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FULL PAPER

Divergent diagnosis from arthroscopic findings and identification of CPII and C2C for detection of cartilage degradation in horses

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Abstract

The objective of this study was to investigate the changes in synovial fluid concentration of collagen type II cleavage site (C2C) and procollagen II C-propeptide (CPII), markers of joint cartilage degeneration and synthesis, respectively, in horses with intraarticular fracture or osteochondrosis dissecans (OCD), and to examine the relationship between arthroscopic findings and these biomarker levels. Synovial fluid was collected from 36 joints in 18 horses (6 fractures and 12 OCDs). Samples from contralateral normal joints, when available, served as controls (n = 12). Concentrations of C2C and CPII were measured using enzyme-linked immunosorbant assays. Moreover, the severity of the cartilage degradation was graded arthroscopically in 16 horses, and the correlation between the C2C and CPII levels and the arthroscopic scores were investigated. Compared to the control, the concentration of C2C was increased in OCD joints but not in fracture joints, whereas the concentration of CPII was increased in fracture joints but not in OCD joints. Within each disease group there was no correlation between biomarker levels and arthroscopic findings. Therefore, although C2C and CPII have diagnostic potential further knowledge is required to provide accurate analysis.

Key words: arthroscopy, biomarker, cartilage, horse, synovial fluid

Introduction

The most common abnormality occurring in the carpal joint is degenerative joint disease, which often results in chip or slab fractures in racing horses[27)] and osteochondrosis dissecans (OCD) remains a prominent and debilitating musculoskeletal syndrome among growing horses[20)]. Recent interest has been devoted to measuring the concentration of

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chondrodegeneration products in biological fluid for an early diagnosis of joint pathology in horses[7,12,13,17,25]. Such tests have some advantages over the current gold standard diagnosis, arthroscopy, as they are faster, less invasive and less expensive. However, the correlation between arthroscopic findings and various joint biomarker concentrations has not been fully established[17].

Several biomarkers of joint alterations have been used successfully in research settings including bone alkaline phosphatase (BAP)[25], osteocalcin[7], keratan sulfate (KS)[4,14], chondroitin sulfate 846 epitope[16], collagen type II cleavage site (C2C)[5,14] and procollagen II C-propeptide (CPII)[23,24]. Bello *et al*.[2] also reported a significant negative correlation between the levels of 3B3(-) epitope and glucosaminoglycan (GAG) in synovial fluid and the degree of chondral damage scores determined by arthroscopic evaluation in humans with acute knee injury. Shinmei *et al.*[23] showed that CPII correlated positively with arthroscopic cartilage damage also in human joints. Some studies showed a greater concentration of particular markers in diseased joints when compared to normal ones (CPII in young OCD horse cases[15], C2C and 3B3[5], BAP)[25]. On the other hand, some show no significant difference between concentrations of biomarkers in either joint (C2C[16], C2C, CIIC and GAG[13], C2C[8]). Even though much has been investigated about joint biomarkers, information concerning the relationship of biomarkers and arthroscopic findings is limited in horses as well as in humans.

Upon formation of type II collagen fibril, carboxy and amino propeptides are released extracellularly from the extremities of the procollagen. CPII is the carboxy portion of the procollagen and its content in the body fluid indicates collagen type II synthesis[11,19]. Collagen type II is actually formed by a meshwork of 3 identical αchains, which are cleaved and destroyed once arthritis is present. Such a cleavage site exposes a neoepitope at the C terminus portion of type II collagen fibrils, therefore, the presence of C2C represents collagen degradation[5,21].

The purpose of this study was to evaluate CPII and C2C concentrations in horses with intraarticular fracture or OCD, and furthermore to investigate how the levels of these biochemical markers were related to the arthroscopic findings in a subset of the horses, and also to find out if the levels of CPII and C2C reflect the severity of the chondral damage.

Materials and Methods

Animals and Samples: Synovial fluid samples were aseptically collected from 36 joints of 18 Thoroughbred racehorses, which underwent arthroscopic surgery for OCD or intraarticular fracture. The median age of the horses was 25 months (ranging from 8 to 42 months). There were 10 males and 8 females.

Synovial fluid samples were obtained from 17 OCD joints, 7 fracture joints and from 12 contralateral clinically and radiographically normal joints. All OCD lesions were affecting the distal tibia: intermediate ridge (n = 11) or medial malleolus (n = 6). All fractures were concerning carpal bones. Samples were aseptically collected (0.2–3.0 ml) from each joint, and kept frozen at −20°C until used for analysis. Digestion was performed prior to aliquoting samples and freezing once again (amounting to 2 cycles of freeze and thaw, i.e. collection, freezing, thawing, taking to the lab, digestion, aliquoting, freezing, thawing, assay). Prior to aliquoting, the samples were treated with hyaluronidase SD (Hyaluronidase purified from broth of Streptococcus dysgalactiae, Seikagaku Corporation, Tokyo, Japan) in a final concentration of 5 U/l of synovial fluid, along with protease inhibitor.

CPII assay: A commercially available competitive ELISA (CPII immunoassay, IBEX, Montreal, QC, Canada) for CPII, previously applied in

horses[3,10,11], was used. The assay included a bovine CPII standard and rabbit polyclonal antibody specific for CPII. Even though this antibody was produced specifically against human CPII, it also has broad cross reactivity in samples obtained from horses as well[11]. Synovial fluid was diluted 1 : 1 with buffer solution and all samples were measured in duplicate. Absorbance was read at 450 nm.

C2C assay: A commercially available competitive ELISA (C2C immunoassay, IBEX, Montreal, QC, Canada) for C2C was used. The assay included a mouse primary C2C antibody (also previously called col 2 3/4 long antibody) which was produced against a synthetic peptide representing the neoepitope. The assay has been applied and shown cross reaction in synovial fluids obtained from horses[13]. Synovial fluid was diluted 1:1 with buffer solution and all samples were measured in duplicate. Following reaction, absorbance was read at 450 nm.

Arthroscopic grading: Arthroscopic photographs were examined in 22 joints in 16 horses, being 7 fractures and 15 OCD joints. These were taken at the time of surgery before and after repair for OCD lesions or removal of fragments (Fig. 1). There was no image evaluation of the contralateral joints which were considered normal, being graded as 0 ($n = 10$). A modified arthroscopic scoring system[22,25] was applied that could be used in any joint of either dissecant injury or intraarticular fracture (Tables 1 and 2). A total of 4 categories were graded and summed to give a scale of arthroscopic scores of 0–16.

Statistical analysis: Concentrations of C2C and CPII obtained in each disease group were compared with their controls using Mann-

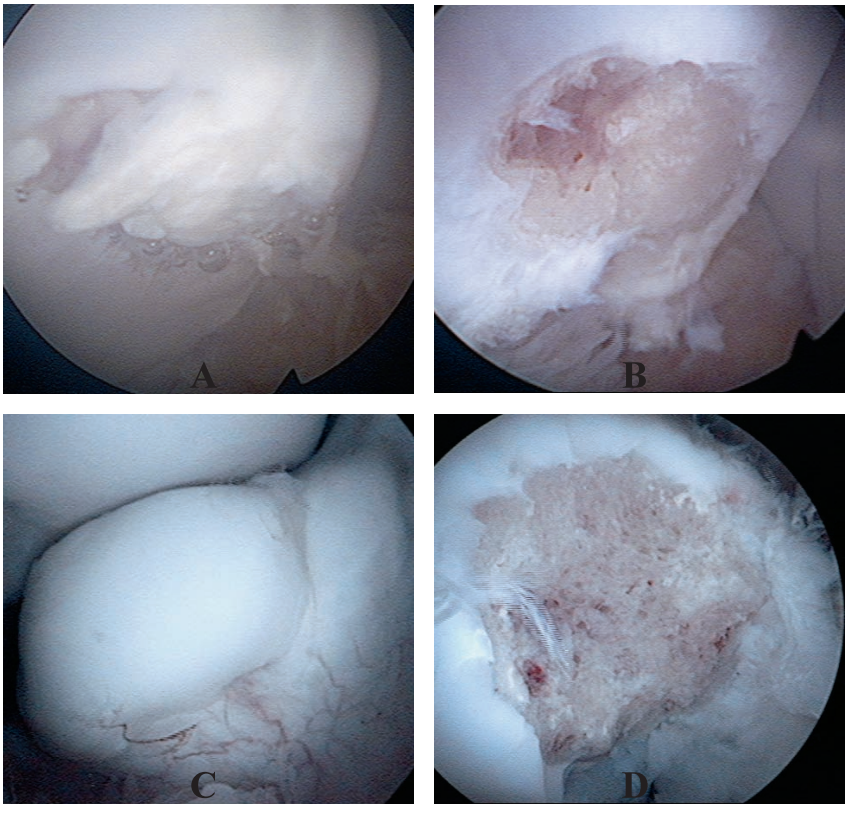

Fig. 1. Arthroscopic images. Fracture of the distal aspect of the radial carpal bone in horses before (A) and after (B) surgery; OCD of the lateral aspect of the throclea before (C) and after (D) surgical intervention. Scores for chondromalacia and synovitis performed as follows; 6 and 1 (fracture) and 5 and 2 (OCD), respectively.

Table 1. Scoring system used for the assessement of degree of intraarticular damage regarding synovitis based on arthroscopic images.

Synovitis	
Criteria	Grade
no synovitis	1
mild	
thin and opaque villi	2
moderate	
opaque, thicker and more squat villi	3
reasonable	
discrete petequation and proliferation	4
severe	
hypervascularization and intense proliferation	5

Table 2. Scoring system used for the assessement of degree of intraarticular damage regarding chondromalacia based on arthroscopic images.

Chondromalacia	
Criteria	Grade
softening and superficial degradation	
as well as superficial flake formation	1
superficial fragmentation/erosion, fissures or “crab meat” formation	2
moderate fissures/erosion, detached fragments	
as well as ulceration with remains of cartilage	3
deep fisures but not yet exposure of subchondral bone	4
deep erosion, complete destruction of cartilage surface	
with eroded subchondral bone	5
Erosion site or Osteochondral fragment	
Criteria	Grade
number	0
1	1
2	2
3	3
size none	0
small	1
medium	2
large	3

Whitney U tests. The arthroscopy scores were also compared in this way. Because this results in multiple comparisons only small values of p were considered significant.

The diagnostic variables were correlated within disease groups to test for relationships between them. In particular, it was examined whether C2C and CPII concentrations showed a clear relationship with arthroscopy scores. Spearman’s correlation test was used because this rank correlation detects curved and lower triangle correlations as well as canonical ones.

Only values of $p \leq 0.05$ were considered significant.

Results

Concentration of each marker in arthritis group and its control category

The median concentrations of C2C epitope was 121 (58–197) ng/ml in OCD, 239 (93–414) ng/ml in fracture, and 108 (75–140) ng/ml in controls. For CPII, the median concentration was 1,316 (4,641–164) ng/ml in OCD, 5,803 (8,624–2,656) ng/ml in fracture and 853 (407–1579) ng/ml in normal joints.

CPII values obtained from synovial fluid samples from joints with fracture were significantly higher than the values in the normal contralateral joints ($p = 0.008$) (Fig. 2B). C2C values in OCD joints were also significantly greater than those in OCD contralateral joints ($p = 0.037$) (Fig. 2A). CPII concentrations in the OCD group were not significantly different from the normal contralateral joints ($p = 0.057$), nor was C2C in the fracture group ($p = 0.0732$) (Fig. 2).

Arthroscopic image scores

Upon examination of arthroscopic photographs, OCD cases presented median scores for chondromalacia of 6 (1–9), synovitis of 2 (1–4) and combined score of 8 (3–11). For cases regarding intraarticular fracture, scores were as follows: 7 (1–7) for chondromalacia, 2 (1–3) for synovitis and 9 (2–11) for combined category. General data of all horses are shown in Table 3.

Correlations between diagnostic variables

There were no significant correlations between either of the biomarkers and any of the arthroscopic scores. Correlations are shown in Table 4.

Discussion

No correlation between C2C and CPII values and those for arthroscopy were found in the

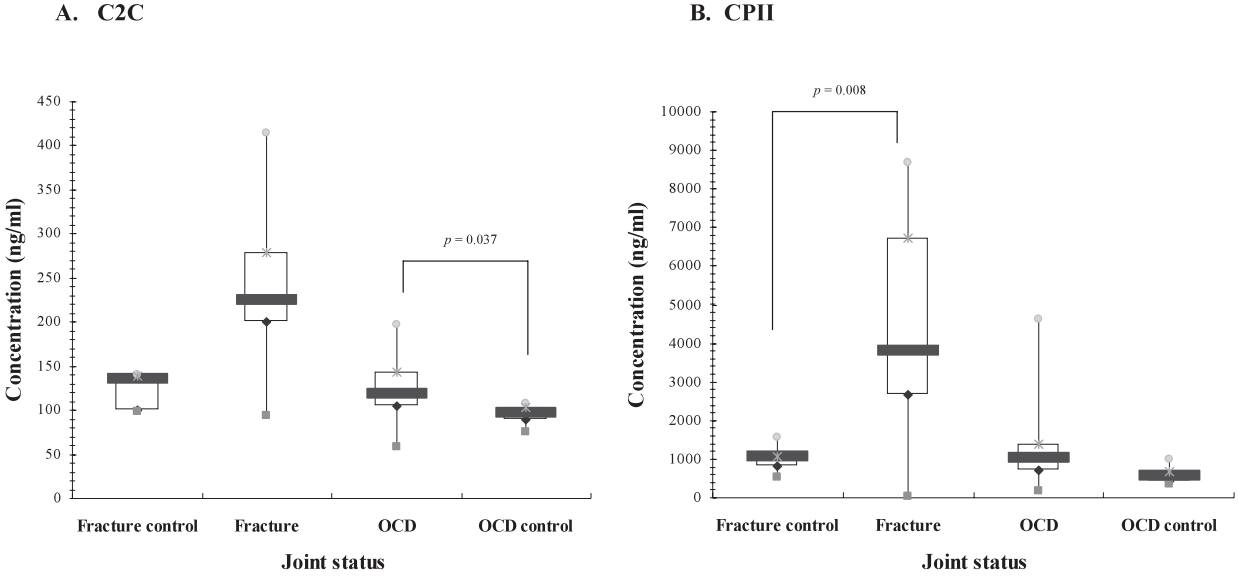

Fig. 2. Synovial fluid levels of collagen type II turnover markers in arthritic and normal joints. Synovial fluid concentrations of (A) collagenase-cleavage neotpitope of type II collagen (C2C) and (B) carboxypropeptide of type II collagen epitope (CPII) in intraarticular fracture of the carpal joint, OCD of the stifle joints of horses and its respective normal contra lateral joint (n = 34). Data are shown as box plots (expressed in ng/ml). Each box represents the 25th and 75th percentiles. Lines outside the boxes represent the 10th and 90th percentiles (minimum and maximum limits, respectively). Lines inside the boxes represent the median.

Table 3. Correlation of biomarkers and arthroscopic image scores.

OCD	Chondro score	Synovitis score	Combined score
CPII	R = −0.404	R = −0.469	R = −0.504
	$P = 0.135$	$P = 0.078$	$P = 0.055$
C2C	R = 0.037	R = −0.126	R = 0.39
	$P = 0.897$	$P = 0.654$	$P = 0.883$
Fracture	Chondro score	Synovitis score	Combined score
CPII	R = 0.25	R = 0.054	R = 0.18
	$P = 0.54$	$P = 0.895$	$P = 0.965$
C2C	R = 0.035	R = −0.036	R = 0.232
	$P = 0.381$	$P = 0.931$	$P = 0.569$

present study. This means that the evaluated biomarkers values might not reflect consistently the state of joints as revealed by direct arthroscopic examination.

C2C and CPII responded differently in synovial fluids from horses with two different pathologies. CPII in intraarticular fracture joints were higher than in contralateral control joints but C2C did not differ. The situation for OCD was reversed with C2C being higher in diseased joints than in controls. This is explicable if CPII represents increased synthesis of cartilage extracellular matrix (ECM)[11,19]. Fracture is an acute joint insult after which the synthesis of new replacement of cartilage ECM is likely. Even though no significant difference was observed in C2C concentrations between fractured joints and their controls, a higher C2C value is expressed in those samples, which could be intensified and indicate cartilage damage once a larger number of cases are being evaluated. Besides, there is the chance that chemical degradation of cartilage could begin after the time at which our tests were conducted.

C2C, in contrast, represents collagen type II degradation, not synthesis. It is therefore more likely for C2C to be highly expressed in OCD, which is a chronic pathology[9]. In addition, regarding the correlation observed between C2C and CPII concentrations in OCD synovial fluids, this could possibly express that in this pathology articular cartilage catabolism and anabolism are changing in parallel. The production of pro-inflammatory cytokines leads to articular cartilage destruction and C2C epitopes are exposed resulting from collagen fibrils degradation[6,21]. Some studies found either no relation of C2C and CPII epitope concentration in OCD versus normal joints[13] or no significant difference between CPII found in OCD affected joints and normal ones[15].

In guinea pigs synovial C2C and CPII were correlated with histological osteoarthritis[14]. However macroscopic grading of osteoarthritis is not adequately correlated with histologically determined damage to cartilage structure[22]. Therefore there is no reason to expect a correlation between immunoassay and arthroscopy on the basis of Huebner and Kraus[14]. The relationship in guinea pigs may, in any case, be different from that in horses.

A further correlation between morphological features, in this case radiographic scores, and Coll2-1 and Coll2-1NO_2 concentrations, was found in Dutch Warmblood stallions[26]. Such markers are specific for the sequence ^{108}HRGYPGLDG^{116}, the former derived from the triple helical region of type II collagen (Coll 2-1), and the latter from its nitrated form (Coll 2-1 NO_2). However, radiographic scores may not behave in the same way as those from arthroscopy. Furthermore, the biomarker used by these authors was Coll 2-1 NO_2, not the same

Table 4. General data from each horse, identification, diseased joint, markers concentration and arthroscopic scores.

Data on horses			Immunoassayscores		Arthroscopic image grading scores		
sex	age	diseased ID	C2C (ng/ml)	CPII (ng/ml)	Chondro score	Synovitis score	Combined score
M	7	OCD	120.42	728.67	4	2	6
M	7	OCD	106.46	425.11	8	2	9
F	24	OCD	145.19	2781.41	1	2	3
F	24	OCD	142.13	2174.1	5	3	8
M	9	OCD	197.12	4640.82	5	2	7
M	9	OCD	87.49	259.73	no image	no image	no image
F	42	Fracture	268.44	3585.87	1	1	2
F	42	None - Fracture control	139.77	1083.2	/	/	/
F	30	Fractue	227.42	8624.44	6	1	7
F	30	None - Fracture control	135.59	1579.55	/	/	/
F	7	OCD	149	824.18	8	4	11
F	7	None - OCD control	99.26	365.85	/	/	/
M	42	Fracture	221.28	2655.86	7	2	9
M	42	None - Fracture control	98.81	539.68	/	/	/
M	19	OCD	109.91	1543.49	6	2	8
M	19	OCD	122.45	1192.62	6	2	8
M	17	OCD	111.093	1042.3	6	2	8
M	17	OCD	93.69	987.62	5	2	7
M	30	Fracture	414.12	8691.09	7	2	9
M	30	None - Fracture control	100.78	817.86	/	/	/
F	42	None - Fracture control	138.93	1083.2	/	/	/
F	42	Fracture	307.82	6099.33	8	3	11
F	7	OCD	99.11	725.87	5	4	9
F	7	None - OCD control	75.37	407.48	/	/	/
M	42	Fracture	139.56	4040.32	7	3	10
M	42	Fracture	93.69	50.2	5	2	7
M	20	OCD	58.37	164.28	7	4	11
M	20	None - OCD control	86.96	677.28	/	/	/
F	8	OCD	116.28	1134.41	5	2	7
F	8	None - OCD control	95.12	980.05	/	/	/
F	9	None - OCD control	104.21	664.37	/	/	/
F	9	OCD	127.78	900.47	9	3	12
M	8	OCD	187.76	1313.09	7	2	9
M	8	None - OCD control	108.25	492.06	/	/	/

as those used in our study, and may therefore respond differently. Cibere *et al*.[6], in their study carried out in human subjects, showed that levels of different cartilage biochemical markers in serum, namely C2C, collagen type I and II cleavage site (C1 - 2C), CPII, chondroitin sulfate cleavage site (CS846), hyaluronic acid (HA), and cartilage oligomeric matrix protein (COMP), did not correlate with radiographically defined OA (ROA), opposing the association found between

increasing levels of urinary C2C and C1 - 2C with pre-ROA defined by magnetic resonance imaging. Herein it was suggested that serum and urine measurements are likely related to the metabolism and clearance of fragments in different body compartments; the usage of such assays as diagnostic markers was significantly associated with pre-ROA, rather than were associated with ROA, meaning that they depended on the stage of OA and also calculated ratios combining biomarker concentrations. No significant correlation of C2C and CPII with joint morphology is implied by a study of synovial fluid from horses in relation to clinical signs and radiographic diagnosis[13]. Likewise, there was no evidence of a correlation between C2C concentration and several different types of arthritis in horses[8]. Thus, there is some evidence already that correlations between biomarkers and joint condition may not be frequent.

A lack of correlation between C2C and CPII concentrations and arthroscopic results would arise if our arthroscopic grading did not accurately represent joint damage. However, the grading scheme is widely applied and accepted in the diagnosis of joint changes[12,22]. A similar scoring scheme correlates well with the Societé Française d'Arthroscopie grading system. This system has being extensively validated[1]. It is therefore unlikely that it fails to reflect joint damage. One other possibility for lack of correlation between C2C and CPII concentrations and arthroscopic scores would be if the arthroscopically visible damage either does not liberate C2C or CPII, or these epitopes are not present in synovial fluid. However the kind of damage visible does liberate C2C or CPII. Epitopes could be lost to serum from synovial fluid as reported by Myers *et al*.[18] where even mild synovitis, as seen in osteoarthritis, could significantly increase clearance of a marker protein (^{131}l-labeled albumin - RISA) from the joint.

In this study, it was observed that C2C and CPII values could not consistently reflect real joint damage and should thus be used with prudence. Certainly biomarkers have diagnostic potential in equine joint disease, but much more has to be understood to refine diagnosis as well as diagnosis of distinct joint diseases in order to provide accurate analysis.

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