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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

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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