Osteoarthritis and Cartilage (2008) **16,** 779–786

doi:10.1016/j.joca.2007.11.008

Osteoarthritis and Cartilage

International Cartilage Repair Society

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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.

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].

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

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Received 14 August 2007; revision accepted 17 November 2007.

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

the fixed effects on the model were determined using Hotelling's Trace. Spearman's rank correlation was used to determine direct correlations. Contingency tables were analyzed using Fisher's exact test to determine the sensitivity, specificity, predictive values and an odds ratio. $P < 0.05$ was considered significant.

Results

BAP ASSAY VALIDATION

The OD values of the standards exhibited acceptable inter-assay precision over 10 plates with an overall mean CV of 8.1% (range 4.8–12.3%). The inter-assay precision of the low control (mean CV of 7.4%) was better than the high control (mean CV of 10.8%). No intra-assay CV was determined for the standards. Samples exhibited acceptable inter-assay precision over three plates with an overall mean CV of 7.2% (range 5.7–9.6%) for the serum and 7.1% (range 4.8–8.8%) for SF. Samples exhibited acceptable intra-assay precision over three plates with an overall mean CV of 3.3% (range 0.1–8.1%) for the serum and 2.6% (range 0.2–7.5%) for SF. Figure 1(A) demonstrates parallelism of equine serum and SF sample dilutions when compared to the standard curve. The lowest detection limit of the assay was determined to be 1.42 U/L. The percent recoveries ranged from 103.1 to 116.1% for the serum with the most accurate recoveries recorded at the high concentrations, whereas percent recoveries for SF ranged from 64.8 to 91.2% with highest recoveries recorded at the low concentrations (Table I). Figure 1(B) demonstrates linearity of equine serum and SF sample dilutions. Serum and SF stability results demonstrated that overnight sample processing was better if performed at 4°C rather than at room temperature (Table II). For the serum QCM and QCL samples, there was an increase in BAP after three freeze/thaw cycles, whereas the QCH sample had minimal loss of BAP out to three freeze/thaw cycles. For the SF and QCM samples, there was minimal loss of BAP out to two freeze/thaw cycles, whereas there was minimal loss for the QCH and QCL samples out to four freeze/thaw cycles.

BAP CONCENTRATIONS IN EQUINE SF AND SERUM

Significantly lower BAP concentrations ($P < 0.01$) were measured in serum of horses with OC injury of carpal and MP joints than in serum of normal horses [Fig. 2(A)]. However, serum BAP concentrations were not significantly different when compared between horses with carpal and MP OC injury. When comparing the serum concentrations between normal horses and OC injured horses, 91% of the horses that had serum BAP concentrations < 30 U/L had MP or carpal OC injury (Table III).

SF BAP concentrations in the carpal joints of normal horses were 2.2 times higher than SF BAP concentrations in the MP joints of normal horses [$P < 0.05$; Fig. 2(B)]. SF BAP concentrations from OC injured carpal joints were 2.7 times higher than concentrations from OC injured MP joints ($P < 0.001$). In addition, SF BAP concentrations from the OC injured carpal joints were significantly higher than from normal carpal joints ($P < 0.001$). When comparing SF concentrations between normal carpal joints and OC injured carpal joints, 80% of the horses that had synovial BAP concentration > 22 U/L had carpal OC injury (Table III). However, there was no significant difference between SF BAP concentrations in normal and OC injured MP joints. When combining the OC injured carpal and MP samples, SF BAP concentrations were positively correlated to the joint that was affected (Table IV).

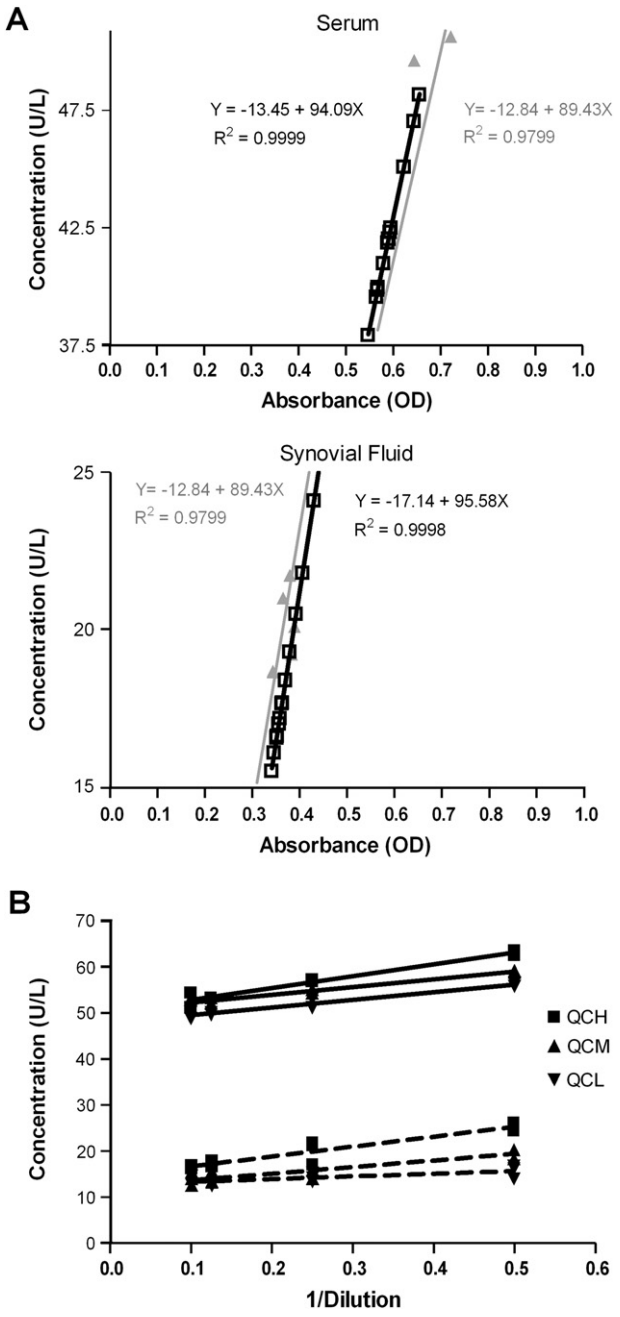

Fig. 1. Validation of BAP immunoassay for use in equine serum and SF. (A) Demonstration of parallelism: absorbance vs concentration of diluted (1:2, 1:4, 1:8, and 1:10) pooled serum and SF samples (squares) is compared with the curve derived from standards provided by the manufacturer (triangles). Coefficient correlations for the samples (black) and standards (gray) are listed next to each corresponding line. (B) Demonstration of linearity: dilutions of serum (solid lines) and SF (dashed lines) samples at 1:2, 1:4, 1:8, and 1:10 for each QCH, QCM, and QCL samples. 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).

Multivariate analysis of all factors demonstrated that serum and SF BAP concentrations varied between the joints that were sampled ($R = 0.717$; $P = 0.008$). There was a significant interaction of the joint sampled with age ($R = 0.828$;

Table I
Percent recovery from spiked samples (measured concentration/expected concentration × 100) for serum and SF BAP concentrations

Serum			SF		
Expected concentration (U/L)	Measured concentration (U/L)	% Recovery	Expected concentration (U/L)	Measured concentration (U/L)	% Recovery
63.965	65.921	103.057	46.784	30.295	64.755
53.965	60.242	111.632	32.784	28.211	86.053
41.965	48.702	116.054	18.884	17.223	91.208

$P = 0.005$). This interaction resulted from horses ≤2 years of age having higher serum BAP concentrations associated with MP joints than older horses. When the interaction was removed from the analysis, joint sampled (carpal or MP) was the only factor that had a significant effect on the serum and SF BAP concentrations ($R = 0.315$; $P = 0.02$).

Serum BAP concentrations were significantly higher ($P < 0.001$) than SF BAP concentrations from either joint from normal horses. In addition, horses with OC injured MP joints had serum BAP concentrations that were significantly higher than their concurrent SF concentrations ($P < 0.001$). However, serum and SF BAP concentrations from horses with OC injured carpal joints were not significantly different. SF:serum BAP was the same between normal horses and horses with OC injured MP joints [Fig. 2(C)], but was significantly increased in horses with OC injured carpal joints ($P < 0.001$). Horses with OC injury in the carpus had 180 times greater likelihood of having an SF:serum BAP ≥ 0.5 compared to normal horses (Table III). In addition, SF:serum BAP correlated with arthroscopic score for both joints ($R = 0.494$; $P = 0.006$; Table IV). When analyzed by joint, SF:serum BAP from horses with OC injured MP joints showed correlation with arthroscopic scores ($R = 0.610$; $P = 0.007$).

Total MP and carpal radiographic scores could be used to identify differences between normal and OC injured joints (Table III). Total radiographic score for OC injured MP joints (mean ± SD; 7.8 ± 4.6) was 5.5 times higher ($P < 0.001$) than normal MP joints (1.4 ± 1.4). Total radiographic score for OC injured carpal joints (8.5 ± 4.0) was 3.9 times higher ($P < 0.001$) than normal carpal joints (2.2 ± 1.4). However, there was no significant difference in radiographic scores between OC injured MP and carpal joints. As an approximate indicator of the severity of lesions, radiographic scores ≥ 10 (out of a maximum score of 30) were identified in 7/18 (38.9%) of the OC injured MP joints and 9/20 (45%) of OC injured carpal joints. In addition, there was no significant difference in arthroscopic scores between OC injured MP (12.4 ± 4.6) and carpal (15.3 ± 6.9) joints. Similarly, arthroscopic scores ≥ 20 (out of a maximum score of 37) were identified in 1/18 (5.6%) of the OC injured MP joints and 4/16 (25%) of OC injured carpal joints. There was a positive correlation ($R = 0.645$; $P = 0.0001$) between radiographic and arthroscopic scores for OC injured MP and carpal joints. For OC injured joints, both radiographic and arthroscopic scores increased with age, however, both decreased as the serum BAP concentrations increased (Table IV).

Discussion

The aims of this study were to validate the use of a commercially available immunoassay for the measurement of BAP in equine serum and SF and to investigate the effects of OC injury on serum and SF BAP concentrations in horses. Other assays for BAP activity in horses have been reported[27,28]. The enzyme immunoassay used in the present study has essentially no cross-reactivity with the liver isoform (3–8%)[26]. Our results indicated that this assay has cross-reactivity with equine serum and SF by demonstrating precision, specificity, sensitivity, accuracy, linearity, and stability.

Intra- and inter-assay variations are indicators of assay precision, with CV < 10–15% usually considered as acceptable. Our intra-assay CV was <9% and the inter-assay variation was <10% for both the serum and SF. SF was not digested with hyaluronidase prior to analysis. This apparently did not affect pipetting accuracy, based on precision of the assay. In addition, the elimination of hyaluronidase digestion allowed the analysis of fresh samples and prevented any potential breakdown of BAP. Spiking recoveries ranged from 103.1 to 116.1% for serum and 64.8 to 91.2% for SF. Although percent recovery was less for SF, highest recoveries were recorded at low concentrations, where most of the biological activity was present in the samples (Table I). BAP activity in serum was more labile than in SF (Table II). In fact, serum BAP activity actually increased when the samples were incubated at room

Table II
Stability data for the QCH, QCM, and QCL samples for serum and SF demonstrating BAP concentrations (U/L) after samples were either processed immediately (Fsh), after 24 h at room temperature (Rm Tmp), after 24 h at 4° C, or after one to four freeze/thaw cycles (F/T 1–4). Percent difference (% Diff) represents difference in concentrations between the fresh samples vs the room temperature, 4° C, or F/T samples

	Fsh	Rm Tmp	% Diff	4°C	% Diff	F/T 1	% Diff	F/T 2	% Diff	F/T 3	% Diff	F/T 4	% Diff
Serum													
QCH	59.7	63.4	6.3	58.8	−1.6	54.1	−9.4	47.4	−20.6	48.2	−19.2	62.9	5.4
QCM	49.2	58.8	19.6	49.4	0.5	45.5	−7.6	53.9	9.5	59.2	20.4	54.1	10.0
QCL	39.6	48.0	21.3	40.6	2.6	37.3	−5.8	38.8	−2.0	41.7	5.5	42.1	6.5
SF													
QCH	40.5	45.4	12.2	41.5	2.6	41.8	3.3	44.8	10.8	35.1	−13.3	36.1	−11.0
QCM	32.7	32.1	−1.8	31.3	−4.2	29.8	−8.6	28.6	−12.3	24.8	−24.1	25.1	−23.1
QCL	16.6	20.0	20.6	19.1	14.7	18.5	11.3	15.2	−8.6	15.0	−9.4	15.3	−8.1

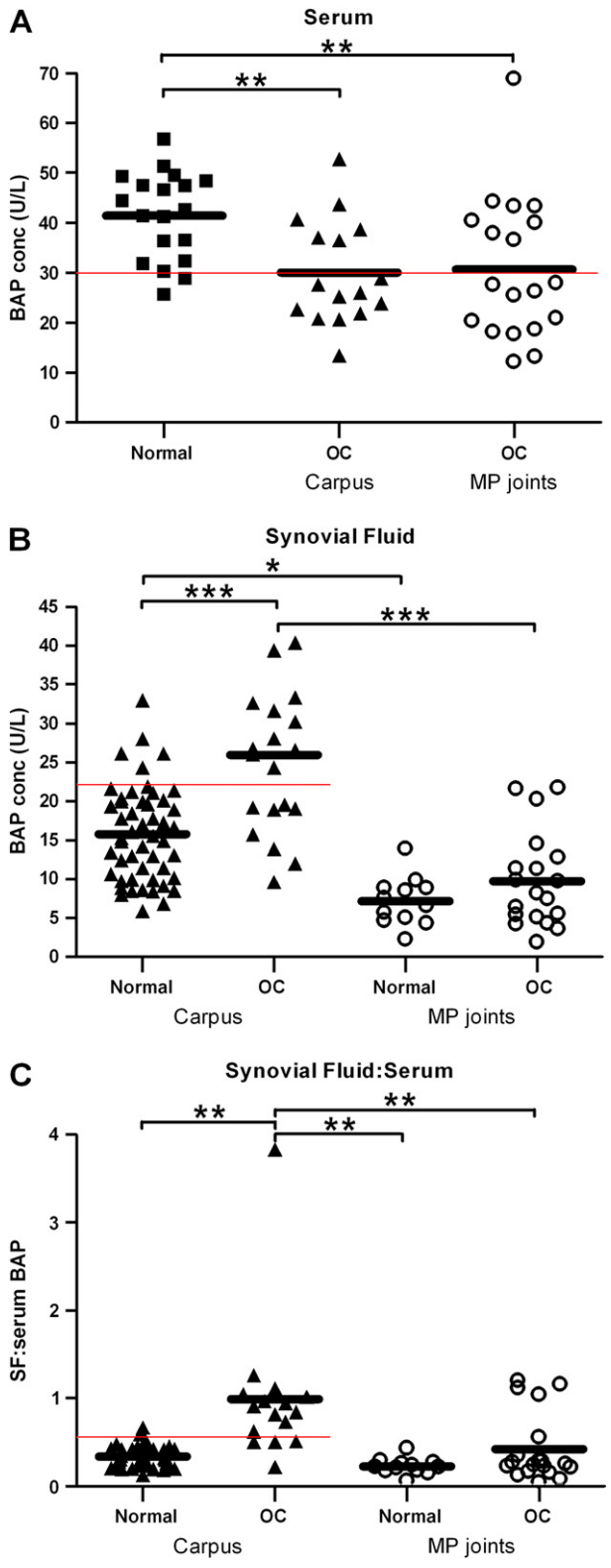

Fig. 2. Scattergram with mean BAP concentrations in (A) serum, (B) SF, and (C) the ratio of SF to serum for normal horses and horses with OC injury of the carpal (triangles) or MP (circles) joints. Significant differences between groups are represented as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Red lines represent concentrations (<30 U/L for serum, >22 U/L for carpal SF, and ≥0.5 for carpal SF:serum) for which there is predictive value in determining OC injury from normal.

Table III
Sensitivity, specificity, positive predictive value, negative predictive value and odds ratio to discriminate between OC injured and normal horses when serum BAP concentration is <30 U/L, carpal SF BAP concentration is >22 U/L, carpal SF:serum ratio ≥0.5, or radiographic score ≥4 for the MP joints, or ≥5 for the carpus

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Odds ratio
Serum BAP <30 U/L	60	88	91	50	10.5
Carpal SF BAP >22 U/L	60	92	80	82	18.0
Carpal SF: serum BAP ≥0.5	94	92	83	97	180.0
MP joint radiographic score ≥4	83	92	94	79	55.0
Carpal joint radiographic score ≥5	80	93	84	90	49.3

temperature, 4°C, and after three freeze/thaw cycles for the QCM and QCL samples. The reason for this phenomenon is not known, but it has been previously reported regarding stability of human alkaline phosphatase activity in serum[29,30].

Most studies of biomarkers in humans are limited by the availability of fluids. Urine and serum are the most commonly analyzed fluids because of ease of collection. SF is rarely analyzed because it is difficult to obtain adequate volumes and consent for collection. However, SF should be the best fluid for characterization of events occurring in a specific joint. Therefore, differential analysis of both serum and SF from the same patient is extremely valuable[31]. The horse is a good species to use for this type of analysis. Horses are a good translational model of OA in humans[32], and SF can be regularly obtained in high volumes (usually >2 mL) without lavage.

To the authors' knowledge, the current study is the first to demonstrate significantly lower serum BAP concentrations from horses with OC injury compared to normal horses. We also demonstrated a concurrent significant increase in SF BAP concentrations from OC injured horses compared to normal horses. In addition, we found a joint-dependent difference in SF BAP concentration. Carpal joints had significantly higher SF BAP concentrations than MP joints in both normal horses and those with OC injury [Fig. 2(B)]. *In vivo* and *in vitro* equine studies have reported differences in SF biomarker concentrations between joints from normal horses[33–36]. Most of these studies have demonstrated that the synovial structures distal to the MP joints normally had higher biomarker concentrations compared to more proximal joints[33–35], as well as greater response to cytokines[33]. Findings may be similar when analyzing SF from different joints in humans because differences have been demonstrated in knee and ankle cartilages in response to cytokines and cytokine antagonists[37]. These discrepancies between joints may be due to intrinsic differences, such as joint size, biomechanical properties of the cartilage and bone, amount of force transferred across the joint, as well as biochemical composition and gene expression of articular cartilage in response to anabolic and catabolic factors[33–37]. Therefore, differences in biomarker concentrations between different joints are important to consider regardless of the biomarker studied.

Table IV

Correlations for variables studied in OC injured horses. Significant correlations are represented by an asterisk. Age, gender, and joint affected are represented as categorical variables. Age groups: 1 = ≤2 years, 2 = >2 to ≤5 years, and 3 = >5 years. Gender: 1 = intact male, 2 = female, and 3 = castrated male. Joint affected: 1 = MCP, 2 = MTP, 3 = MCJ, and 4 = RCJ

	Age	Gender	Joint affected	SF BAP concentration	Serum BAP concentration	SF:serum	Radiographic score	Arthroscopic score
Age	1.00	$R = 0.308$ $P = 0.057$	$R = 0.005$ $P = 0.976$	$R = 0.131$ $P = 0.428$	***R = −0.428** **P = 0.01**	$R = 0.287$ $P = 0.095$	***R = 0.467** **P = 0.003**	***R = 0.492** **P = 0.003**
Gender		1.00	$R = 0.177$ $P = 0.281$	$R = 0.039$ $P = 0.815$	$R = -0.232$ $P = 0.179$	$R = 0.032$ $P = 0.854$	$R = 0.176$ $P = 0.920$	$R = 0.227$ $P = 0.197$
Joint affected			1.00	***R = 0.554** **P = 0.0001**	$R = 0.032$ $P = 0.856$	$R = 0.316$ $P = 0.065$	$R = 0.046$ $P = 0.782$	$R = 0.038$ $P = 0.830$
SF BAP concentration				1.00	$R = -0.130$ $P = 0.458$	***R = 0.845** **P = 0.0001**	$R = 0.241$ $P = 0.145$	$R = 0.290$ $P = 0.096$
Serum BAP concentration					1.00	***R = −0.592** **P = 0.0001**	***R = −0.414** **P = 0.015**	***R = −0.451** **P = 0.012**
SF:serum						1.00	$R = 0.293$ $P = 0.093$	***R = 0.494** **P = 0.006**
Radiographic score							1.00	***R = 0.645** **P = 0.0001**
Arthroscopic score								1.00

Significant correlations are represented as bolded R and *P* values.

In general, serum concentrations of bone biomarkers depend upon the number of osteoblasts required for bone formation as well as clearance rate of the biomarker[38]. Serum BAP concentrations in the present study were 26–28% lower in horses with OC injury when compared to normal horses [Fig. 2(A)]. Our findings were similar to other osteoblastic biomarker studies in humans where there was a decrease in serum BAP (19%)[39] or osteocalcin (15%)[40] concentrations in women with OA compared to normal women. However, Campion *et al.* reported high concentrations of osteocalcin in serum of people with destructive OA[41]. Therefore, our results support the hypotheses that lower than normal concentrations of osteoblastic biomarkers in the serum are indicative of non-destructive OA[39–41]. The cause for this decrease is unknown, but may be related to lower bone turnover[39,40].

Decrease in serum BAP concentrations in early OA may be associated with the structure of BAP when it is released into serum. Most BAP in serum is in the soluble dimer form[42,43]. However, in OA there may be greater release of the hydrophobic domain of the BAP enzyme that anchors it to the plasma membrane of the osteoblast. If this anchor-bound form is released into serum, it can form aggregates with other proteins and lipids[42,43]. These complex formations may prevent the immunoassay from recognizing BAP.

Our findings that serum concentrations decreased while SF concentrations increased raise the question whether BAP in serum may be filtrated into the SF. Previous reports have suggested that a basic way to examine the relationship between serum and SF is to calculate a ratio of SF:serum[44,45]. The assumption was that a ratio of SF:serum >1 would be suggestive of local production of the bone biomarker in the joint[45], whereas ratios <1 would indicate that BAP in the joint was due to diffusion from the blood[44]. Using this approach in the present study would suggest that most of the SF BAP concentration was derived from the blood [Fig. 2(C)], because SF:serum BAP was <1 for all normal horses and the majority of horses with OC injury. Normal horses in our study generally had lower ratios than horses with OC injury. This suggests that there was not an increase in the amount of filtration of BAP from the blood to the joint when OC injury was present. On the contrary, it suggests that with OC injury, there was greater local production of BAP in the joint. This was most obvious in horses with carpal OC injury where many ratios were ≥0.5. In fact, a ratio of ≥0.5 for the horses with OC injury in their carpal joints was predictive of OC injury. Using this ratio assumes passive diffusion of BAP across the synovial membrane. None of these studies have investigated the kinetic movement of the biomarker of interest across the synovial membrane and therefore have not confirmed that the site of origin was either blood or SF. Nonetheless, the concept of a ratio between SF and serum BAP concentrations is useful.

Fuller *et al.* reported increased SF BAP concentrations in TB racehorses with OC injury compared to contralateral joints. Although these investigators concluded that the increased concentration supported the view that bone is involved in the early process of OA, they offered no explanation for the source of the BAP in SF[14]. Alkaline phosphatase activity has been shown to be present around the cell surfaces of hypertrophic chondrocytes in growth plates as well as matrix vesicles, allowing it to participate in mineralization and subsequent calcification[46]. Therefore, the most likely source for increased SF BAP concentrations after OC fragmentation is the subchondral bone. Studies of osteochondritis dissecans have demonstrated that BAP can be immunolocalized just below the tidemark and can be released toward the surface of the joint when a fissure is present in the articular cartilage[47]. The results of the present study may support the subchondral bone as a major source of locally produced BAP in OC injured joints, considering that the extent of subchondral bone injury and exposure is usually greater in carpal joints than in MP joints.

Another likely reason for injury-related increase in SF BAP concentrations is the formation of osteophytes and enthesophytes. Osteophytes have been shown to go through the same morphogenetic process as growth plate cartilage by the development of hypertrophic chondrocytes[48]. It has been demonstrated that osteophytes have high alkaline phosphatase activity[49]. In a previous canine cranial cruciate

transection model of OA, a positive correlation ($R = 0.6705$; $P < 0.0001$) was identified between the SF BAP concentration and the radiographic osteophyte score[16]. A direct correlation to osteophyte production could not be made in the present study, but most OC injured horses had some degree of osteophytosis, making osteophytes a likely contributory source of SF BAP. Immunohistochemical studies in rheumatoid arthritis have suggested that BAP could be produced in the synovium, however, there was little support for this in OA patients[12].

To the authors' knowledge, there are no veterinary radiographic or arthroscopic scoring systems for OC fragmentation that can be applied to different joints. As a modification of previously described scoring systems, we devised radiographic and arthroscopic scoring systems for OC injury that could be used in both the carpal and MP joints of horses. The radiographic scoring system accounted for the presence of soft tissue swelling, number of OC fragments, osteophytes, enthesophytes, and subchondral bone changes such as lysis and sclerosis[16,50]. The arthoscopic scoring accounted for the presence of OC fragments, inflammation, and extent of cartilage damage[22–25].

These scoring systems appeared to cover the spectrum of injury that was visible either radiographically or arthroscopically and were a good indicator of the degree of pathologic change present. In support of this, radiographic scores were good predictors of the severity of joint damage associated with OC injury compared to normal carpal and MP joints, in which no OC injury was present (Table III). This same comparison could not be made with the arthroscopic scoring system because the control horses were not examined arthroscopically. For OC injured joints, the highest severity scores tended to occur more often in the carpus than the MP joints for both scoring systems. In addition, we identified a significant positive correlation between the radiographic score and the arthroscopic score ($R = 0.645$; $P = 0.0001$), indicating that the two scoring systems could identify increasing pathologic change. Serum BAP concentrations negatively correlated with both radiographic and arthroscopic scores indicating that as serum BAP decreased, the radiographic and arthroscopic scores increased (Table IV). Interestingly, SF BAP concentrations did not correlate with either the radiographic or arthroscopic scoring systems. However, SF:serum BAP showed a positive correlation with arthroscopic score (Table IV). SF:serum BAP was the only indicator for MP joints that BAP concentrations correlated with the degree of pathologic change present in the joint ($R = 0.610$; $P = 0.007$).

Potential sources of variability for bone biomarkers in horses include age[27,51], gender[52,53], circadian rhythms[54], and seasonal effects[55]. We attempted to minimize sources of variability, but had some limitations. All horses were of the same breed and groups were age-matched as closely as possible. Similar to previous studies[27,51], age was a factor in the present study, with younger horses ($\leq$2 years) having higher serum BAP concentrations than horses $>$ 2 years of age. However, we saw no effect of age on SF BAP concentrations. Age also correlated with radiographic and arthroscopic scores, with scores tending to increase with age (Table IV). Gender did not correlate with BAP concentrations or radiographic or arthroscopic scores. The effects of circadian rhythm and seasonal differences were minimized in the normal horses by sampling all horses in the morning as well as in the spring. However, the time and season of sampling could not be controlled for the OC injured horses because samples were collected at the time of arthroscopic surgery. However, based on previous reports[54,56], it is anticipated that the effects of these sources of variation would not heavily contribute to the results.

This study had some additional limitations. With regards to the validation, no standard stock solution could be obtained from the manufacturer, so internal QC samples were prepared using the highest concentration standard provided by the manufacturer (140 U/L). Because of this, only the lower half of the standard curve was analyzed extensively. Although it would be ideal to examine the entire curve by dividing it into thirds, examination of the lower half of the curve was acceptable because that is where values for our equine serum and SF samples were detected. When used with human serum, this assay shows only 3–8% cross-reactivity with the liver isoenzyme. However, it has been suggested that horse serum may have slight differences in the carbohydrate component of the liver isoenzyme compared to humans[27]. We did not examine the cross-reactivity of the assay with other isoenzymes and therefore the amount of cross-reactivity with the liver isoenzyme in horses is unknown.

Some factors are difficult to control when collecting clinical samples from horses with OC injury. This includes exercise and medication history. Racehorses may be trained and treated in many different ways that could affect concentrations of BAP. Controlled, longitudinal studies of experimentally created OC injury would allow control of these factors.

This study validated the use of a commercially available immunoassay for the measurement of BAP in equine serum and SF. The assay was used to identify joint and injury dependent differences in the SF concentration of BAP when comparing normal and OC injured carpal and MP joints. In addition, serum and SF concentrations, as well as the ratio of the two, demonstrated a strong predictive value of BAP concentration for identifying OC injury. Based on the results of this study, determination of serum and SF BAP concentrations may be beneficial in the investigation of early joint injury.

Conflict of interest

None of the authors of this manuscript have any financial and personal relationships with other people or organisations that could inappropriately influence (bias) our work.

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