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Joint dependent concentrations of bone alkaline phosphatase in serum and synovial fluids of horses with osteochondral injury: an analytical and clinical validation

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Summary

Objectives: Validate use of a commercially available immunoassay for measurement of bone alkaline phosphatase (BAP) in equine serum and synovial fluid (SF), and investigate the effects of osteochondral (OC) injury in horses on BAP concentrations in serum and SF.

Methods: SF was collected from 37 joints of 34 Thoroughbred (TB) racehorses undergoing arthroscopic surgery for the removal of OC fragments from either the carpal joints ($n = 18$) or the metacarpo-/metatarsophalangeal (MP) joints ($n = 19$). SF was also obtained from 52 joints of 16 normal TB horses, collected bilaterally from carpal joints of 10 horses ($n = 40$), and MP joints of six horses ($n = 12$). Blood was obtained from all 50 horses. A commercially available immunoassay was validated and subsequently used to determine equine serum and SF BAP concentrations. Correlations to radiographic and arthroscopic scores were assessed.

Results: BAP concentrations were significantly lower in serum from horses with OC injury in their carpal or MP joints than in serum from normal horses. SF BAP concentrations in normal and OC injured carpal joints were significantly higher than MP joints. BAP concentrations were significantly higher in SF from OC injured carpal joints than normal. BAP concentrations were affected by joint sampled, with age having a significant interaction. Concentrations of BAP in the serum (<30 U/L), SF (>22 U/L) and a ratio of SF to serum ≥0.5 were predictive of OC injury. Radiographic and arthroscopic scores significantly correlated with serum BAP concentrations, and SF:serum BAP correlated with arthroscopic scores.

Conclusions: Determination of serum and SF BAP concentrations may be beneficial in the investigation of early joint injury. Joint and injury dependent differences in BAP concentrations allowed the estimation of predictive value for identifying OC injury.
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Key words: Bone alkaline phosphatase, Validation, Biomarker, Joint, Synovial fluid, Serum, Osteochondral injury, Horse.

Introduction

Osteoblasts synthesize and secrete a variety of non-collagenous proteins, of which, bone alkaline phosphatase (BAP) is the most abundant[1]. BAP is a bone specific isoenzyme of the alkaline phosphatases that is coded by the same gene product as the hepatic, placental, intestinal, and renal isoenzymes, but differs from these as a result of post-translational modifications[2]. Analysis of total alkaline phosphatase activity has demonstrated the liver- and bone-specific isoenzymes as the most abundant forms (>90%) identified in human serum[3]. The exact role of BAP is still unknown, but it may contribute to calcification of bone matrix[4,5] because it is a membrane-bound glycoprotein that is anchored to the matrix vesicle[6,7]. It is released into the circulation after phospholipase cleavage from the membrane in physiopathologic conditions[8].

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Even though the health of subchondral bone may be critical in the development of osteoarthritis (OA)[9], few studies have measured bone-specific biomarkers to examine bone turnover associated with OA. In human OA patients, the presence and location of total alkaline phosphatase and/or bone-specific isoenzyme in the joint have been described *in situ*[10–12]. The activity levels in human and canine OA have primarily been determined in serum, with minimal examination of SF[13]. However, the examination of BAP concentrations in both dogs and horses has demonstrated correlation of SF concentrations with pathologic change[14–16].

Biomarkers have been examined in horses to identify injury-and/or exercise-induced metabolic changes that occur in both bone and joints[14,17–21]. Most examine the response of bone turnover in relationship to skeletal development and/or exercise, independently of the joint, or the resultant metabolic changes of the extracellular cartilage matrix and synovium in response to joint injury and/or exercise, independently of the bone. As a result of high-speed exercise, horses will commonly sustain injury to subchondral bone. This may result in an osteochondral (OC) fragment, exposing subchondral bone to the synovial cavity. The resultant debris, inflammation, and instability can eventually progress to OA; most horses with OC injury have

evidence of OA. Therefore, OC injury in the horse is a useful model to examine the relationship of concurrent injury to bone and cartilage and the resulting development of OA.

The aims of the present study were to: (1) validate use of a commercially available immunoassay for the measurement of BAP in equine serum and SF, (2) investigate the effects of OC injury on serum and SF BAP concentrations in horses, (3) establish BAP as a biomarker for non-invasive investigation of OA by comparing BAP concentrations to radiographic and arthroscopic scores, and (4) evaluate the influences of age, gender, and joint injured on the BAP concentrations in equine serum and SF.

Materials and methods

STUDY ANIMALS AND SAMPLES

Serum and SF were aseptically collected from 37 joints of 34 Thoroughbred (TB) racehorses (1–7 years of age; median age 3 years) undergoing arthroscopic surgery for the removal of exercise-induced OC fragments. These fragments were removed from the dorsoproximal, medial or lateral aspect of the first phalanx in 14 metacarpophalangeal (MCP) joints and five metatarsophalangeal (MTP) joints, as well as from the dorsal articular borders of the third, radiocarpal, or intermediate carpal bones in 13 middle carpal (MC) joints and the dorsal articular borders of the distal radius, proximal intermediate or radiocarpal bones in five antebrachiocarpal (ABC) joints. Sixteen horses were females, 12 were intact males, and six were castrated males. Serum and SF were also aseptically collected from 16 normal TB horses (2–6 years of age; median age 4 years) to use as controls. Control horses were determined to be free of orthopedic disease based on their clinical, lameness, and radiographic examinations. Ten horses were castrated males and six were females. SF was collected bilaterally from the MC and ABC joints of 10 horses ($n = 40$), and bilaterally from the MCP joints of six horses ($n = 12$).

Blood was collected from the jugular vein *via* needle venipuncture. After being allowed to clot, serum samples were centrifuged and decanted. SF was collected by aseptic needle arthrocentesis. If SF samples were contaminated with blood, they were also centrifuged and decanted. All clinical and control samples were stored at −80°C until assayed. Sample collection was approved by the University of Florida Institutional Animal Care and Use Committee.

RADIOGRAPHIC AND ARTHROSCOPIC SCORES

All joints used in the study were radiographed prior to SF sampling. A numerical scoring system was developed for radiographic scoring of the carpal and MCP joints. There were 10 categories of radiographic changes that were each graded by two blinded surgeons (TNT and MPB) from 0 to 3 to make up a total radiographic score of 0–30. Joint space narrowing, soft tissue swelling/effusion, subchondral bone sclerosis, and subchondral bone lucency were all graded as: 0 = none, 1 = mild, 2 = moderate, 3 = severe. The number of osteophytes and enthesophytes that were present in each joint was determined and graded as: 0 = none, 1 = 1–2 present, 2 = 3–4 present, or 3 = >4 present. The size of the largest osteophyte or enthesophyte was determined and subjectively graded as: 0 = none, 1 = small, 2 = medium, or 3 = large. OC fragments were graded according to the number of fragments present: 0 = none, 1 = 1 fragment, 2 = 2 fragments, 3 = >2 fragments. The size of the largest OC fragment was determined and subjectively graded as: 0 = none, 1 = small, 2 = medium, or 3 = large.

Medical records, including surgery reports, arthroscopic photographs and videos, were examined for all 34 horses undergoing arthroscopic surgery for the removal of fragments. A modified arthroscopic scoring system was developed that could be used in any joint and would specifically account for injury associated with OC fragmentation. A total of 11 categories were graded by two blinded surgeons (TNT and MPB) and summed to make up a total arthroscopic score of 0–37. The total numeric score takes into account five categories of inflammation (graded 0–3)[22,23], two categories associated with the fragments (graded 0–3), and, four categories of degenerative cartilage changes related to the fragments (graded 0–4)[23–25]. Inflammation was graded as: 0 = none, 1 = mild, 2 = moderate, 3 = severe for the following categories: hyperemia, petechiation, increase in synovial villi density/thickening, presence of new villi/rice body formation, and villi atrophy/flattening with fibrin and adhesion formation. The OC fragment was graded from 0 to 3 as described above in the radiographic grading and was based on the number and size of the largest fragment. The degree of cartilage damage was graded based on the worst lesion present. The relationship of cartilage damage to the OC fragment was graded as: 0 = normal, 1 = localized to fragment, 2 = kissing (opposing articular surface) lesion present, 3 = extends onto cartilage surface of the affected bone, with or without a kissing lesion, or 4 = extensive, including large parts of the articular surface. Extension of cartilage damage from the fragment was graded as: 0 = localized to fragment, 1 = minimal fibrillation or fragmentation at the edge of defect left by fragment, extending no more than 5 mm from fracture line, 2 = cartilage degeneration extending more than 5 mm from the defect, including up to 30% of articular surface of bone, 3 = loss of 50% or more of articular cartilage from affected bone, or 4 = significant loss of subchondral bone[24]. Depth of cartilage damage surrounding the fragment was graded as: 0 = normal, 1 = swelling/softening, 2 = superficial fibrillation, 3 = deep fibrillation down to bone, 4 = exposure of subchondral bone. Depth of cartilage damage of the kissing lesion was also graded as: 0 = normal, 1 = swelling/softening, 2 = superficial fibrillation, 3 = deep fibrillation down to bone, 4 = exposure of subchondral bone.

PROCEDURE FOR THE BAP IMMUNOASSAY

Concentrations of BAP were measured in equine serum and SF using a commercially available immunoassay (Metra BAP, Quidel Corporation, San Diego, CA). This immunoassay uses a purified murine monoclonal anti-BAP antibody that has high affinity for the bone-specific isoform and low cross-reactivity with the liver isoform of alkaline phosphatase (3–8%)[26]. Serum and SF samples were analyzed without digestion or dilution.

VALIDATION OF THE BAP IMMUNOASSAY FOR EQUINE USE

The assay was validated for use in equine serum and SF by determining the precision, specificity, sensitivity, accuracy, linearity of dilution, and stability for each fluid. From six of the normal horses, fresh serum was aseptically collected from the jugular vein and SF was aseptically collected from 12 MC and 12 ABC joints. Each fluid was pooled together for further processing and analysis. Internal quality control (QC) samples were prepared utilizing the highest concentration standard provided by the manufacturer (140 U/L). Pooled samples were spiked with high (QCH), medium (QCM) and low (QCL) levels of BAP. For each fluid, the prepared QCH, QCM, and QCL samples were pooled together for further analyses.

The reproducibility of the standard curve was evaluated by computing the mean optical density (OD) and the percent coefficient of variation (CV) at each standard point as well as with the included low and high controls from the manufacturer. The intra-assay CV was determined using three of the same QCH, QCM, and QCL analyzed in duplicate on the same plate. The inter-assay CV was determined using the same QCH, QCM, and QCL samples analyzed in duplicate across three different plates. To demonstrate parallelism, dilutions of 1:2, 1:4, 1:8, and 1:10 were compared with the standard curve that was derived using the standards provided by the manufacturer by comparing the absorbance to the concentration. Detection limit of the assay was analyzed using the assay buffer. The lowest limit of detection was defined as two standard deviations above the mean of the assay buffer.

Each of the QC samples (high, medium, and low) was assayed along with the original pooled sample (background) and the percent recovery for each was determined to identify whether BAP measurement agreed with the actual amount present in the sample. Percent recovery was calculated as: 100 × (amount of BAP recovered from QCH, QCM, or QCL)/(amount of BAP added to the pooled sample + background BAP amount in the pooled sample). The linearity of the BAP assay was analyzed by serially diluting the samples at 1:2, 1:4, 1:8, and 1:10 to determine whether the results were directly proportional to the concentration of BAP in the sample. The concentration of each dilution was determined from the standard curve, and the observed concentration was plotted against the reciprocal of the dilution (1/dilution). Stability of the BAP analyte was analyzed across two plates by comparing fresh QCH, QCM, and QCL samples analyzed immediately after sample collection as well as after 24 h at room temperature, after 24 h at 4°C, and then after one to four freeze/thaw cycles at −80°C.

STATISTICAL ANALYSIS

Statistical evaluation was performed by the use of personal computer-based statistical software (SPSS 15.0 for Windows, SPSS, Inc., Chicago, IL). Normal distribution of the data was determined by producing normality plots. A one-way ANOVA was performed on serum and SF BAP concentrations using Tukey's pairwise test for multiple comparisons. A Kruskal–Wallis analysis was performed on the SF:serum BAP and radiographic scores using Dunn's test for multiple comparisons. A Mann–Whitney unpaired *t* test was performed on the arthroscopic scores. A multivariate general linear model was used to determine what factors contribute to the serum and SF BAP concentrations. The model included serum and SF BAP concentrations from horses undergoing arthroscopic surgery as dependent variables. Age (grouped into three ranges: ≤2, >2 to ≤5, and >5 years of age), gender (intact male, female, and castrated male), location (left fore, right fore, left hind, and right hind limbs), and joint sampled (MCP, MTP, MC, and ABC) were analyzed as fixed effects and total radiographic and arthroscopic scores were analyzed as covariates. The main effects and interactions of

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