Arq. Bras. Med. Vet. Zootec., **v.63, n.1, p.40-45, 2011**

Tartrate-resistant acid phosphatase as a biomarker of bone turnover in dog

[*Fosfatase ácida resistente ao tartarato como biomarcador do metabolismo ósseo no cão*]

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ABSTRACT

Values of serum tartrate-resistant acid phosphatase **(**TRAP) activity were obtained in adult dogs and its biological variability was assessed. Nine healthy skeletally mature Portuguese Podengo dogs were used for the determination of TRAP, total and bone alkaline phosphatase serum activities, and also to study their relationship with serum minerals, namely calcium (Ca), phosphorous (P), and magnesium (Mg). The serum TRAP activity was 2.19±0.56IU/mL, with intra-individual variation of 18.3% and inter-individual variation of 25.6%. Significant correlations were observed between serum TRAP activity and Ca (r=-0.3431; P<0.05), Ca and Mg (r=-0.787; P<0.01), and TRAP and Mg (r=0.397; P<0.05). The results indicate that serum TRAP activity in dog could be of great value in research and in clinical practice, providing complementary non-invasive information on bone metabolism.

Keywords: dog, bone metabolism, bone resorption, osteoclast

RESUMO

Determinaram-se os valores da atividade da fosfatase ácida resistente ao tartarato (FART) e avaliou-se a sua variabilidade biológica. Neste estudo, foram utilizados nove cães adultos e saudáveis de raça Podengo Português para as determinações das atividades da FART, da fosfatase alcalina total, da isoenzima óssea da fosfatase alcalina e da concentração dos minerais séricos – cálcio, fósforo e magnésio. A atividade sérica obtida da FART foi de 2,19±0,56 UI/mL, com uma variação intra-individual de 18,3% e interindividual de 25,6%. Foram observadas correlações significativas ao longo do tempo entre FART e cálcio (r=-0,3431; P<0,05), entre FART e magnésio (r=0,3974; P<0,05) e entre cálcio e magnésio (r=-0,787; P<0,01). Os resultados indicam que este marcador de reabsorção óssea pode ser de grande valor na prática clínica e na investigação e, ainda, ser utilizado como um método auxiliar não invasivo para avaliação do metabolismo ósseo.

Palavras-chave: cão, metabolismo ósseo, reabsorção, osteoclaste

INTRODUCTION

Bone modelling and remodelling are the result of osteoblastic and osteoclastic cell activity. For this reason, it has been suggested that changes in bone metabolism could be monitored by the determination of specific enzymes and structural proteins of extracellular matrix (ECM), produced by bone cells and that are released into the bloodstream during this cellular activity (Seebeck et al., 2005). These molecules are considered as biochemical markers of bone metabolism and are usually divided into formation and resorption biomarkers. Formation markers are enzymes secreted by osteoblasts, such as total alkaline phosphatase (tALP), bone-

Recebido em 24 de maio de 2010
Aceito em 10 de janeiro de 2011
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specific isoform of alkaline phosphatase (BALP), osteocalcin (OC), and propeptides of type I collagen, like procollagen type I C-and N-terminal propeptides (PICP and PINP) formed during the bone formation process. Bone resorption markers are those resulting from the breakdown of type I collagen during the bone resorption process, namely serum carboxyterminal telopeptide of type I collagen (ICTP), type I collagen cross-linked C-and N-telopeptide (CTX and NTX), urinary hydroxyproline, total and free urinary pyridinoline and deoxypyridinoline, and specific enzymes expressed in bone-resorbing osteoclasts, like tartrate-resistant acid phosphatase (TRAP) (Garnero et al., 2002).

Bone-derived biochemical markers have been widely studied to assist in diagnosis and prognosis of metabolic bone diseases, such as osteoporosis in women after menopause and Paget's disease, and also to monitor the response to the treatment of these diseases. More recently, they have been applied to assess changes in bone turnover due to the presence of prostate and breast cancer metastases (Seibel, 2006). Furthermore, bone-derived biomarkers have also been proposed by some researchers to assess the process of bone fracture healing. In fact, several parameters seem to be suitable for reflecting the bone healing process allowing differentiation between normal and delayed or non-union processes of fracture healing (Joerring et al., 1992; Kurdy, 2000; Laurer et al., 2000; Herrmann et al., 2002; Komenou et al., 2005). Some parameters have also been presented as an aid in osteomyelitis diagnosis after osteosynthesis (Philipov et al., 1995; Southwood et al., 2003).

TRAP in particular, specifically its 5b isoform, is considered the most sensitive and specific biomarker of bone resorption, since it is only produced by bone-resorbing osteoclasts. The value of this enzyme has already been demonstrated as a reliable predictor of pathological fractures in a population of elderly women (Gerdhem et al., 2004) to monitor the response of osteoporosis antiresorptive treatments (Hallen et al., 2002; Nenonen et al., 2005) and as an auxiliary in the diagnosis of breast and prostate cancer metastases (Chao et al., 2004). There are six (0-5) acid phosphatase isoenzymes identified by electrophoresis, one of which is the isoenzyme 5, which is resistant to tartrate. This TRAP is a glycoprotein produced by osteoclasts, activated macrophages, and dendritic cells. Additionally, this band 5 has two isoforms (a, b), in which 5b isoenzyme is produced only by osteoclasts. It has been proposed that this marker describes the number of osteoclasts in addition to their activity and, due to this fact, it could be safely used to replace the determination of the osteoclasts number by bone histomorphometry in *in vivo* and *in vitro* studies using animal models or bone cell cultures, respectively (Hallen, 2006; Leeming et al., 2006). To the author's knowledge, there is only one work published in the scientific literature reporting on the serum activity of TRAP in the dog. Lee et al. (2008) assessed the activity of this enzyme after the induction of an experimental model of osteoarthritis, in order to analyze this parameter as a possible biomarker for diagnosing early stages of osteoarthritis.

The main goal of the present study was to obtain the value of serum TRAP activity in the adult healthy dog and to evaluate its biological variability, since there is no information concerning this parameter in the canine species. Besides this, TRAP would be an inexpensive analytical parameter that could be routinely performed in the large majority of clinical biochemistry laboratories. The degrees of correlation of this enzyme with concentration levels of serum minerals, namely – calcium (Ca), phosphorus (P), and magnesium (Mg) – also were studied.

MATERIAL AND METHODS

This study was conducted with nine healthy Portuguese Podengo dogs (four males and five females), with an average weight of 12kg, and aging 2 to 6-year-old. Females were not pregnant or in a sexual stage of estrus. Selection of animals was based on observance of proper prophylactic and health controls. All dogs had normal renal and hepatic functions and none of them showed evidence of pre-existing or ongoing orthopedic disease, history of any bone lesion, or administration of any drug that could affect bone metabolism. All the animals were fed with the same standard canine diet, water was provided *ad libitum*, and they were submitted to a weekly clinical examination during the study period.

Jugular venous blood samples were collected into plain tubes containing a clot activator (Venosafe®, gel and clot activator, ref. VF – 054SAS Terumo). All samples were collected at the same time in the morning, in order to minimize possible diurnal enzymatic activity variation, and repeated every 10 days, with a value of four samples per animal. The specimens were processed within 30 minutes after the blood collection, centrifuged at 2000*g* during 10 minutes, and the resulting serum decanted into 2mL polypropylene cryovials. Serum samples were frozen and stored at -80ºC until assayed, since it is reported that the serum activity of TRAP declines rapidly during storage at room temperature or even at -20ºC, but is stable at -70ºC or lower. All samples were analysed in duplicate and the mean value was used for statistical analysis.

Total ALP and TRAP serum activities were measured by molecular absorbance spectrophotometry, using an enzymatic method. TRAP serum activity was determined with 1-naphthyl phosphate as substrate and tartrate in the reaction mixture with a commercial reagent kit (Roche ACP®, ref. 04375351190), and enzymatic activity was measured at 37ºC and 415nm by an automated biochemistry analyzer (Hitachi 917® Roche). Total ALP serum activity was also measured with a commercial reagent kit (Aeroset AlkPo® E-3230), at 37ºC and 405nm by an automated biochemistry analyzer (Abbott Aeroset®). BALP serum activity was measured by ELISA (MicroVue BAP EIA, Quidel Corporation). Commercially available kits were also used for the determination of serum minerals, namely Ca (Calcium Abbott®, ref. 7d61-20) and P (Phosphorus Abbott®, ref. 7d71-2), by means of colorimetric test using a molecular absorbance spectrophotometer and reading carried out by automated analyser (Abbott Aeroset®).

All data are reported as mean ± standard deviation (SD) and the corresponding minimum and maximum values, respectively. The intra- and inter-individual variations were determined by calculating the percentage of SD from the mean value for each one of the biochemical parameters. The degree of correlation between the different biochemical parameters was assessed using the Fisher test. All statistical analyses were performed with the statistical software program JMP (JMP Version 7, SAS Institute, Inc.®, NC, USA). The *r*-value of correlations was considered significant at $P<0.05$.

RESULTS

The values of serum biochemical parameters values are presented in Table 1.

Table 1. Mean values and estimated analytical average, intra- and inter-individual variations of bone-derived markers, and serum minerals in adult dogs

Parameter	Mean ± SD	Range (Min.-Max.)	Intra-individual CV (%)	Inter-individual CV (%)	Intra-assay CV (%)
tALP (IU/L)	44.89±21.9	7.0-100.0	17.3	48.8	4.33
BALP (IU/L)	14.62±5.44	6.8-25.7	14.9	37.2	5.8
TRAP (IU/L)	2.19±0.56	1.4-3.9	18.3	25.6	3.2
Ca (mg/dL)	10.49±1.06	5.2-11.7	14.4	10.1	1.4
P (mg/dL)	4.21±0.70	2.76-6.0	21.0	16.6	9.3
Mg (mg/dL)	2.04±0.37	1.72-4.0	5.3	18.1	4.6

tALP: total alkaline phosphatase; BALP: bone alkaline phosphatase; TRAP: tartrate-resistant acid phosphatase; Ca: calcium; P: phosphorous; Mg: magnesium; CV: coefficient of variation

There was significant correlation between serum TRAP activity and Mg ($r=0.397$; $P<0.05$), Ca and Mg ($r=-0.787$; $P<0.01$), and TRAP and Ca ($r=-0.3431$; $P<0.05$) which could evidence and reinforce the serum TRAP activity parameter as a resorption biomarker.

DISCUSSION

The value for the serum TRAP activity was 2.19±0.56IU/mL, which is slightly lower than the one found in the sham group in the work of Lee et al. (2008). However, in that work, the animals

underwent a surgical procedure in which the stifle joint was fully opened and the cranial cruciate ligament and meniscus exposed, which does not exclude the influence of the surgery in stimulating serum TRAP activity. In the present study, the serum tALP and BALP activities and the values of serum minerals were found to be within the reference ranges published by Allen et al. (1998) and Kaneko et al. (2008) (tALP: 20-156IU/L; BALP: 0.9-24.5IU/L; Ca: 9.0-11.3mg/dL; P: 2.2-6.2mg/dL; Mg: 1.8-2.4mg/dL).

The recent interest in the bone metabolism serology arises from its possible application in the evaluation of metabolic bone diseases and in the possible evaluation of the bone fracture healing process. Bone biochemical markers could allow a dynamic assessment of bone healing which could facilitate early decision making in the treatment of bone fractures (Herrman et al., 2002; Komenou et al., 2005). However, some experimental studies showed no effectiveness with a selected panel of bone-derived markers chosen to monitor the fracture healing process (Paskalev et al., 2005; Seebeck et al., 2005; Theyese et al., 2006). This fact might be related to the choice of biomarkers, since not all bone-derived biomarkers have the same sensitivity to assess specific changes in bone metabolism, so that for each bone disease it is necessary to select a specific parameter panel, including its analytical test, to obtain the most useful clinical information (Hallen et al., 2002; Seibel et al., 2008). In the study of Seebeck et al. (2005), using an ovine fracture healing model, although the authors had suggested that the bone turnover parameters were unsuitable for monitoring the course of the fracture healing process, they also concluded that some of these bone-derived biomarkers were suitable to estimate the extent of the osteogenic response after a bone fracture. Additionally, with reference specifically to the serum TRAP activity, these authors found a significant degree of correlation between the decrease of this biomarker of bone resorption and the bone callus formation, caused by the higher level of bone formation than bone resorption, in the initial phase of the bone fracture healing process. In a posterior phase, those authors observed an increase in the serum TRAP activity related to the remodelling of the newly formed bone. The study also concluded that bone biomarkers could be useful as indicators of healing disturbances due to systemic deficiencies at a very early stage of the fracture healing process, which could facilitate early specific intervention in clinical situations.

The clinical application of TRAP as an aid to the diagnosis and prognosis of bone disturbances in dogs is dependent on the ability to detect significant changes from baseline values in healthy state. This discriminatory power is related with the variability underlying the analytical assay and the variability inherent to the study population. However, if the biological variability of a marker is high, the variability of the analytical test is not as restrictive. For a potential clinical application, it has been suggested that the variability of the analytical test to measure a specific biochemical marker should be less than or equal to half of the underlying biological variability for that parameter within the study population (Ladlow et al., 2002; Allen, 2003). The main limitation for clinical application of these parameters has been related to the difficulty in the interpretation of the individual results due to the high biological variability associated with factors like age; gender; nutrition; exercise; systemic disease; and circadian, day-to-day, and seasonal rhythms (Ladlow et al., 2002).

Biological variation could be divided into an intra-individual component, reflecting changes occurring in the same individual over time, and an inter-individual component, representing the differences between individuals (Panteghini and Pagani 1995). In the present work, the analytical variability of the assay used for serum TRAP activity determination was less than half of the intra-individual biological variation of the studied population, which could indicate that the discriminatory power of this assay justifies its routine clinical use in dogs. Among the factors that may contribute to the intra-individual variability in this work, the authors studied the influence of the day-to-day rhythm, since, unlike circadian variation which can be minimized by controlling the time of sampling, the day-to-day-variability is not capable of control, contributing greatly to the overall biological variability of biomarkers of bone metabolism and, therefore, a main problem in the interpretation of individual results. In this study, when analyzing the biological variability of these two biomarkers,

the serum activity of tALP and BALP presented a lower value of within-subject variability than serum TRAP activity and this last enzyme showed the lowest inter-individual variation, a fact also observed by Panteghini and Pagani (1995) in man. Moreover, the high rate of inter-individual variation found for the serum tALP activity was similar to previously data published for the dog (Allen et al., 2000; Kaneko et al., 2008). Additionally, the intra- and inter-individual variations found for TRAP was similar to that found for other resorption biomarkers for this species such as ICTP in the study of Ladlow et al. (2002), and similar to that found in humans in the study of Panteghini and Pagani (1995). In accordance with results obtained in studies published for man concerning the activity of the two enzymatic parameters studied in the present work, the inter-individual variability was higher than the intra-individual variation. Finally, regarding the mean values obtained for the serum TRAP activity, no statistically significant differences between males and females were found ($P>0.05$).

The clinical usefulness of conventional reference values could be objectively measured by the index of individuality (II), and the ratio between intra- and inter-individual biological variabilities (Panteghini and Pagani, 1995). If this ratio is lower than 0.6, the use of reference values as an aid in the interpretation of individual results appears to be quite limited, as with the case of serum tALP activity in this study (II=0.4). However, the reference intervals of serum TRAP activity showed usefulness for the interpretation of individual results (II=0.7), with the small exception that certain results, even when within the reference limits, could be abnormal for the concerned individual.

Determination of bone-derived biomarkers can be a simple and non-invasive alternative to bone biopsy to assess bone disease in dogs. Furthermore, these parameters can also be used in research to evaluate skeletal safety in dietary and drug formulations for this species. The analytical variability in the assay and the biological variability of serum TRAP activity presented within the dog population in this work justify additional studies to evaluate the sensitivity and clinical utility of this bone resorption biomarker.

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