

Circadian variation in biochemical markers of bone cell activity and insulin-like growth factor-I in two-year-old horses¹

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ABSTRACT: Studies in humans have found circadian changes to be one of the most important sources of controllable preanalytical variability when evaluating bone cell activity using biochemical markers. It remains unclear whether similar circadian changes influence bone marker concentrations in the horse. The aim of this study was to characterize changes in serum concentrations of three biochemical markers of bone cell activity over a 24-h period in six 2-yr-old Thoroughbred mares, and to determine circadian variability in IGF-I, which regulates bone turnover. Three bone markers were measured in serum: osteocalcin, a marker of bone formation, the carboxy-terminal propeptide of type-I collagen (a marker of bone formation), and the carboxy-terminal telopeptide of type-I collagen (a marker of bone resorption). Data were analyzed using the cosinor technique, which fits a 24-h cycle to each dataset. A significant circadian rhythm was observed for osteocalcin ($P = 0.028$), with an estimated amplitude of 7.6% of the mean (95% confidence interval 1.3% to 16.3%), and an estimated peak time of 0900. However, the observed

rhythm for the carboxy-terminal telopeptide of type-I collagen (amplitude = 7.4%) was not significant ($P = 0.067$), and there were no significant changes in concentrations of the carboxy-terminal propeptide of type-I collagen over the 24-h study period ($P = 0.44$). There was a small but significant circadian rhythm for IGF-I ($P = 0.04$), with an estimated amplitude of 3.4% (95% confidence interval 0.2 to 7.1%) and peak at 1730. Further studies are now required to determine the potential association between circadian changes in IGF-I and osteocalcin in the horse. Although no significant circadian variation was found in concentrations of the carboxy-terminal propeptide of type-I collagen and the carboxy-terminal telopeptide of type-I collagen, this may in part be a result of the age of the animals that were still skeletally immature. Future studies should aim to determine whether these markers develop a circadian rhythm at a later age when growth is complete. In the meantime, consistency in time of sampling should continue to be considered best practice when measuring biochemical markers of bone turnover in the horse.

Key Words: Bone Tissue, Circadian Rhythm, Horses, Insulin-Like Growth Factor, Osteocalcin

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Introduction

Biochemical markers can be used to noninvasively monitor bone cell activity, and a number of studies have explored the potential applications of bone markers in horses (Price 1999; Lepage et al., 2001). Markers studied include osteocalcin, a noncollagenous protein produced by osteoblasts and a marker of bone formation; the carboxy-terminal propeptide of type-I collagen (PICP), a marker of type-I collagen formation; and the carboxy-terminal telopeptide of type-I collagen (ICTP), a marker of type-I collagen degradation.

Studies in humans (reviewed by Hannon and Eastell 2000) and animals (Liesegang et al., 1999; Srivastava et al., 2001; Ladlow et al., 2002) have identified circadian rhythms as an important source of preanalytical variability in these markers. However, whereas some studies have reported a circadian rhythm in osteocalcin in horses (Lepage et al., 1991; Black et al., 1999), others have found no change (Hope et al., 1993; Geor et al., 1995). To date, no studies have established whether PICP and ICTP show circadian variability in the horse as they do in humans (Hassager et al., 1992; Heshmati et al., 1998). If a bone marker displays circadian variability it is important that samples are collected at precise times for results to be meaningful.

Studies in humans and animals have suggested endogenous factors, including hormones, may play an important role in regulating daily rhythms in bone metabolism (Nielsen et al., 1991; Ostrowska et al., 2002). In

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horses, GH administration increases circulating IGF-I (Noble et al., 2000) and increases bone turnover, as determined by measuring bone markers (Price et al., 2000). Circadian changes in bone cell activity in the horse could therefore potentially be regulated by IGF-I.

The aim of this study was to characterize changes in serum concentrations of three biochemical markers of bone cell activity over a 24-h period in six 2-yr-old Thoroughbred mares, and to determine circadian variability in IGF-I.

Materials and Methods

Horses

Six 2-yr-old (average age = 20 ± 1 mo) Thoroughbred, unbroken mares with no history of orthopedic disease were used for this study. The horses were part of a 20-wk multicenter project designed to evaluate the adaptation of musculoskeletal tissues to athletic training and formed the control group for this study. The horses were kept stabled throughout the study, given 40 min of walking exercise 6 d/wk, and fed 1 kg of a concentrate plus hay ad libitum each day. Animals remained under constant fluorescent lighting throughout the sampling period.

Sample Collection

On the day of the study, baseline blood samples were collected from each horse at 0946, 1032, 1116, 1205, 1255, and 1329. Further samples were then taken from each horse at intervals of 30 min and at 1, 2, 4, 6, 8, 12, 18, and 24 h after the initial sample. Samples were drawn from indwelling catheters, allowed to clot for 30 min, and then centrifuged (4°C , $1,000 \times g$) to separate serum. Timing of baseline samples was staggered to enable individual samples to be processed rapidly and to ensure accurate timing of subsequent samples.

Osteocalcin

The concentration of osteocalcin was measured in serum samples using a competitive immunoassay (Meta Osteocalcin; Quidel Corp., San Diego, CA) that has been validated previously in horses (Hoyt and Siciliano, 1999). In order to ensure that the concentration measured fell within the range of the assay (0 to 32 ng/mL), all samples were diluted 1:2 with $\times 10$ wash. The limit of detection of the assay was 0.45 ng/mL. The intraassay CV for osteocalcin was 6.2% and the corresponding interassay CV 8.3%, within the concentration range in this study (all assay CV were determined in-house).

Assay for Carboxy-Terminal PICP in Serum

The concentration of PICP was determined using a competitive RIA (Orion Diagnostica, Oulunsalo, Finland) that was validated previously for use in horses (Price et al., 1995). Serum was diluted 1:5 with PBS

when concentrations were above the upper assay limit of 500 $\mu\text{g/L}$. The intraassay CV was 4.7% in the concentration range in the study. The corresponding interassay CV was 8.2%.

Crosslinked Telopeptide of Type I Collagen (ICTP)

The concentration of ICTP was measured in serum samples using a commercially available RIA (Orion Diagnostica) previously validated for use in horses (Price et al., 1995). The limit of detection of the assay was 0.5 $\mu\text{g/L}$. The intraassay CV was 4.5% in the concentration range in this study. The corresponding intraassay CV was 5.6%.

Insulin Like-Growth Factor Type-I

The concentration of IGF-I was measured using a two-site immunoenzymometric assay (OCTEIA IGF-1; Immunodiagnostic Systems Ltd, Boldon, U.K.). Samples were initially incubated with a releasing agent to inactivate binding proteins and then diluted for assay. The assay was noncompetitive, with an excess of antibody directed against a specific site on the IGF-I molecule coated on the surface of microtiter wells, whereas a second antibody that recognizes a different site was labeled with horseradish peroxidase. After incubating the test sample with both antibodies and a wash step, remaining activity in the wells was determined by the addition of substrate. Because the assay was not previously validated for use in horses, cross-reactivity with IGF-I in horse sera was assessed by serially diluting serum from one animal and plotting a standard curve. This showed parallel dilution with the assay standard curve (not shown). The limit of detection was 1.9 $\mu\text{g/L}$. The intraassay CV for IGF-I was 6.2% and the corresponding interassay CV of 2.4% was in the concentration range in this study.

Statistics

Circadian rhythms were assessed using a separate cosinor analysis for each subject. This model fits a 24-h cycle to each subject using two parameters estimated from the data for each subject: a peak time and the magnitude of the diurnal variation from the mean (Nelson et al., 1979). The test allows the probability that a sine wave fits the data better than a straight line. Rhythm characteristics derived from the analysis of individuals were used as input statistics for calculation of the population statistics. The method also allows for the fact that there were different sampling times for each subject.

Results

Circadian Variability

A circadian rhythm was observed for serum osteocalcin concentrations ($P = 0.028$), with an estimated ampli-

Table 1. Mean 24-h concentrations and circadian variation in osteocalcin, carboxy-terminal propeptide of type-I procollagen (PICP), carboxy-terminal telopeptide of type-I collagen (ICTP), and IGF-I in 2-yr-old Thoroughbred mares

Item	Mean	SEM	Amplitude, % ^a	Probability ^b	Time of peak ^a
Osteocalcin, ng/mL	35.06	0.65	7.6	0.028	0900
PICP, µg/L	933	17.57	2.8	0.443	—
ICTP, µg/L	12.97	0.22	7.4	0.067	—
IGF-I, µg/L	217	4.85	3.4	0.043	1730

^aBest estimate of amplitude, expressed as a percentage of the 24-h mean.

^bProbability that the data are better expressed as a percentage of the 24-h mean.

^cEstimated time of fitted peak.

tude of 7.6% of the 24-h mean (95% confidence interval 1.3 to 16.3%) and an estimated peak time of 0900 (Table 1 and Figure 1). However, the observed rhythm for ICTP (amplitude 7.4%) did not quite reach statistical significance ($P = 0.067$), and there were no significant changes in PICP concentrations over the 24-h study period ($P = 0.44$). There was a small but significant circadian rhythm in IGF-I concentrations ($P = 0.04$), with an estimated amplitude of 3.4% of the 24-h mean (95% confidence interval 0.2 to 7.1%), with a peak at 1730.

Discussion

In the present study, we characterized changes in serum concentrations of three biochemical markers of bone cell activity and IGF-I over a 24-h period in 2-yr-old Thoroughbred mares kept under closely controlled conditions. As a result, we observed a significant circadian rhythm in osteocalcin and IGF-I. However, there was no significant 24-h variation in serum markers of type-I collagen formation and degradation.

Circadian variation is one of the most important sources of variability in markers of bone metabolism that can be controlled for. However, previous studies aimed at describing circadian rhythms in osteocalcin in the horse have not produced consistent results. Lepage et al. (1991) observed significant 24-h variation in osteocalcin concentrations in adult Standardbreds, and Black et al. (1999) also found serum osteocalcin concentrations in adult geldings (predominantly Standardbreds) to exhibit a significant circadian pattern. In contrast, Hope et al. (1993) reported no significant changes in 24-h osteocalcin 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-yr-old Thoroughbreds. In the present study, a significant circadian rhythm was observed for osteocalcin, with a peak at 0900, which is consistent with the findings of Black et al. (1999), although the method of analysis in that study did not define an estimated peak time. In the same study, Black et al. (1999) failed to find a circadian rhythm for osteocalcin when Standardbred weanlings (average age of 4.6 mo) were used rather than adults and suggested that high rates of skeletal

modeling and remodeling during growth and the resulting greater variability in marker concentrations were likely to explain the lack of a circadian rhythm compared to adults. In agreement with this, a more recent study in quarter horse foals and yearlings also failed to observe any detectable change in osteocalcin over a 24-h sampling period (Fletcher et al., 2000). There is also evidence to suggest that circadian rhythms in other biochemical variables develop gradually and at different rates during early life, so that adult patterns only start to emerge as animals mature (Komosa et al., 1990). In the present study, the amplitude of the rhythm in osteocalcin concentrations was 7.6% of the 24-h mean. In humans, the reported amplitude of the rhythm in osteocalcin concentrations is 5 to 20% of the 24-h mean (Hannon and Eastell 2000), whereas in a similar study in dogs, the reported amplitude was 21% (Ladlow et al. 2002). The observed rhythm in the present study was therefore toward the lower end of what has been reported in other species and may reflect the fact that animals in this study were, on average, 20 mo old and thus still skeletally immature at the time of sampling. Species differences may also be an important factor. For example, osteocalcin exhibits no circadian rhythm in sheep (Corlett et al., 1990), and whereas serum cortisol concentrations play an important role in determining the circadian pattern of osteocalcin in humans (Heshmati et al., 1998), Geor et al. (1995) failed to find any similar effect in horses.

In humans, PICP has been shown to exhibit a circadian rhythm comparable to that of osteocalcin, with a peak between 0200 and 0500, and an amplitude of approximately 20% of the 24-h mean (Hassager et al., 1992; Pedersen et al., 1995). Surprisingly, the present study found no evidence of a circadian rhythm for this marker in 2-yr-old Thoroughbred mares, which may reflect species differences in the regulation of type-I collagen turnover. The difference in circadian variability between osteocalcin and PICP in the horse may also be explained, at least in part, by the fact that synthesis of osteocalcin and type-I collagen are controlled by different local mechanisms in osteoblastic cells (Schlemmer et al., 1997). Whereas osteocalcin is synthesized by fully differentiated osteoblasts, type-I collagen is synthesized by proliferating osteoblasts (Stein et al.,

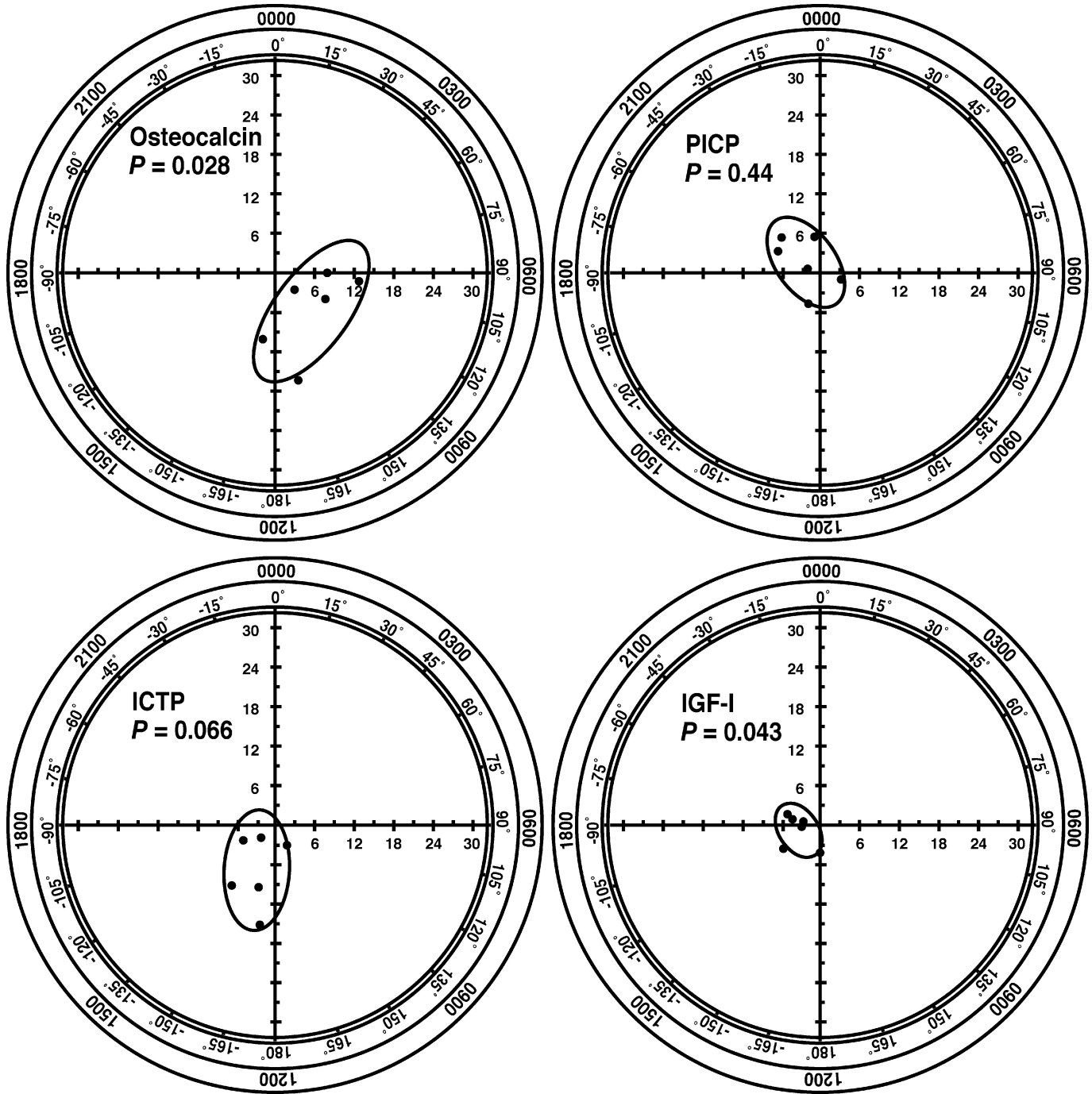


Figure 1. Cosinor analysis showing the circadian variation of osteocalcin, the carboxy-terminal propeptide of type-I collagen (PICP), the carboxy-terminal telopeptide of type-I collagen (ICTP), and IGF-I. Each data point represents the best-fit cosine curve for each individual horse. The distance of each point from the center represents the amplitude (expressed as a percentage of the 24-h mean, scale given on horizontal and vertical axes), and the angle of the vector from the center to the data point represents the timing of the individual peak. The ellipse represents the joint 95% confidence interval for the population mean amplitude and phase.

1990). The absence of a circadian pattern in PICP could also be due to the fact that type-I collagen is found in tissues other than bone, and that synthesis in extra-skeletal sites can contribute to the circulating pool. Studies in humans indicate that there is a significant nonskeletal contribution to circulating PICP concentra-

tions (Parfitt et al., 1987), and previous studies have shown this may also be an important factor in horses, particularly following soft tissue injury (Jackson et al., 2003a) or at times of rapid growth (Price et al., 2001). The fact that horses in the present study were still skeletally immature may also be a reason for the ab-

sence of a circadian rhythm for PICP. Future studies should aim to determine whether this marker develops a circadian rhythm at a later age when growth is complete.

Significant circadian variations in urinary markers of bone resorption have been described in humans (Blumsohn et al., 1994; Schlemmer et al., 1994; Aoshima et al., 1998), dogs (Liesegang et al., 1999; Ladlow et al., 2002), and horses (Black et al., 1999). In humans, this variation is much more pronounced than that seen in markers of bone formation. For example, urinary excretion of pyridinoline and deoxypyridinoline peaks between 0200 and 0800, with an amplitude that may be as much as 100% of the 24-h mean (Schlemmer et al., 1992). Assays have also been developed for the measurement of bone resorption markers in serum, including ICTP, and a significant circadian pattern has been described for this marker in humans (amplitude 20% of 24-h mean) (Hassager et al., 1992). There is also a significant circadian variability in ICTP in dogs, with a reported amplitude of 32% of the 24-h mean, and estimated peak time of 0300 (Ladlow et al., 2002). However, the observed rhythm in ICTP concentrations in the present study did not reach statistical significance (amplitude 7.4%). The reason for this is unclear, but again may reflect species differences. It should also be taken into account that whereas ICTP appears to be a relatively specific marker of bone resorption in the horse because ICTP concentrations are correlated with serum deoxypyridinoline (B. F. Jackson, A. E. Goodship, R. Eastell, J. S. Price, unpublished results), there are concerns relating to the use of ICTP as a specific marker of bone resorption in humans (Hassager et al., 1994; Filipponi et al., 1995). The results of the current study may also have been influenced by the age of the animals because Black et al. (1999) found no circadian changes in urinary excretion of the bone resorption markers pyridinoline and deoxypyridinoline in weanling colts, whereas skeletally mature adult animals exhibited a significant circadian variation. There is greater biological variability in marker concentrations in growing animals, and thus a larger sample size could result in the circadian rhythm for ICTP becoming statistically significant.

It should also be considered that factors such as photoperiod might influence circadian changes in bone markers, although this warrants further investigation. The present study was carried out under conditions of constant lighting, but these results are not consistent with those of Geor et al. (1995), who found no circadian rhythm for osteocalcin in a study of adult Thoroughbreds exposed to constant fluorescent lighting. In contrast, it was noted in a study of adult standardbreds that circadian changes in osteocalcin appeared to be related to photoperiod (Lepage et al., 1991). More recently, the circadian rhythm in concentrations of a smaller carboxy-terminal telopeptide fragment of type-I collagen (known as CTX), which is often measured as an alternative to ICTP in human studies, was found to

be unaffected by the absence of a normal light cycle in a study of adult men and women (Qvist et al., 2002).

Time of year could also potentially influence the outcome of studies of circadian variability as seasonal changes in concentrations of bone markers have been described in some human (Woitge et al., 1998, 2000) and yearling Thoroughbred (Price et al., 2001) studies. However, it was recently shown that season has no significant effect on bone marker concentrations in 2-yr-old Thoroughbreds in training (Jackson et al., 2003b), whereas in adult humans, the circadian rhythm in osteocalcin is not acutely affected by season (Nielsen et al., 1990).

Several studies in humans have attempted to define other causes of circadian variation in bone turnover and have found the circadian rhythm in osteocalcin to be unaffected by age, sex, or sleep deprivation, (Nielsen et al., 1990, 1991). Furthermore, the variation in bone resorption has been shown to be independent of age, posture (Schlemmer et al., 1994), and serum cortisol concentrations (Schlemmer et al., 1997). More recently, studies have shown that part of the circadian variation seen in humans may be influenced by food intake (Schlemmer and Hassager 1999; Bjarnason et al., 2002). This has also been shown to be important in animal studies (Muhlbauer and Fleisch, 1995), and the influence of food intake on bone cell activity should now be studied in horses.

A number of hormones with effects on bone turnover, including parathyroid hormone (PTH) and GH, also exhibit circadian rhythms and could be candidates for mediating circadian changes in bone turnover. However, Ledger et al. (1995) found that abolishing the circadian variation in serum PTH in women had no effect on the circadian variation in the urinary excretion of the crosslinked N-telopeptide of type-I collagen, a marker of bone resorption. The complex effects of GH on bone remodeling are thought to be partially mediated by IGF-I, a peptide structurally and functionally related to insulin, which plays an important role in regulating bone growth and the maintenance of bone mass (Ohlsson et al., 1998; Zhang et al., 2002). Previously, it was shown that IGF-I concentrations correlate with osteocalcin in light breed horses (Davico et al., 1994), and changes in IGF-I concentrations correlate with changes in osteocalcin and ICTP in treadmill-exercised 2-yr-old Thoroughbreds (Jackson et al., 1998). In addition, it was also shown that increased IGF-I following GH administration is associated with a significant increase in osteocalcin in horses (Noble et al., 2000; Price et al., 2000). We therefore measured IGF-I in the present study in order to determine whether this hormone displays circadian variability.

We observed a small but statistically significant circadian rhythm for IGF-I (estimated amplitude was 3.4% of the 24-h mean), with a peak in the late afternoon (1730), and further intervention studies are now required to determine the potential association between circadian changes in IGF-I and osteocalcin in the horse.

In contrast to these results, a recent study in three 10-yr-old horses found no significant variation in IGF-I concentrations over a 16-h period between 0500 and 2100 (Popot et al., 2001). The reason for this difference is unclear but may be related to the age of the animals used. It is generally accepted that there is no significant circadian variation in total IGF-I concentrations in healthy human adults. However, a significant nocturnal decrease in free IGF-I has been reported in healthy subjects, and this corresponds to a nocturnal increase in IGFBP-1 (Skjaerbaek et al., 2000). Clearly, IGFBP may also play an important role in regulating IGF-I activity in the horse, but unfortunately, no assays were available to us for measuring equine IGFBP at the time this study was undertaken.

In conclusion, this study shows that serum osteocalcin and IGF-I concentrations exhibit a significant circadian rhythm in 2-yr-old Thoroughbred mares. Although no significant circadian variation was found in markers of type-I collagen formation (PICP) and degradation (ICTP), evidence from previous studies suggests this may in part be due to the age of the animals, which were still skeletally immature.

Implications

Further studies are required to identify the effects of age and other factors such as photoperiod and diet on circadian variability in bone cell activity in the Thoroughbred. In the meantime, consistency in time of sampling should continue to be considered best practice when measuring biochemical markers of bone turnover in the horse.

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