum activity of wheat-germ lectin-precipitable alkaline phosphatase: temporal relationships with the diurnal rhythm of serum osteocalcin. *Scand J Clin Lab Invest* 50:851–856, 1990.
- Ohtsuka M, Saeki S, Igarashi K, Shinoda H: Circadian rhythms in the incorporation and secretion of ^3H -proline by odontoblasts in relation to uncremental lines in rat dentine. *J Dental Res* 77:1889–1895, 1998.
- Ostrowska Z, Kos-Kudla B, Marek B, Kajdaniuk D, Ciesielska-Kopacz N: The relationship between the daily profile of chosen biochemical markers of bone metabolism and melatonin and other hormone secretion in rats under physiological conditions. *Neuro Endocrinol Lett* 23:417–425, 2002.
- Pastoret V, Carstanjen B, Lejeune JP, Farnir F, Remy B, Reginster JY, Serteyn D, Gabriel A: Evaluation of serum osteocalcin and CTX_I in ardenner horses with special reference to Juvenile interphalangeal joint disease. *J Vet Met A Physiol Pathol Clin Med* 54:458–463, 2007.
- Piccione G, Fazio F, Giudice E, Grasso F, Morgante M: Nycthemeral change of some haematological parameters in horses. *J Appl Biomed* 3:123–128, 2005.
- Piccione G, Giannetto C, Fazio F, Giudice E: Daily rhythm of tear production in normal horse. *Vet Ophthalmol* 1(Suppl. 1):57–60, 2008a.
- Piccione G, Fazio F, Caola G, Refinetti R: Daily rhythmicity of glycemia in four species of domestic animals under various feeding regimes. *J Physiol Sci* 58:271–275, 2008b.
- Price JS, Jackson B, Eastell R, Goodship AE, Blumsohn A, Wright I, Stoneham S, Lanyon LE, Russell RG: Age related changes in biochemical markers of bone metabolism in horses. *Equine Vet J* 27:201–207, 1995.
- Refinetti R: Non-stationary time series and the robustness of circadian rhythms. *J Theor Biol* 227:571–581, 2004.
- Refinetti R: *Circadian Physiology*. 2nd ed. Taylor & Francis Group, Boca Raton 2006.
- Schlemmer A, Hassager C: Acute fasting diminishes the circadian rhythm of biochemical markers of bone resorption. *Eur J Endocrinol* 140:332–337, 1999.
- Vervuert I, Winkelsett S, Christmann L, Bruns E, Hoppen HO, Distl O, Hertsch B, Coenen M: Evaluation of the influences of exercise, birth date, and osteochondrosis on plasma bone marker concentrations in Hanoverian Warmblood foals. *Am J Vet Res* 68:1319–1323, 2007.