Osteoarthritis and Cartilage (2008) **16,** 1196–1204

doi:10.1016/j.joca.2008.03.008

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses

D. D. Frisbie D.V.M., Ph.D., Dr., F. Al-Sobayil B.V.Sc., Ph.D., Dr., R. C. Billinghurst D.V.M., Ph.D., Dr.[a], C. E. Kawcak D.V.M., Ph.D., Dr. and C. W. McIlwraith B.V.Sc., Ph.D., D.Sc., Professor*

Orthopaedic Research Center, Colorado State University, 300 West Drake, Fort Collins, CO 80523, United States

Summary

Objective: To discriminate between changes in biomarkers with exercise compared to changes in biomarkers with osteoarthritis (OA) in exercising horses.

Method: Sixteen, 2-year-old horses were randomly assigned either to an exercise-alone ($n = 8$) or OA-affected (also exercised) ($n = 8$) group. All horses had both mid-carpal joints arthroscoped and OA induced in one mid-carpal joint in the OA-affected joints of OA-affected horses. Two weeks after surgery all horses commenced a strenuous exercise program on a high-speed treadmill. Clinical outcomes and synovial fluid and serum biomarkers, were evaluated weekly. Synovial and serum biomarkers evaluated were epitope CS846 (CS846), epitope CPII (CPII), glycosaminoglycans (GAGs), epitope Col CEQ (Col CEQ) (a marker of type II collagen degradation), type I and II collagen degradation fragments (C1,2C), osteocalcin, C-terminal of bone type I collagen (CTX1), type I collagen (Col I) and (synovial fluid only of cartilage) prostaglandin E2 (PGE2) levels. Horses were euthanized at day 91 and their joints assessed grossly, histopathologically, and histochemically.

Results: Exercise induced a significant increase in synovial fluid CS846, CPII, GAG, Col CEQ, C1,2C, osteocalcin and Col I concentrations. There was a significant increase in synovial fluid CS846, CPII, Col CEQ, C1,2C, osteocalcin, Col I and PGE2 concentrations in OA-affected joints compared to exercise-alone joints. The concentration of serum CS846, CPII, GAG, osteocalcin, C1,2C and Col I increased with exercise. For each of these biomarkers there was also a statistically significant increase in serum biomarker levels in OA-affected horses compared to exercise-alone horses.

Conclusions: Six synovial fluid and serum biomarkers were useful in separating early experimental OA from exercise alone but synovial fluid CTX1 and serum Col CEQ and CTX1 were not.

Key words: Horse, Synovial fluid, Serum, Biomarkers, Exercise, Osteoarthritis.

Abbreviations: EDTA Ethylenediaminetetraacetic acid, ELISA Enzyme linked immunosorbant assay, GAGs Glycosaminoglycans.

Introduction

Release of different macromolecules and their fragments into synovial fluid and serum follow the anabolic and catabolic processes in the cartilage[1–9]. Biomarkers that provide specific information about alterations in cartilage matrix anabolism or catabolism are designated as direct biomarkers[10]. As biochemical alterations caused by osteoarthritis (OA) involve dynamic processes within all elements that constitute the joint, these mediators and products of tissue metabolism originate from cartilage, synovial membrane, and subchondral bone. Biomarkers can potentially be used to: (1) clarify pathobiological processes in the joint; (2) differentiate diagnostically between affected and non-affected joints and distinguish the degree of degradation in articular cartilage; (3) monitor the response to therapy; and (4) prognosticate. An experimental model of OA has been used by the authors for all four purposes[11–13].

Synovial fluid and serum biomarkers have proven useful in the diagnosis of equine bone and joint disease[14]. Initial work in the authors' laboratory used biomarkers developed in Dr Robin Poole's laboratory[15] and showed synovial fluid epitope CS846 and total protein levels were significantly higher in joints in clinical cases of osteochondral fragmentation (OCF) in the carpus and that synovial fluid total protein and CS846 epitope concentrations were linearly related to the grade of fragmentation[16]. Of equal significance serum epitope CS846 and CPII concentrations were significantly higher in horses with OCF than in control horses.

Biomarkers have been used to evaluate the response to therapy in an equine OA-exercise model but the confounding effects of exercise require clarification. Other studies have shown both increases in certain markers with exercise[17–19] as well as decreases with other biomarkers with exercise[20–22]. In order to discriminate between changes in biomarkers with exercise compared to changes of biomarkers with OA in exercising horses the following study was designed in which both synovial fluid and serum biomarkers were assessed.

[a]Present address: Billinghurst is at the School of Health Sciences, St Lawrence College, Kingston, NO, Canada K7L5A6.

*Address correspondence and reprint requests to: Prof. C. W. McIlwraith, Orthopaedic Research Center, Colorado State University, 300 West Drake, Fort Collins, CO 80523, United States. Tel: 1-970-297-0348; Fax 1-970-297-4118; E-mail: wayne.mcilwraith@colostate.edu

Received 9 August 2007; revision accepted 9 March 2008.

Methods

EXPERIMENTAL DESIGN

Following approval by the Colorado State University Animal Care and Use Committee 16 2-year-old horses free of lameness and with radiographically clinically normal carpal joints were used in the study. The horses were randomly assigned to either an exercise-alone ($n = 8$) or OA-affected (also exercised) ($n = 8$) group. All horses were exercised (see below) for the first 21 days. On day 21, horses in the exercise control had both mid-carpal joints arthroscopically examined under general anesthesia. Horses in the OA-affected group had both mid-carpal joints arthroscoped and OA induced in one middle carpal joint (briefly an 8-mm osteochondral fragment is created on the distal radial carpal bone and then the defect burred back to a 15-mm defect with the debri left in the joint as previously described[11]); the other sham operated joint was designated as the control joint. The mid-carpal joints in the exercise-alone groups were also arthroscoped to confirm that they were normal.

EXERCISE

Horses were housed in a stall (3.65×3.65 m) unless otherwise noted. Horses were exercised on a high-speed treadmill 5 days each week for the first 21 days; treadmilling was recommenced at day 35 (stall rested after surgery days 22–34) and continued until the end of the study (day 91). Each day, the horses underwent trotting (16–19 km/h) for 2 min, galloping (approx. 32 km/h) for 2 min, followed by trotting (16–19 km/h) for 2 min to simulate the strenuous exercise of race training.

ASSESSMENT OF CLINICAL OUTCOMES

For each horse, clinical examinations of both forelimbs were performed bi-weekly from prior to day 0 (baseline) throughout the study period. These included lameness graded on a scale of 0–5[23], flexion of the carpal joint followed by the horse trotting as an indicator of increase in pain at the trot, response to flexion graded on a scale of 0–4, and mid-carpal joint effusion graded on a scale of 0–4 as previously described[11]. All outcome parameters were assessed by a board certified large animal surgeon (a specialist in equine lameness) who was unaware of treatment assignments.

IMAGING

For each horse, there were radiographic evaluations of both carpi as well as nuclear scintigraphy, computed tomography and magnetic resonance imaging studies done. The results will be presented in a separate manuscript.

COLLECTION OF SYNOVIAL FLUID AND SERUM

Beginning on day 0 until the end of the study (day 91), a jugular vein blood sample as well as synovial fluid sample (both middle carpal joints) was aseptically aspirated once per week (except week 7, 9, and 11) from each horse in both groups. Serum from the blood sample was stored at -80°C for further analysis. Synovial fluid (2–4 mL) was directly aspirated from the joints by use of a 20-gauge needle and syringe. Samples were placed in tubes containing ethylenediaminetetraacetic acid (EDTA) for routine synovial fluid analysis (total protein concentration, cytologic evaluation, and total WBC count) or stored at -80°C for biochemical/biomarker protein analysis.

SYNOVIAL FLUID AND SERUM BIOMARKERS

Seven biomarker protein assays were performed on both serum and synovial fluid samples collected. Concentrations of the epitope CS846 were measured by a commercial enzyme linked immunosorbant assay (ELISA) kit (IBEX Diagnostics, Montreal, Quebec, Canada) as a marker of aggrecan synthesis[6,24]. Concentrations of the epitope CPII were measured by using a commercial ELISA kit (IBEX Diagnostics, Montreal, Quebec, Canada) as a measure of type II collagen synthesis[25,26]. Both of these assays were previously validated in the investigator's laboratory for use in the horse[27]. A modified 1,9-dimethylmethylene blue dye-binding assay was used on papain digested samples to determine glycosaminoglycan (GAG) concentration as a marker of cartilage matrix degradation (GAG)[28]. Concentrations of the epitope Col CEQ were measured using an ELISA developed for the horse to measure type II collagen degradation[27]. Concentrations of osteocalcin were estimated using a Metra™ Osteocalcin EIA Kit (Quidel Corporation, San Diego, CA, USA), which has been validated as a serum marker of bone formation[29]. Bone turnover in serum was estimated based on the release of the C-terminal of type I collagen (CTX1), a measure of bone specific type I collagen (Col I). This assay has previously been used successfully in horses (CrossLaps ELISA Nordic Bioscience Diagnostics, Denmark)[30,31]. A competitive ELISA was used to estimate concentrations of the epitope Col 2-3/4Cshort which has been validated as measuring both type I and II collagen degradation fragments[25] (now called C1,2C, IBEX Diagnostics, Montreal, Quebec, Canada[32]). By subtracting the concentration of Col CEQ from the concentration of C1,2C, Col I degradation in cartilage could indirectly be determined[15]. Synovial fluid concentration of PGE2 was assessed following extraction of PGE2 from synovial fluid and estimated by use of a commercially available high-sensitivity enzyme immunoassay kit (PGE2 ELISA, Assay Design, Ann Arbor, MI, USA).

GROSS OBSERVATION OF JOINTS

At the end of the study, all horses were euthanized by the use of sodium pentobarbital overdose. For each horse, a necropsy examination was performed during which both middle carpal joints were specifically examined for degree and location of articular cartilage fibrillation or erosion and synovial membrane hyperemia as previously described (gross pathology score)[11].

HISTOLOGIC EXAMINATIONS

At necropsy, samples of synovial membrane and joint capsule were collected from the dorsomedial region of the joint, placed in neutral-buffered 10% formalin and 5-μm sections of the tissue samples were prepared as previously described[11]. An evaluator, who was unaware of treatment assignments, evaluated the sections of synovial membrane and fibrous joint capsule for cellular infiltration, synovial intimal hyperplasia, subintimal edema, subintimal fibrosis, and subintimal vascularity (each outcome graded 0–4)[20].

Articular cartilage specimens were collected from an area directly adjacent to the osteochondral fragment (or equivalent in control joints), a portion of the opposing articulating surface (third carpal bone), and a remote location (fourth carpal bone). Half of the 5-μm sections were stained with haematoxylin and eosin (H&E) and the remainder was stained with Safranin O-Fast Green (SOFG). Sections stained with H&E were evaluated blindly for articular cartilage fibrillation, chondrocyte necrosis, chondrone formation (chondrocyte division within a lacuna), and focal loss of cells (each outcome graded 0–4)[11].

Articular cartilage sections stained with SOFG were evaluated blindly for intensity of staining in the tangential, intermediate, radiate territorial, and radiate interterritorial zones. Numeric values ranging from 0 to 4 were assigned to each variable (0 indicated no stain uptake and 4 indicated normal stain uptake).

ARTICULAR CARTILAGE MATRIX EVALUATION

To estimate articular cartilage proteoglycan content, the total articular cartilage GAG content was measured by use of a previously reported 1,9-dimethylmethylene blue technique[11]. Radiolabeled SO_4 ($^{35}SO_4$) incorporation was also measured by use of previously reported methods[11]. Samples were processed in duplicate and the results were reported as counts per minute (cpm) per mg of dry weight.

STATISTICAL ANALYSIS

The data from the exercise-alone group was analyzed to compare any differences between left and right sham operated limbs using a general linear model analysis of variance with the horse serving as a random effect. Because no significant differences were noted in these analyses the limbs from the exercise-alone group were considered similar. Thus, for synovial fluid biomarkers, three groups were defined for statistical analysis of limb related outcome parameters: exercise-alone (sham operated limbs) ($n = 16$), OA-affected ($n = 8$), exercise-alone joints of OA-affected horses ($n = 8$). This model allowed determination of a systemic effect within each group (i.e., does the OA-affected joint influence outcome parameters from the contralateral sham operated joint). A split plot with repeated measures design was used as the statistical model to evaluate the dependent variables over time. Outcome variables recorded at a single time point were subjected to a general linear model procedure analysis of variance. In all cases analyzing independent variables for both the main and the interaction effects on the dependent variables were performed.

For outcome parameters measured in the serum, the effect of exercise and OA were considered in the analyses. A split plot design with repeated measures analysis was used as the statistical model to evaluate the dependent variables. Furthermore, proc Mixed (SAS, version 8e, Carey, NC, USA) was used to perform a general linear mixed model analysis of variance for statistical comparisons. The independent variables in this model were day of sample collection and exercise-alone operated or OA-affected groups. The subject within-exercise/exercise OA was used as a random effect variable. In all analyses the highest interaction of the independent variables with $P < 0.05$ was considered the most significant and was reported. When there was a significant interaction effect, a Least Square Means test was used to make individual comparisons. A Pearson correlation coefficients

analysis was used to study the relationship among various dependent variables. Values of the correlation coefficients were classified into four grades: slight ($r \leq 0.25$), mild ($0.25 < r \leq 0.50$), moderate ($0.50 < r \leq 0.75$), and strong ($0.75 < r \leq 1$). A protected *F*-test was used on main effect variables as well as their interactions to reduce multiple comparison issues. Only significant *F*-tests in the analyses of variance table were looked at further for individual comparisons using a least square means procedure.

Results

CLINICAL ASSESSMENTS

A significant increase in lameness, response to carpal flexion and effusion were demonstrated in the OA-affected limbs/joints. The exercise-alone joints in the OA-affected horses had a significant increase in response to flexion by the termination of the study when compared to pre-study values, as did the exercise-alone joints of the exercise-alone horses, but this response was significantly lower compared to the exercise OA-affected joints. The degree of joint effusion increased (but not significant) in all horses following the onset of treadmill exercise; exercise-alone joints returned to pre-study effusion levels by day 84, while exercise-alone joints in OA-affected horses maintained a similar effusion level post surgery [Fig. 1(a)]. The OA-affected joints maintained a constant level of effusion post surgery that was significantly elevated compared to both other groups throughout the remainder of the study.

SYNOVIAL FLUID BIOMARKERS

Results of routine synovial fluid analysis indicated no significant differences in synovial fluid color or clarity. The total WBC for all joints increased significantly in the 2 weeks following surgery. Exercise-alone joints of the OA-affected horses and the exercise-alone horses returned to pre-study levels but the OA-affected joints remained significantly elevated compared to the other groups and pre-study levels [Fig. 1(b)]. Similarly, within 14 days of surgery the total protein level in OA-affected joints was significantly elevated when compared to both exercise-alone joints in both OA-affected and exercise-alone joints, and these values remained significantly elevated for the duration of the study [Fig. 1(c)]. Exercise induced a significant increase in synovial fluid CS846, CPII, GAG, Col CEQ, C1,2C, osteocalcin and Col I with exercise which persisted once it increased but no increase in PGE2 (except day 14) and CTX1 (Fig. 2). The levels of CS846, CPII, GAG, Col CEQ, C1,2C, osteocalcin, Col I and PGE2 in the OA-affected joints were significantly elevated (within 14, 7, 42, 56, 56, 91 and 35 days, respectively) and remained elevated throughout the study when compared to both the exercise-alone joints in exercise-alone and OA-affected horses at similar study periods (Fig. 2).

Concentrations of Col I were significantly elevated in the OA-affected joints compared to exercise-alone joints and these differences remained throughout the duration of the study. There was no increase in PGE2 levels with exercise but within 35 days the PGE2 concentrations were significantly elevated in the OA-affected joints compared to the exercise-alone joints; this difference remained significant for the duration of the study. There were no significant increases in CTX1 levels with exercise or with OA and therefore these data are not presented graphically.

Significant correlations were observed between various biomarkers. Specifically, CS846 levels showed significant mild correlations with CPII ($r = 0.50$), GAG ($r = 0.46$), Col CEQ ($r = 0.37$) and PGE2 ($r = 0.36$). Concentrations of CPII demonstrated a strong significant correlation with Col CEQ ($r = 0.82$) and a moderate correlation with PGE2 ($r = 0.68$) as well as GAG ($r = 0.64$) concentrations. The concentrations of GAG had mild significant correlations with Col CEQ ($r = 0.49$) and PGE2 ($r = 0.36$). A significant strong correlation both type II collagen degradation (Col CEQ) ($r = 0.92$) and Col I degradation products ($r = 0.90$) and the combination of C1,2C was demonstrated. A moderate significant correlation between Col CEQ and PGE2 ($r = 0.59$) was seen. All other biomarker to biomarker correlation comparisons were not shown to be statistically significant.

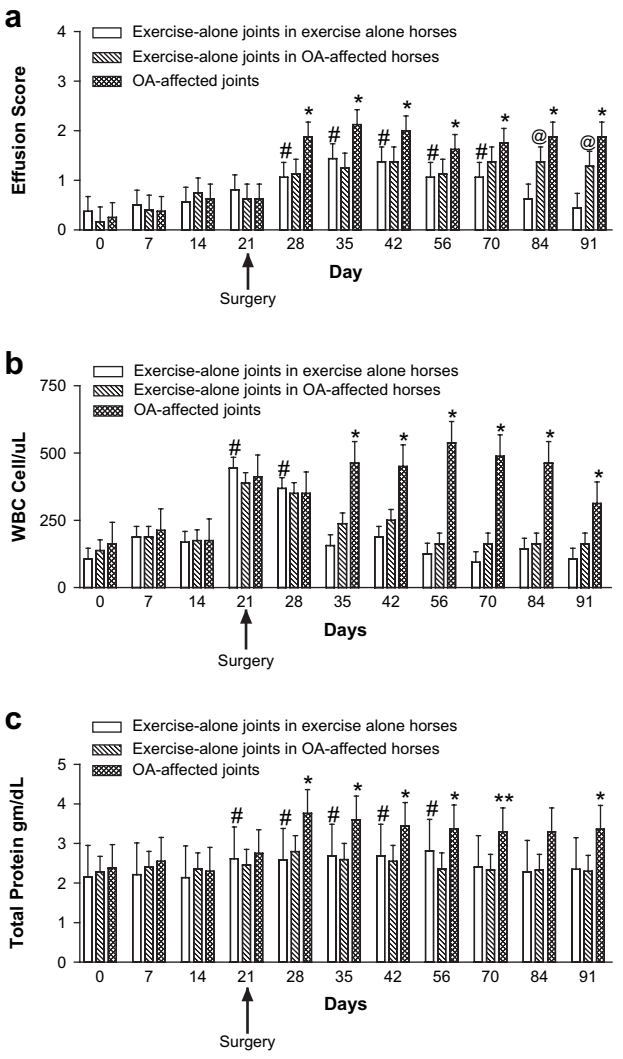

Fig. 1. (a) Plot of average effusion score ± S.E.M. by study day. Study days that have any bar with ''#'' symbols represent a significant change in all three groups (exercise-alone joints in exercise-alone horses, exercise-alone joints in OA-affected horses, OA-affected joints) compared to day 0 value. Bars with ''@'' symbol represent significant change compared to exercise-sham operated. Bars with ''*'' symbol represent significant change compared to both exercise-alone joints in exercise-alone horses and exercise-alone joints in OA-affected horses. (b) Plot of average WBC ± S.E.M. and (c) total protein ± S.E.M. by study day. Study days that have any bar with ''#'' symbols represent a significant change in all three groups (exercise-alone joints in exercise-alone horses, exercise-alone joints in OA-affected horses, OA-affected joints) compared to day 0 value. Bars with ''*'' symbol represent significant change compared to both exercise-alone joints in exercise-alone horses and exercise-alone joints in OA-affected horses.

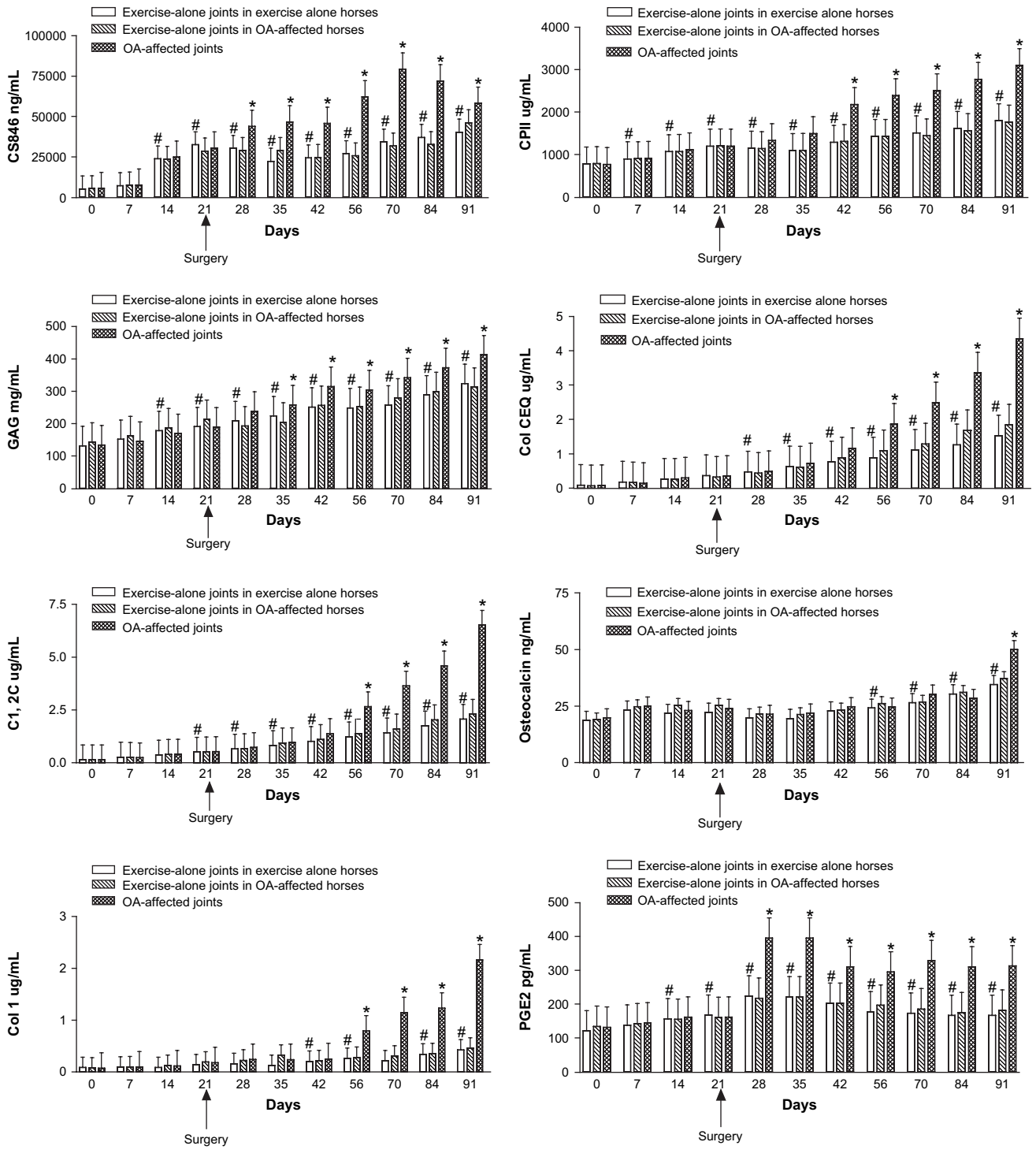

Fig. 2. Plot of average synovial fluid CS846, CPII, GAG, Col CEQ, C1,2C, osteocalcin, Col I and PGE2 levels ± S.E.M. by study day. Study days that have any bar with "#" symbols represent a significant change in all three groups (exercise-alone joints in exercise-alone horses, exercise-alone joints in OA-affected horses, OA-affected joints) compared to day 0 value. Bars with "*" symbol represent significant change compared to both exercise-alone joints in exercise-alone horses and exercise-alone joints in OA-affected horses.

SERUM BIOMARKERS

The concentrations of CS846, CPII, GAG, C1,2C, osteocalcin and Col I (within 7, 14, 7, 42, 42 and 42 days, respectively) increased significantly following the onset of exercise. Col CEQ only increased significantly with exercise in the last week of the study and there was no clear pattern with CTX1. There was also a significant increase in CS846, CPII, GAG, C1,2C, osteocalcin and Col I levels (days 35, 42, 49, 84, 91 and 63, respectively) in OA-affected horses compared to exercise-alone horses that persisted beyond

these initial increases. There was no significant increase in Col CEQ or CTX1 levels in OA-affected horses. A strong significant correlation between CS846 and CPII concentrations was also observed ($r = 0.77$) (Fig. 3).

COMPARISON OF SYNOVIAL FLUID AND SERUM BIOMARKER LEVELS

Significant correlations were demonstrated between synovial fluid and serum biomarker levels (Table I). The levels of osteocalcin, C1,2C, and Col I in serum compared to synovial fluid were 10%, threefold and ninefold higher, respectively, for exercise-OA horses serum compared to the exercise OA-affected synovial fluid. This is in comparison to the mean levels of CPII, Col CEQ, CS846, and GAG which were 50%, 10-fold, 20-fold, and threefold higher, respectively, in the synovial fluid compared to the serum for exercise-sham operated horses and 70%, 23-fold, 22-fold and threefold higher in exercise OA-affected synovial fluid compared to serum for the same horses. The levels of CPII, Col CEQ, CS846, and GAG in exercise OA-affected synovial fluid compared to serum for the same horses was 70%, 23-fold, 22-fold, and threefold higher, respectively.

GROSS PATHOLOGIC OBSERVATIONS AND CORRELATION WITH BIOMARKERS

At necropsy examination, full thickness articular cartilage fibrillation, synovial membrane hemorrhage and the total

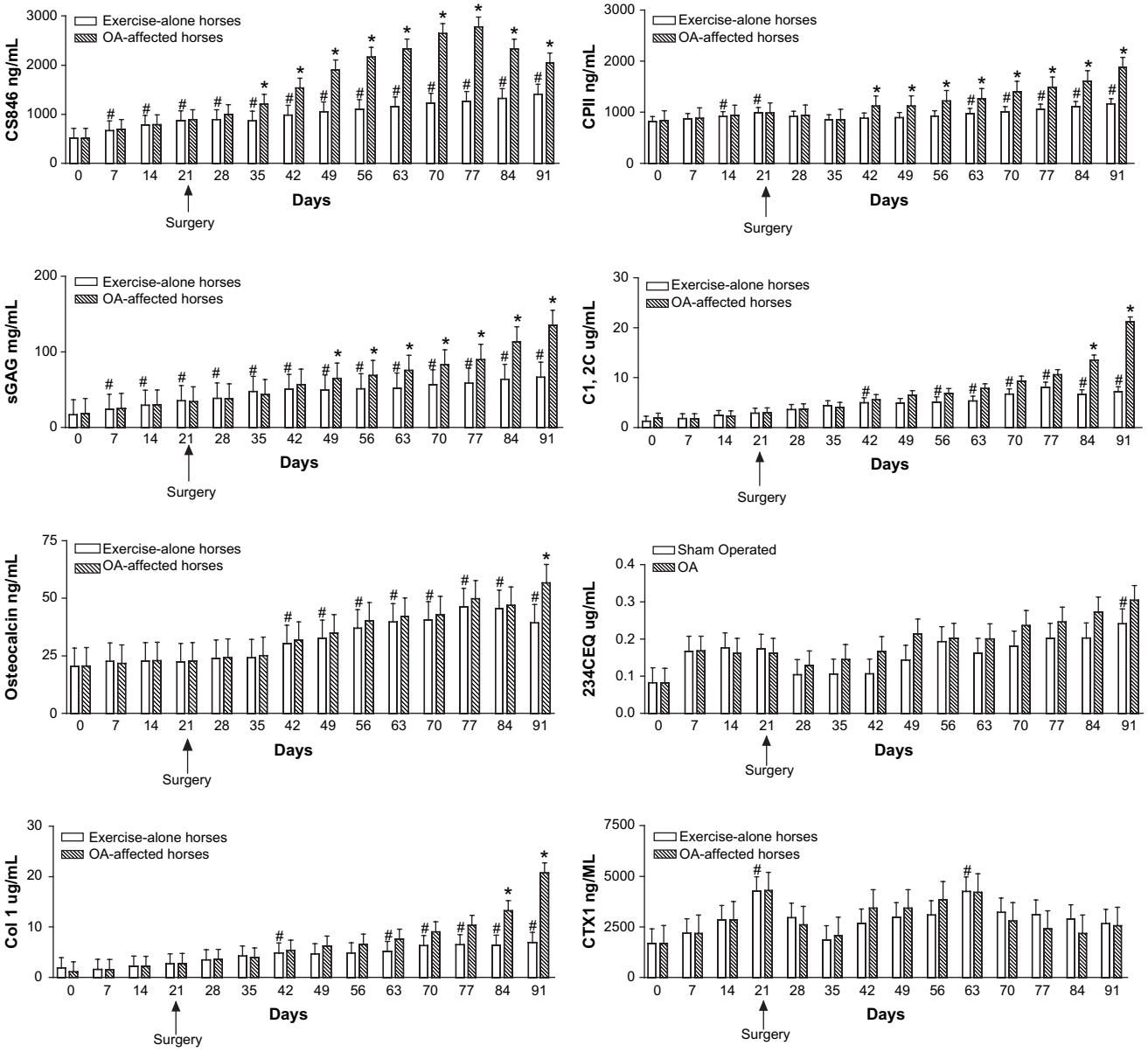

Fig. 3. Plot of average CS846, CPII, GAG, C1,2C, osteocalcin, Col CEQ, Col I, and CTX1 serum levels ± S.E.M. by study day. Study days that have any bar with "#" symbols represent a significant change with exercise groups (exercise-alone horses, and OA-affected horses) compared to day 0 value. Bars with "*" symbol represent significant change in OA-affected horses compared to exercise-alone horses.

Table I
The values of R^2 between the synovial fluid and serum biomarkers among the groups (exercise-alone joints in exercise-alone horses, exercise-alone joints in OA-affected horses, and OA-affected joints)

Biomarker	Synovial fluid OA-affected joints vs serum OA-affected horses	Synovial fluid exercise-alone joints in OA-affected horses vs serum	Synovial fluid exercise-alone joints in exercise-alone horses vs serum
Osteocalcin	0.14[b]	0.1[b]	0.06[b]
C1,2C	0.81[a]	0.56[a]	0.5[a]
Col 1-3/4	0.52[a]	0.22[a]	0.27[a]
CPII	0.53[a]	0.48[a]	0.15[a]
234CEQ	0.18[a]	0.18[a]	0.01[b]
CS846	0.44[a]	0.22[a]	0.5[a]
sGAG	0.79[a]	0.67[a]	0.69[a]

Superscript letters denote a *P*-value less than 0.05 for the slope of the best fit line when = a, conversely a *P*-value of greater than 0.05 when = b.

pathology score were significantly elevated in the OA-affected when compared to the exercise-alone joints (Table II). When these pathologic observations were compared to biomarker levels at day 91, strong significant correlations were observed for the degree of total articular cartilage erosion and GAG ($r = 0.79$), CS846 ($r = 0.79$), and CPII ($r = 0.77$) concentrations in the synovial fluid. Moderate significant correlations were observed for total erosion scores and synovial fluid C1,2C ($r = 0.68$) concentrations. A strong significant correlation was observed for the degree of total articular cartilage erosion and serum GAG ($r = 0.77$) concentrations. Moderate significant correlations were observed for total erosion scores and serum CS846 ($r = 0.73$), CPII ($r = 0.65$) and C1,2C ($r = 0.74$) concentrations. No other significant correlations were noted between synovial fluid and serum biomarkers and gross outcome parameters.

HISTOLOGIC EXAMINATIONS

Synovial membrane stained with H&E from exercise OA-affected exercise-alone joints in OA-affected horses showed significantly greater cellular infiltration and subintimal vascularity when compared to exercise-alone joints in exercise-alone horses. There were no other significant differences in these outcome parameters and no significant correlations between any synovial membrane outcome parameters and biomarker concentrations.

When articular cartilage samples from three separate locations were averaged a significant increase in articular cartilage fibrillation, chondrocyte necrosis, chondrone formation, and focal chondrocyte loss was noted when OA-affected joints were compared to exercise-alone joints. Differences in the individual locations are also detailed in Table II.

When all locations were pooled and correlated to biomarker concentrations, significant correlations that ranged from mild to moderate in strength were noted for each of the outcome parameters (Table III).

When the articular cartilage was assessed using histochemical methods (SOFG) a significant reduction in Safranin-O stain was noted in three of the four cartilage zones when OA-affected joints were compared to exercise-alone joints. Samples obtained from the radial and third carpal bones demonstrated decreased GAG (SOFG) staining, in the tangential, intermediate and radiate interterritorial zones when OA-affected joints were compared to exercise-alone joints and samples from the third carpal bones showed decreased GAG staining in the tangential and radiate interterritorial zones when the OA-affected joints were compared to the exercise-alone joints in the OA-affected horses (Table II). No other significant differences were observed with any comparison or when samples from the fourth carpal bone were assessed. Only synovial fluid GAG concentrations showed significant correlations with SOFG scores; these correlations were mild in strength; tangential ($r = 0.45$), intermediate ($r = 0.47$), radiate interterritorial ($r = 0.36$) and radiate territorial ($r = 0.36$). No significant correlation between histochemical outcome parameters and serum biomarker concentrations was found.

Discussion

This study was designed to differentiate changes in synovial fluid and serum biomarkers with exercise compared to horses with experimental OA. Exercise is part of this OA model that has been used to evaluate a number of treatments for equine OA[11–13]. This equine model develops OA rather quickly and it is recognized that the normal process in human clinical cases is longer. However, clinical OA in the equine athlete also develops quite quickly particularly when associated with OCF in the same location that the fragment is created in the model. The authors wanted to incorporate evaluation of biomarkers as additional outcome parameters in the model but because of various changes seen with exercise previously it was considered important to differentiate the effects of exercise from exercise plus concurrent disease.

It was therefore possible to identify seven synovial fluid biomarkers that could be used to differentiate experimentally induced OA in the horse from non-diseased joints and six serum biomarkers that could differentiate experimental OA-affected horses from exercise-alone horses.

Whereas there were correlations between synovial effusion and synovial fluid biomarkers, serum biomarker levels showed significant correlations with lameness and response to flexion but not synovial effusion. Synovial effusion is generally considered a reflection of synovitis but these serum biomarkers are not considered markers of synovitis.

This study involved repeated arthrocenteses. The influence of repeated arthrocentesis has been previously examined as a potential confounding factor with the synovial fluid markers NO, PGE2 and GAG concentrations[33]. Those authors noted that an increase in NO, PGE2 and GAG concentrations with repeated arthrocentesis. An earlier study also showed an increase in matrix metalloproteinase 1 (MMP-1) was significantly associated with repeated arthrocentesis (within 60 h)[34]. Overall these effects were short term in that NO levels in the metacarpophalangeal (MCP) joint peaked 12 h after previous arthrocentesis, the increase in PGE2 persisted for 60 h and the authors concluded it had passed after 2 weeks. We feel that any effect of arthrocentesis was minor in our study due to arthrocentesis being 1 week apart.

There has been some attention to changes in biomarkers with exercise in horses. Levels of carboxy terminal propeptide (PICP) biomarker of Col I synthesis have been shown to decrease significantly with age and increase with exercise when compared to non-exercised horses[18,19]. On the other hand osteocalcin levels were lower at the end of

Table II
Outcome variable data (mean ± S.E.M.) of gross pathologic observations and histologic examinations for comparison of exercise-alone joints in exercise-alone horses, exercise-alone joints in OA-affected horses, and OA-affected joints

Outcome variable	Exercise-alone joints in exercise-alone horses (mean ± S.E.M.)	Exercise-alone joints in OA-affected horses (mean ± S.E.M.)	OA-affected joints (mean ± S.E.M.)
Gross pathologic observations			
Full thickness articular cartilage fibrillation	$0.20^{b} \pm 0.3$	$0.50^{b} \pm 0.3$	$1.71^{a} \pm 0.3$
Hemorrhage	$0.80^{b} \pm 0.3$	$0.66^{b} \pm 0.3$	$1.28^{a} \pm 0.3$
Total pathology score	$0.30^{b} \pm 0.3$	$0.50^{b} \pm 0.3$	$1.28^{a} \pm 0.3$
Histologic examinations			
Synovial membrane Cellular infiltration	$0.18^{b} \pm 0.3$	$0.50^{a} \pm 0.3$	$0.50^{a} \pm 0.3$
Synovial membrane Subintimal vascularity	$0.68^{b} \pm 0.3$	$1.12^{a} \pm 0.3$	$1.00^{a} \pm 0.3$
Synovial membrane intimal Hyperplasia	$1.31^{a} \pm 0.3$	$1.37^{a} \pm 0.3$	$1.50^{a} \pm 0.3$
Synovial membrane Subintimal edema	$1.12^{a} \pm 0.3$	$1.00^{a} \pm 0.3$	$1.00^{a} \pm 0.3$
Synovial membrane Subintimal fibrosis	$1.31^{a} \pm 0.3$	$1.37^{a} \pm 0.3$	$1.50^{a} \pm 0.3$
Cartilage morphology – radial carpal bone			
Fibrillation	$0.00^{b} \pm 0.3$	$0.25^{a} \pm 0.3$	$0.37^{a} \pm 0.3$
Chondrocyte necrosis	$0.37^{a} \pm 0.3$	$0.37^{a} \pm 0.3$	$0.50^{a} \pm 0.3$
Chondrone formation	$0.12^{b} \pm 0.3$	$0.12^{b} \pm 0.3$	$0.50^{a} \pm 0.3$
Focal chondrocyte loss	$0.50^{b} \pm 0.3$	$0.50^{b} \pm 0.3$	$1.37^{a} \pm 0.3$
Cartilage morphology – third carpal bone			
Fibrillation	$0.00^{b} \pm 0.4$	$0.46^{b} \pm 0.4$	$1.12^{a} \pm 0.5$
Chondrocyte necrosis	$0.62^{b} \pm 0.4$	$0.62^{b} \pm 0.4$	$1.62^{a} \pm 0.5$
Chondrone formation	$0.12^{b} \pm 0.4$	$0.25^{b} \pm 0.4$	$1.37^{a} \pm 0.5$
Focal chondrocyte loss	$0.75^{b} \pm 0.4$	$0.87^{b} \pm 0.4$	$1.87^{a} \pm 0.5$
Cartilage morphology – fourth carpal bone			
Fibrillation	$0.00^{a} \pm 0.3$	$0.00^{a} \pm 0.3$	$0.12^{a} \pm 0.3$
Chondrocyte necrosis	$0.37^{a} \pm 0.3$	$0.50^{a} \pm 0.3$	$0.50^{a} \pm 0.3$
Chondrone formation	$0.00^{a} \pm 0.3$	$0.00^{a} \pm 0.3$	$0.00^{a} \pm 0.3$
Focal chondrocyte loss	$0.50^{a} \pm 0.3$	$0.62^{a} \pm 0.3$	$0.62^{a} \pm 0.3$
SOFG staining – radial carpal bone			
Tangential	$1.42^{a} \pm 0.6$	$1.25^{a} \pm 0.6$	$0.62^{b} \pm 0.6$
Intermediate	$3.00^{a} \pm 0.6$	$2.50^{a} \pm 0.6$	$1.62^{b} \pm 0.6$
Radiate territorial	$2.85^{a} \pm 0.6$	$2.75^{a} \pm 0.6$	$2.50^{b} \pm 0.6$
Radiate interterritorial	$2.85^{a} \pm 0.6$	$2.62^{a} \pm 0.6$	$1.87^{b} \pm 0.6$
SOFG staining – third carpal bone			
Tangential	$1.62^{a} \pm 0.6$	$0.75^{b} \pm 0.6$	$0.25^{c} \pm 0.6$
Intermediate	$2.50^{a} \pm 0.6$	$2.37^{a} \pm 0.6$	$1.50^{c} \pm 0.6$
Radiate territorial	$2.62^{a} \pm 0.6$	$2.50^{a} \pm 0.6$	$2.50^{a} \pm 0.6$
Radiate interterritorial	$2.62^{a} \pm 0.6$	$1.87^{b} \pm 0.6$	$1.25^{c} \pm 0.6$
SOFG staining – fourth carpal bone			
Tangential	$1.37^{a} \pm 0.6$	$1.50^{a} \pm 0.6$	$1.25^{a} \pm 0.6$
Intermediate	$2.75^{a} \pm 0.6$	$2.75^{a} \pm 0.6$	$2.25^{a} \pm 0.6$
Radiate territorial	$2.87^{a} \pm 0.6$	$3.00^{a} \pm 0.6$	$2.12^{a} \pm 0.6$
Radiate interteritorial	$2.50^{a} \pm 0.6$	$2.50^{a} \pm 0.6$	$2.50^{a} \pm 0.6$

Superscript letters that are different show a significant difference (P-value < 0.05) between treatment groups for each outcome variable.

Table III
Values of R^2 between articular cartilage histopathologic variables and synovial fluid biomarkers

Biomarker	Fibrillation	Cell necrosis	Chondrone	Cell loss
CS846	0.36^{a}	0.04^{b}	0.03^{b}	0.49^{a}
CPII	0.46^{a}	0.41^{a}	0.44^{a}	0.01^{b}
sGAG	0.53^{a}	0.1^{b}	0.04^{b}	0.41^{a}
C1,2C	0.49^{a}	0.41^{a}	0.58^{a}	0.04^{b}
234CEQ	0.48^{a}	0.38^{a}	0.58^{a}	0.04^{b}
PGE2	0.58^{a}	0.53^{a}	0.52^{a}	0.52^{a}

Superscript letters denote a P-value less than 0.05 for the slope of the best fit line when = a, conversely a P-value of greater than 0.05 when = b.

a conditioning period in training horses than in day 1 of the study[20]. Serum levels of type I collagen nonhelical telopeptide (ICTP) were significantly lower in treadmill exercised horses than controls[22] and in a second study in 2-year-old Thoroughbreds in race training high speed exercise led to a decrease in serum concentrations in both osteocalcin and ICTP[21]. The authors are unaware of any clinical studies depicting changes with exercise with the biomarkers tested in this study (other than osteocalcin mentioned above). The finding that there were differences with OA compared to exercise only in seven synovial fluid biomarkers and six serum biomarkers suggests the possibility of using these biomarkers to diagnose early disease in horses in training.

However, these studies need to be done in the field to ascertain if this is feasible. At this stage the biomarker changes can only be applied to the equine OA model that was tested. It is also to be noted that the histological and histochemical changes in the articular cartilage in this model are quite mild and would be less than typically seen in clinical equine OA. Having identified these series of markers as useful in an early equine model of OA where the changes in the articular cartilage are relatively mild a more detailed study in clinical cases of OA using these biomarkers would definitely be appropriate.

It is noteworthy that PGE2 levels were significantly increased with experimental OA. Synovial fluid PGE2 levels have consistently increased in other studies with this experimental model and have been presumed to have been correlated with synovitis. Based on the lack of correlation between synovitis and serum biomarker concentrations as previously noted (PGE2 is not one of them) PGE2 increase is probably associated with more than synovial inflammation. With regard to the increase in CS846 and CPII levels seen in this study it should be noted that a previous study with clinical OCF in horses had shown an increase in synovial fluid epitope CS846 but no increase in synovial fluid CPII levels[16].

It is particularly useful to find serum biomarkers in the horse that will differentiate OA from exercise alone. This knowledge can be of major value in screening clinical cases for OA when the horses are in active training. Routine synovial fluid acquisition is difficult to obtain in elite athletes but serum sampling is well tolerated. Based on the study the use of serum CS846, CPII, GAG and C1,2C would be useful for detecting early OA. On the other hand osteocalcin, Col CEQ and CTX1 are not.

Conflict of interest

None of the authors have financial or personal relationships with other people or organizations that could inappropriately influence their work.

Acknowledgment

Funding provided as a charitable gift from the Thoroughbred Corp. The study sponsor did not play a role in study design, collection, analysis and interpretation of data or in writing and submission of this manuscript.

References

1. Sumer EU, Schaller S, Sondergaard BC, Tanko LB, Qvist P. Application of biomarkers in the clinical development of new drugs for chondroprotection in destructive joint diseases: a review. Biomarkers 2006; 11(6):485–506.
2. Sandell LJ, Aigner T. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. Arthritis Res 2001;3(2):107–13.
3. Heinegard D, Inerot S, Wieslander J, Lindblad G. A method for the quantification of cartilage proteoglycan structures liberated to the synovial fluid during developing degenerative joint disease. Scand J Clin Lab Invest 1985;45:421–7.
4. Lohmander LS. Molecular markers of cartilage turnover: a role in monitoring and diagnosis of osteoarthritis. Articular Cartilage and Osteoarthritis 1992;653–67.
5. Lohmander LS, Felson DT. Defining the role of molecular markers to monitor disease, intervention, and cartilage breakdown in osteoarthritis. J Rheumatol 1997;24(4):782–5.
6. Poole AR, Ionescu M, Swan A, Dieppe P. Changes in cartilage metabolism in arthritis are reflected by altered serum and synovial fluid levels of the cartilage proteoglycan aggrecan: implications for pathogenesis. J Clin Invest 1994;94:25–33.
7. Saxne T, Heinegard D, Wollheim FA. Therapeutic effects on cartilage metabolism in arthritis as measured by release of proteoglycan structures into the synovial fluid. Ann Rheum Dis 1986;45:491–7.
8. Ratcliffe A, Doherty M, Maini RN, Hardingham TE. Increased concentrations of proteoglycan components in the synovial fluids of patients with acute but not chronic joint disease. Ann Rheum Dis 1988;47:826–32.
9. Caterson B, Flannery CR, Hughes CE, Little CB. Mechanisms involved in cartilage proteoglycan catabolism. Matrix Biol 2000;19(4):333–44.
10. Thonar EJ-MA, Lenz ME, Masuda K, Manicourt D-H. Body fluid markers of cartilage metabolism. In: Seibel MJ, Robins SP, Belezikian JP, Eds. Dynamics of Bone and Cartilage Metabolism. San Diego: Academic Press; 1999:453–64.
11. Frisbie DD, Ghivizzani SC, Robbins PD, Evans CH, McIlwraith CW. Treatment of experimental equine osteoarthritis by *in vivo* delivery of the equine interleukin-1 receptor antagonist gene. Gene Ther 2002;9(1):12–20.
12. Frisbie DD, Kawcak CE, Trotter GW, Powers BE, McIlwraith CW. The effects of triamcinolone acetate on an *in vivo* equine osteochondral fragment exercise model. Equine Vet J 1997;29(5):349–59.
13. Kawcak CE, Frisbie DD, McIlwraith CW, Trotter GW, Powers BE. Effects of intravenously administered sodium hyaluronate on equine carpal joints with osteochondral fragments under exercise. Am J Vet Res 1997;58:1132–40.
14. McIlwraith CW. Use of synovial fluid and serum biomarkers in equine bone and joint disease: a review. Equine Vet J 2005;37(5):473–82.
15. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, *et al*. Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. J Clin Invest 1997;99:1534–45.
16. Frisbie DD, Ray CS, Ionescu M, Poole AR, Chapman PL, McIlwraith CW. Measurement of synovial fluid and serum concentrations of the 846 epitope of chondroitin sulfate and of carboxy propeptides of type II procollagen for diagnosis of osteochondral fragmentation in horses. Am J Vet Res 1999;60:306–9.
17. Mathiason-Kochan KJ, Potter GD, Michael EM, Terrell KM, Morris EL. Biochemical markers of bone activity in the juvenile racehorse. In: Equine Nutrition and Physiology Society Meeting, 2001; Lexington, Kentucky; 2001.
18. Price JS, Jackson B, Eastell R, Goodship AE, Blumsohn A, Wright I, *et al*. Age related changes in biochemical markers of bone metabolism in horses. Equine Vet J 1995;3:201–7.
19. Price JS, Jackson B, Eastell R, Wilson AM, Russell RG, Lanyon LE, *et al*. The response of the skeleton to physical training: a biochemical study in horses. Bone 1995;17(3):221–7.
20. Hiney KM, Potter GD, Gibbs PO, Bloomfield SM. Response of serum biochemical markers of bone metabolism to training in the juvenile racehorse. J Equine Vet Sci 2000;20:851–7.
21. Jackson B, Eastell RE, Wilson AM, Lanyon LE, Goodship AE, Price JS. The effects of exercise on biochemical markers of bone metabolism and insulin-like growth factor-I in two year old thoroughbreds. J Bone Miner Res 1998;13:221–7.
22. Jackson BF, Goodship AE, Eastell R, Price JS. Evaluation of serum concentrations of biochemical markers of bone metabolism and insulin-like growth factor I associated with treadmill exercise in young horses. Am J Vet Res 2003;64(12):1549–56.
23. Anonymous. Definition and classification of lameness. In: Guide for Veterinary Service and Judging of Equestrian Events. Lexington: AAEP 1991:19.
24. Rizkalla G, Reiner A, Bogoch E, Poole AR. Studies of the articular cartilage proteoglycan aggrecan in health and osteoarthritis: evidence for molecular heterogeneity and extensive molecular changes in disease. J Clin Invest 1992;90:2268–77.
25. Nelson F, Dahlberg L, Laverty S, Reiner A, Pidoux I, Ionescu M, *et al*. Evidence for altered synthesis of type II collagen in patients with osteoarthritis. J Clin Invest 1998;102:2115–25.
26. Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: a template for tissue repair. Clin Orthop Relat Res 2001;(Suppl 391):S26–33.
27. Billinghurst RC, Buxton EM, Edwards MG, McGraw MS, McIlwraith CW. Use of an anti-neoepitope antibody for identification of type-II collagen degradation in equine articular cartilage. Am J Vet Res 2001;62(7): 1031–9.
28. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883:173–7.
29. Lepage OM, Marcoux M, Tremblay A. Serum osteocalcin or bone Gla-protein, a biochemical marker for bone metabolism in horses: differences in serum levels with age. Can J Vet Res 1990;54(2):223–6.
30. Billinghurst RC, Brama PA, van Weeren PR, Knowlton MS, McIlwraith CW. Significant exercise-related changes in the serum levels of two biomarkers of collagen metabolism in young horses. Osteoarthritis Cartilage 2003;11(10):760–9.

31. Lepage OM, Carstanjen B, Uebelhart D. Non-invasive assessment of equine bone: an update. Vet J 2001;161(1):10–22.
32. Kong SY, Stabler TV, Criscione LG, Elliott AL, Jordan JM, Kraus VB. Diurnal variation of serum and urine biomarkers in patients with radiographic knee osteoarthritis. Arthritis Rheum 2006;54(8):2496–504.
33. van den Boom R, van de Lest CHA, Bull S, Brama PAJ, van Weeren PR, Barneveld A. Influence of repeated arthrocentesis and exercise on synovial fluid concentrations of nitric oxide, prostaglandin E_2 and glycosaminoglycans in healthy equine joints. Equine Vet J 2005;37(3):250–6.
34. Brama PAJ, van den Boom R, DeGroot J, Kiers GH, van Weeren PR. Collagenase-1 (MMP-1) activity in equine synovial fluid: influence of age, joint pathology, exercise and repeated arthrocentesis. Equine Vet J 2004;36(1):34–40.