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CIRCADIAN RHYTHM OF BONE FORMATION BIOMARKERS IN SERUM OF DROMEDARY CAMELS

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SUMMARY

The circadian rhythm of biomarkers of bone formation including osteocalcin and bone alkaline phosphatase (BAP) was studied in the serum of dromedary camels. Blood samples were collected every 60 minutes for 24 hour from 10 healthy adult female camels. ELISA was used to determine the concentrations of serum osteocalcin and BAP. The results showed a marked fluctuation in the concentration of osteocalcin during the 24 hour period with minimum and maximum levels at 13:00 (01:00 pm) and 1800 (06:00 pm), respectively. Slight fluctuation was observed in the concentration of BAP with minimum and maximum levels at 01:00 am and 12:00 pm, respectively. The correlation between the two biomarkers was weak. It was concluded that it is important to fix the time of blood sampling for analysis of osteocalcin concentrations, but not for BAP.

INTRODUCTION

Diagnosis of bone diseases is a real problem in humans and animals. The ability to diagnose several types of bone diseases, such as stress fractures, in early stages is considered a diagnostic challenge. Bone fractures are real problem in dromedary camels (Ramadan 1992, Gahlot and Chouhan 1994). Radiography is the most widely imaging technique used for the diagnosis of bone diseases. However, radiography fails to diagnose early stages of bone diseases. It has been reported that approximately 30-40% changes in bone mineral density are required before bone changes can be detected by radiographs (Greenfield 1986). The development of other new procedures is necessary to accurately assess the progression of bone damage and evaluate the bone healing process. Other modern imaging modalities have been developed to diagnose bone disease in relatively earlier stages. However, these modern imaging

modalities have their disadvantages including lack of specificity (nuclear scintigraphy), or difficulty of application especially in large animals (computed tomography and magnetic resonance imaging) (Park et al 1996). Therefore, more specific, sensitive and applicable methods are needed to identify early changes in bone and to monitor therapeutic response and the healing process.

Biomarkers of bone are molecules of connective tissue matrices released into biological fluids during the process of bone turnover and reflect either bone formation or resorption. The major advantage of using bone biomarker analysis over the other diagnostic techniques is its ability to diagnose early changes in bone and accurately monitor its healing. Not like other diagnostic techniques, biomarkers are not only specific to the tissue but are specific for each structure. For example, many bone biomarkers are specific for collagen type I while others are specific for bone mineralization. In addition, there are biomarkers that are specific either for bone formation (synthesis) or resorption. The assays used to assess the levels of biomarkers are generally very accurate and easy to apply. Recently, it has been reported that the increase of the levels of bone formation biomarker is the early change occurs in some of bone diseases (Alsobayil 2003). Osteocalcin and bone alkaline phosphatase (BAP) are the most commonly bone formation biomarkers that can be easily measured in the serum.

Osteocalcin (gamma carboxy-glutamic acid-Gla) also refered to as Bone Gla protein (BGP), is a small vitamin K-dependent and calcium binding protein that contains 49 amino acids with three Gla residues. It composes 1-20% of the noncollagenous protein of the organic matrix of bone depending on animal species, age, and site (Conn and Termine 1985, Hauschka 1979, Price 1983). Osteocalcin is biosynthesized and secreted by osteoblasts (Beresford et al 1984, Camarda et al 1987, Lian et al 1985) and is therefore considered a specific osteoblastic marker produced during bone synthesis. Most of the secreted osteocalcin is deposited in the extracellular matrix of bone and only a small amount enters the blood. Although the precise biological function of osteocalcin in bone has not been determined yet, it has been suggested that the protein plays a role in the mineralization of bone matrix by regulating the growth of hydroxyapatite crystals (Hauschka et al 1975, Hauschka and Gallop 1977, Boivin, et al 1990). Therefore, it is considered a later marker of osteoblastic differentiation. The circulating levels of osteocalcin have been used as specific biomarkers for osteoblastic activity and bone formation in metabolic bone diseases. The normal concentrations of osteocalcin have been reported in humans (Markowitz et al 1984, Gundberg et al. 1985, Markowitz et al 1987, Nielsen et al 1988, Rosenquist et al 1995) and animals (Lepage et al 1991, Arican et al 1996, Collignon et al 1996). In humans and some animals, serum osteocalcin concentrations have showed diurnal variations

that may be related to circadian rhythms in the rate of bone formation (Markowitz et al 1984, Gundberg et al. 1985, Markowitz et al 1987, Nielsen et al 1988, Lepage et al 1991, Greenspan et al 1997, Heuck et al 1998).

Alkaline phosphatase is a membrane-bound enzyme that hydrolyzes phosphate esters. Its mechanism of release and specific function are still unknown. Although total alkaline phosphatase (TAP) is not specific to bone, its levels in serum have shown correlation with bone formation rate as assessed by calcium kinetics in normal humans (Weaver et al 1997). TAP levels in serum consist of several enzyme isoforms produced by bone, liver, intestine, kidney, spleen and placenta (Moss 1987). The majority of TAP in serum is the liver and bone isoforms. Bone-specific alkaline phosphatase (BAP) is the major component of TAP and its level can be determined in the blood. The normal levels of BAP have been determined in humans (Rosalki & Foo 1984, Garnero and Delmas 1993, Gomez et al 1995, Hata et al 1996, Price et al 1997) and animals (Farley et al 1992, Hank et al 1993, Sanecki et al 1993). The circadian rhythms of the concentrations of TAP have been determined in serum of humans and horses (Lepage et al 1991, Naylor & Eastell 1999).
The normal concentration and diurnal rhythms in the levels of serum osteocalcin and BAP have not been studied in dromedary camels. The purpose of this study was to determine the normal concentrations of osteocalcin and BAP in serum of dromedary camels and to determine their variations during the day.

MATERIALS AND METHODS

Camels

10 adult female camels, 6-9 years old, were used in this study. The camels were in good health as assessed by physical and clinical examinations including body temperature, respiratory rate, heart rate, renal and hepatic functions, gait, and locomotion.

Sample collection

Blood samples were collected in plain vacutainer tubes from the jugular vein of each camel every hour for 24 hours. Blood samples were centrifuged for 10 minutes at 3000 rpm to obtain serum. The serum samples were aliquotted in tubes and immediately stored at -20°C for 5 days.

Biomarker assays

Osteocalcin concentration was determined in serum using a commercial immunoassay kit (Quidel Cor., California, USA) . Briefly, 25 µl of standards, controls, or samples were added to each well of a 96-well plate previously coated with human osteocalcin. The plate was incubated for two hours at room temperature after adding 125µl of

antiosteocalcin antibody to each well. After washing with buffer, 150 µl of enzyme conjugate were added to each well. The plate was incubated for one hour at room temperature and then washed with buffer. After that, 150 µl of working substrate solution were added to each well and the plate incubated for 40 minutes at room temperature. Finally, 50 µl of stop solution were added to each well and the optical density was read at 405 nm using an automated plate reader.

A commercial immunoassay kit was used to assess the concentrations of BAP (Quidel Cor.,California, USA). Briefly, 125 µl of assay buffer and 20 µl of standards, controls, or serum samples were added to each well of the coated 96-well plate. After incubation for three hours at room temperature, the plate was washed with washing buffer. After adding 150 µl of working substrate solution, the plate was incubated for 30 minutes at room temperature. Finally, 100 µl of stop solution were added to each well and the optical density was read at 405 nm using an automated plate reader.

Statistical analysis

A repeated measures design was used as the statistical model to evaluate the concentrations of osteocalcin and BAP over time. A general linear model procedure was used to study the individual and time effect between two hours during the 24 h period using SAS program, and the significance level was set at $P< 0.05$. Pearson correlation coefficient analysis was used to study the relationship between osteocalcin and BAP levels.

RESULTS

Figure 1 shows the mean concentrations of serum osteocalcin. There is fluctuation in the concentration of osteocalcin during the 24 hour period with minimum and maximum concentrations were at 1300h and at 1800 h, respectively.

Figure 2 shows the mean concentrations of serum BAP for 24 hours. There was slight fluctuation in the concentration of BAP. The minimum and maximum concentrations of BAP were recorded at 0100 h and at 1200 h, respectively. Lower correlation was observed between serum osteocalcin

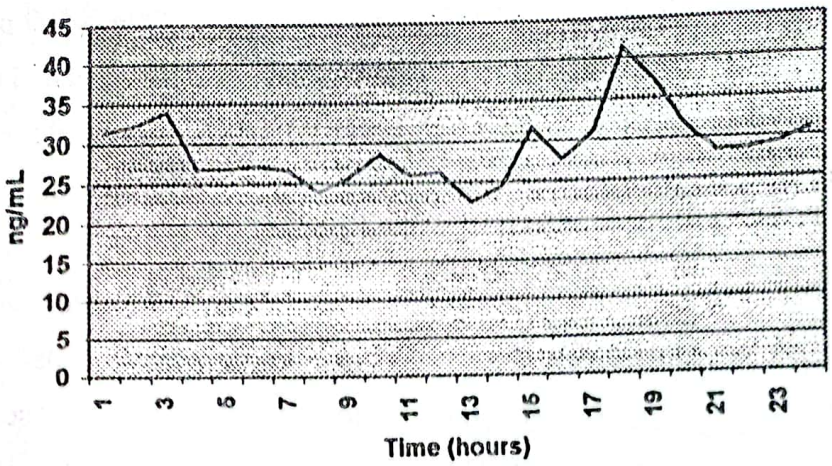

Fig 1: The concentrations of osteocalcin in serum of camels during the 24 hours

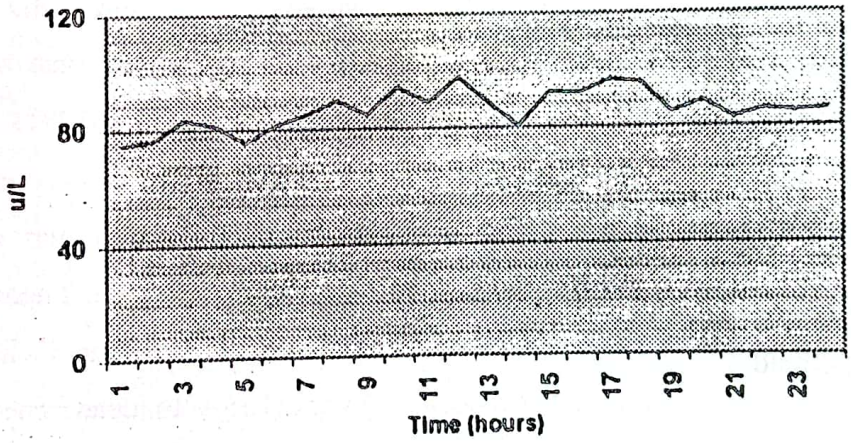

Fig 2: The concentrations of BAP in serum of camels during the 24 hours

and BAP

DISCUSSION

Measurements of serum osteocalcin and BAP concentrations have been used widely to assess skeletal status with emphasis to bone disorders in humans (Markowitz et al 1984, Rosalki & Foo 1984, Gundberg et al 1985, Markowitz et al 1987,

Nielsen et al 1988, Garnero & Delmas 1993, Rosenquist et al 1995, Gomez et al 1995, Hata et al 1996, Greenspan et al 1997, Price et al 1997, Heuck et al 1998) and animals (Lepage et al 1991, Farley et al 1992, Hank et al 1993, Sanecki et al 1993, Arican et al 1996, Collignon et al 1996).

There is no previous report describing the circadian rhythm of osteocalcin in camels. The present study has shown that the concentrations of serum osteocalcin were significantly different over time in camels. The maximum concentrations of osteocalcin were at 1800 h (0600 pm) and the level remarkably decreased for the next three hours. The level began to rise at 2200 h (1000 pm) and gradually increased in for the following 5 hours. At 0400 h, the levels decreased and continued nearly constant until at 1300 h (0100 pm) where it reached the lowest concentrations. This showed that there was a marked fluctuations observed in the concentration of serum osteocalcin during the 24 hour period. Therefore, it is important to adjust the time of blood collection for camels when the goal is to measure the concentrations of osteocalcin. Osteocalcin has a half-life of around 5 minutes and that might explain the high circadian variability of serum osteocalcin of camels (Naylor & Eastell 1999). The diurnal variation in the concentrations of serum osteocalcin in horses showed less fluctuation compared to what was found in camels (Lepage et al 1991). The levels of serum osteocalcin were not significantly different during the day from 0700 to 1900 h (0700 pm) and the minimum concentrations were in the early evening between 1900h (0700 pm) and 2000 h (0800 pm) (Lepage et al 1991). Then, the levels of serum osteocalcin gradually rose during the night and reached the maximum concentrations at 0500 h and finally dropped to a constant daytime level around 0700 h (Lepage et al, 1991). In humans, it has been shown that the levels of serum osteocalcin declined in the morning and reached a nadir after 1200 h, then rose again to reach a maximum concentration around 0400h (Markowitz et al 1984, Markowitz et al, 1987; Nielsen et al 1988).

This study showed that the maximum and minimum concentrations of serum osteocalcin in camels were 41.37 and 22.5 ng/mL, respectively. These were higher than what was reported in horses (Lepage et al 1991). In addition to the species effect, the assay used in the present study was different from what was used in Lepage study (Lepage et al 1991). Lepage et al used radioimmunoassay procedure while the present study used ELISA to determine the concentrations of serum osteocalcin. The serum samples in Lepage et al study were stored at -25°C for three weeks whereas the serum samples of the present study were stored at -20°C for 5 days.

There was slight fluctuation in the concentration of serum BAP during 24 hour period. There was no significant difference in the concentrations of

serum BAP among the samples collected between 1900 h (0700 pm) and 09.00h. Therefore, there is no need to fix the time of blood collection during this period of time . However, there were significant differences in the concentration of serum BAP among the blood samples collected between 1000 h and 1800 h (0600 pm). Therefore, the time of blood collection has to be fixed if the blood is collected between 1000 h and 1800 h (0600 pm). In comparison to osteocalcin, the concentration of serum BAP was more constant during the 24 hour period. This is may be due to the half-life of alkaline phosphatase is around 40 hours (Crofton 1982). The minimum and maximum concentrations of serum BAP in camels were at 0100 and 1200 h, respectively. In horses there were marked fluctuations in the level of total serum alkaline phosphatase over 24 hour period with minimum concentrations at 1400 (0200 pm) and 0200 h and maximum concentrations at 1600 (0400 pm) and 0500 h. A circadian rhythm for BAP has been reported in humans, but this was small and parallel to the reduction in serum albumin at night (Naylor & Eastell 1999).

The present study showed that the mean concentrations of serum BAP were 77.25 U/L. This is higher than what was reported for horses (Hank et al 1993) and less than what was reported for dogs (Sanecki et al 1993) and humans (Gomez et al 1995). The concentration of serum BAP was higher in camels compared to that in humans (Garnero et al 1993). However, different methods in the different studies were used to measure the concentrations of serum BAP. Price et al (1997) compared between two immunoassays for measuring BAP in serum of healthy children and the results showed highly different values between the two assays. This indicated that the type of assay used affects the concentration of the biomarker measured.

Osteocalcin and BAP are biomarker products of osteoblast that can be used as indicators of bone synthesis (Beresford et al 1984, Lian et al 1985, Deftos 1991). Therefore, it was expected that their concentrations in serum will have strong correlation. However, the present study showed weak correlation between serum osteocalcin and BAP in camels. Similar results have been reported in humans (Deftos et al 1982, Delmas et al 1990, Tracy et al 1990, Deftos 1991, Parthemore et al 1993). The lack of having strong correlation between the two biomarkers has been attributed to technical differences in methods of assay or because these biomarkers reflect different stages of osteoblast function (Delmas et al 1990, Tracy et al 1990). In the present study, very similar immunoassay procedures have been used to determine the concentrations of osteocalcin and BAP. Therefore, the expected reason(s) for having poor correlation between the two biomarkers can be attributed to biologic rather than methodologic causes. It has been reported that BAP represents an early

osteoblast biomarker because it presents in preosteoblasts and osteoblasts, whereas osteocalcin is a later biomarker of osteoblast differentiation and bone mineralization (Naylor & Eastell 1999).

In conclusion, the concentrations of osteocalcin and BAP were assessed in the serum of dromedary camels. In camels, it is important to fix the time of blood collection if the concentration of osteocalcin will be assessed, but this is less important when the level of BAP is going to be assessed. Although both osteocalcin and BAP are ostoblast products, a weak correlation between their concentrations was found in the serum of camels.

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