*Connective Tissue Research*, Early Online: 1–11, (2010)

ISSN 0300-8207 print/1607-8438 online
DOI: 10.3109/03008207.2010.523803

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healthcare

# Effects of high mobility group box protein-1, interleukin-1β, and interleukin-6 on cartilage matrix metabolism in three-dimensional equine chondrocyte cultures

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## Abstract

The effects of high mobility group box protein (HMGB)-1, interleukin (IL)-1β, and IL-6 on equine articular chondrocytes were investigated, with emphasis on detecting differences between anatomical sites exposed to different loading in vivo, using three-dimensional (3D) cell cultures established with chondrocytes from dorsal radial facet (DRF, highly loaded) and palmar condyle (PC, less loaded) of the third carpal bone (C3). Expression of important genes involved in cartilage metabolism, presence of glycosaminoglycans and cartilage oligomeric matrix protein (COMP) in pellets, and concentrations of matrix metalloproteinase (MMP)-13 and aggrecan epitope CS 846 were evaluated. Compared to controls, IL-1β treatment increased gene expression of versican, matrix-degrading enzymes, and tissue inhibitor of metalloproteinase (TIMP)-1, and decreased aggrecan and collagen type I and type II expression. In addition, IL-1β-treated pellets showed decreased safranin O staining and increased COMP immunostaining and MMP-13 concentrations in culture supernatants. Effects of IL-6 and HMGB-1 on gene expression were variable, although upregulation of Sry-related high-mobility group box 9 (Sox9) was often present and statistically increased in HMGB-1-treated pellets. Response to cytokines rarely differed between DRF and PC pellets. Thus, site-associated cartilage deterioration in equine carpal osteoarthritis (OA) is not explained by topographically different responses to inflammatory mediators. Differences in gene expressions of structural matrix proteins in untreated DRF and PC pellets were noted in the youngest horses, which may indicate differences in the chondrocytes potential to produce matrix in vivo. Overall, a strong catabolic response was induced by IL-1β, whereas slight anabolic effects were induced by IL-6 and HMGB-1.

**Keywords:** gene expression, HMGB-1, horse, IL-1β, IL-6, osteoarthritis

## INTRODUCTION

Osteoarthritis (OA) is a common finding in the middle carpal joint of sport horses and macroscopic cartilage lesions are frequently seen in the dorsal part of the radial facet of the third carpal bone (C3) [1], whereas the palmar condyle (PC) is often spared. Abnormal mechanical loading and metabolic tissue failure are discussed as interrelated factors in the pathogenesis of OA [2], and production of inflammatory mediators in the synovial membrane or cartilage are likely to have regulatory functions in the development of disease [2,3]. The biochemical composition of equine carpal cartilage shows topographical variation with regard to collagen and proteoglycan content, which is also influenced by mechanical loading (exercise) [4]. In addition, Palmer et al. [5] showed site-specific differences in proteoglycan concentrations of C3 articular cartilage, with significant increases in newly synthesized proteoglycans related to exercise but not to anatomical site. With increased loading, the radial facet, but not the PC, is subjected to a significant increase in contact area and pressure [6], thus the high proportion of cartilage lesions of the radial facet may relate to inappropriate joint loading but also to suboptimal biochemical properties of the loaded articular cartilage. The biochemical composition, and hence the biomechanical properties of articular cartilage, may directly be related to the metabolic function of the chondrocytes, as these cells

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*Received 19 February 2010; revised 2 September 2010; accepted 10 September 2010*

synthesize structural matrix components, as well as matrix-degrading enzymes and enzyme inhibitors. Therefore, possible differences in chondrocyte phenotype in healthy cartilage and the metabolic response to inflammatory mediators may be of importance for the development of cartilage lesions as seen in equine OA. Indeed, a topographical difference in the synthesis of specific proteoglycans of carpal cartilage of strenuously exercised horses [7] has been shown.

The activity of the proinflammatory cytokine interleukin (IL)-1 has been detected in synovial fluid from equine osteoarthritic joints [8,9]. IL-1 has been shown to induce gene expression of matrix-degrading enzymes in equine chondrocytes [10–15], inhibition of proteoglycan synthesis [16–19], and increased release of glycosaminoglycans from cartilage explants [13,16,19–21]. In addition, using cDNA array on equine monolayer chondrocytes, over 100 modulated transcripts were identified after brief exposure to IL-1β [14]. In addition to studies on IL-1, effects of tumor necrosis factor (TNF)α and IL-1β plus oncostatin M on matrix metabolism in equine cartilage explants [13] and of TNFα in suspension cultures of chondrocytes [11,22] have been reported. However, to our knowledge there are no studies on the effects of other proinflammatory cytokines on articular cartilage metabolism in horses.

IL-6 protein and gene expression have been demonstrated in tissues of naturally diseased equine joints [23,24], and we previously found that joints with carpal dorsal osteochondral fragmentation often showed highly increased IL-6 bioactivity in the synovial fluid [25]. Furthermore, we detected the nuclear protein high mobility group box protein (HMGB)-1, also recognized as an important proinflammatory cytokine of inflammatory arthritis in man [26], extracellularly in the synovial membrane in equine joints with dorsal osteochondral fragmentation and/or cartilage pathology, and along the tidemark of osteochondral fragments [24]. Increased synovial fluid concentrations of HMGB-1 were also recently demonstrated in carpal and fetlock joints with osteochondral injury [27]. Thus, both IL-6 and HMGB-1 could potentially affect cartilage metabolism. As dorsal osteochondral fragments have been proposed to contribute to the advancement of equine OA [28], but joints with osteochondral fragments show indications of increased activity with regard to cartilage oligomeric matrix protein (COMP) [29] and aggrecan synthesis [30]; it is of interest to evaluate possible involvement of IL-6 and HMGB-1 on cartilage metabolism by analyzing anabolic as well as catabolic parameters.

The aim of this study was to use a three-dimensional (3D) culture system for equine chondrocytes to investigate the effects of IL-1β, IL-6, and HMGB-1 stimulation on cells derived from normal articular cartilage. Chondrocytes from the dorsal radial facet (DRF) and the PC of C3, representing sites exposed to different mechanical loading in vivo and with different susceptibility to cartilage degeneration in OA, were studied to investigate topographical differences in chondrocyte phenotype and response to cytokines. To determine the involvement of cytokines in cartilage metabolism, we evaluated the gene expressions of several important genes involved in cartilage metabolism including the differentiation factor Sry-related high-mobility group box 9 (Sox9), structural matrix proteins, matrix-degrading enzymes, and the tissue inhibitor of metalloproteinase (TIMP)-1. Results from gene expression studies were followed up by histochemical detection of collagens and glycosaminoglycans and protein detection of COMP in pellets and by determinations of concentration of the aggrecan epitope CS 846 and activated matrix metalloproteinase (MMP)-13 in culture supernatants. Our hypotheses were that IL-6 and HMGB-1 treatment would alter chondrocyte matrix metabolism, including the induction of COMP and aggrecan synthesis, and that IL-1β treatment would induce a catabolic response. In addition, we hypothesized that distinct site-associated metabolic differences would be detected.

## METHODS

### Cartilage sampling

Articular cartilage was aseptically collected from C3 of four euthanized horses (1–3 and 8 years old), with no clinically known disease of the sampled joints. Following arthrotomy of the middle carpal joint, all surfaces of articular cartilage were visually inspected to ensure that no macroscopic cartilage lesions within the sampled joints were present. Using a scalpel, cartilage of the DRF and PC of C3 (Figure 1) was incised down to the calcified cartilage/subchondral bone, and full-thickness articular cartilage samples were collected using forceps. In all but one horse, cartilage samples from the left and right joints were pooled according to sampling site (DRF vs. PC). The tissues were placed in transport medium (sterile 0.9% sodium chloride with 50 mg/l gentamicin sulfate and 250 μg/ml amphotericin B) and transported chilled to the laboratory.

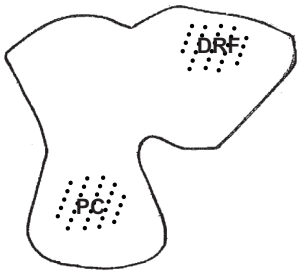


Figure 1. Schematic drawing of the proximal articular surface of the third carpal bone (C3), with dotted areas showing the sampling sites of dorsal radial facet (DRF) and palmar condyle (PC).

### Expansion of chondrocytes in monolayer culture

The procedures of chondrocyte isolation and monolayer culture followed the protocols modified from previously described [31]. Isolated chondrocytes were obtained by mechanical mincing of the cartilage, followed by collagenase digestion (type II collagenase, 0.8 mg/ml, Worthington Biochemicals, Lakewood, NJ, USA) for 20–24 hr at 37°C in 7% $CO_2$. Then the cells were transferred to Primaria™ cell culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) and grown in monolayer in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco, Invitrogen, Paisley, UK) supplemented with 0.1 mg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) 1× penicillin–streptomycin (PAA Laboratories, Linz, Austria and Sigma-Aldrich), 2 mM L-glutamine (Gibco), and 10% equine serum (SVA, Uppsala, Sweden) until 80% confluence. Subculture was performed once with trypsin in phosphate buffer. Cells were frozen at −80°C and then further expanded once for pellet culture.

### Pellet culture

Expanded cells were cultured in a 3D culture system modified from previous studies [32,33]. Briefly, 200,000 cells in 200 µl DMEM-high glucose (PAA Laboratories or Gibco, Invitrogen) supplemented with 14 µg/ml ascorbic acid (Sigma-Aldrich), $10^{-7}$ M dexamethasone (Sigma-Aldrich), 1 mg/ml human serum albumin (Equitech Bio, Kerville, TX, USA), 1× insulin–transferrin–selenium (Gibco, Invitrogen), 5 µg/ml linoleic acid (Sigma-Aldrich), 1× penicillin-streptomycin (PAA Laboratories or Sigma-Aldrich), and 10 ng/ml human transforming growth factor (TGF)β-1 (R&D Systems, Abingdon, UK) were seeded in the wells of flat bottom 96-well culture plates (Corning Life Sciences, Lowell, MA, USA). Cells were centrifuged at 410× g for 5 min and then incubated at 37°C in 7% $CO_2$ for 14 days. Culture medium was changed daily once the pellet formation was established. On Day 14, pellets were divided into four treatment groups, and either treated with recombinant equine IL-1β (5 ng/ml, R&D Systems), recombinant equine IL-6 (5 ng/ml, R&D Systems), and recombinant human HMGB-1 (1 µg/ml, R&D Systems or Sigma-Aldrich), or left untreated. After 24 hr, culture medium was changed and pellets were stimulated for another 24 hr. After a total of 48 hr, culture supernatants were collected and frozen at −80°C. Pellets were washed in phosphate buffered saline (PBS), fixed for light microscopy evaluation or snap frozen in liquid nitrogen, and stored at −80°C.

### Histology and histochemistry

Pellets for histological examination were fixed in Histofix™ (Histolab products AB, Gothenburg, Sweden, dehydrated) and embedded in paraffin. Sections from the central parts of pellets were stained with hematoxylin and eosin (H&E), safranin O, and Masson's trichrome. Stained sections were evaluated using light microscopy and photographed (Nikon Eclipse E600 microscope and Nikon DXM1200 digital camera).

### Quantitative real-time reverse transcriptase polymerase chain reaction

Pellets were homogenized in 1.5 ml polypropylene tubes (SafeSeal micro tubes, Sarstedt AG, Nümbrecht, Germany), each with an added tungsten bead (3 mm, Qiagen, Hilden, Germany), for 10 min prior to RNA isolation using a TissueLyser (Qiagen). Total RNA was extracted using QIAzol lysis reagent (Qiagen) and the RNeasy® Mini Kit (Qiagen) according to the manufacturer's protocol. Genomic DNA was removed from the isolated RNA using DNase I (Qiagen). Total RNA (100 ng) was used for DNA synthesis and reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

For the quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR), equine primers and probes for collagen type I, versican, aggrecan, COMP, MMP-9, MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-5, TIMP-1, and beta glucuronidase (GUSB) were used (Table 1). The National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) and ENSEMBL Genome Browser (ENSEMBL, www.ensembl.org) were used to choose sequences for equine genes. The primers and probes were designed by Applied Biosystems according to the reference genes. Human primers and probes were used for Sox9 (Hs00165814_m1) and collagen type II (Hs01064869_m1) after verification of homology between human and equine sequences using basic local alignment search tool (BLAST). Quantitative PCR was performed with ABI 7900 HT using Taqman® Gene Expression Master Mix. A validation experiment was performed for each equine-specific primer and probe set with a dilution series of cDNA template for the target genes. All reagents were purchased from Applied Biosystems. For all analyses, GUSB was used as housekeeping gene. The relative gene expression was evaluated by the $2^{-\Delta\Delta CT}$ method [34].

### Assays for aggrecan epitope CS 846 and MMP-13

Concentrations of epitope CS 846 were measured in culture supernatants using a commercially available ELISA kit (IBEX Diagnostics, Montreal, Quebec, Canada). Concentrations of MMP-13 were measured using Fluorokine E Human Active MMP-13 Fluorescent Assay (R&D Systems) after adding APMA (aminophenyl mercuric acetate), which activates latent MMP-13. In all assays, analyses were performed on duplicate samples. The lower detection limits were 20 ng/ml for CS 846 and 8 pg/ml for active MMP-13.

Table 1. Equine primer and reporter sequences for investigated genes

| Gene | Forward primer sequence (5′-3′) | Reverse primer sequence (5′-3′) | Reporter sequence (5′-3′) |
|---|---|---|---|
| ADAMTS-5[a] | TGGCTCACGAAATTGGACATC | AGGTCTAGCAAACAGTTACCATG ACC | TGGTCCAAATGCACCTC |
| Aggrecan[b] | GCGAGGCCACCCTAGAG | CGTGGAGATGGCTCTGTAATGG | AACACGATGCCTTTCACC |
| COMP[b] | GGGCAACGGCTCATACTGT | GCGAGGGAAGCAGGGATT | CAACGAGTGCAACGCC |
| Coll I[b] | GAGGCCGTCCTGTATGCA | CAAGAGGAGGGCCAAGAAGAAG | CTGCTGGGATGTCTTC |
| GUSB[b] | GTGACCAACTCCAACTATGAA GCA | AGGAGTAGTAACTATTCACACAG ATGACA | CATATGGCGCCCCTAGGTC |
| MMP-9[a] | TTGGACATGCACGACGTCTT | GAAGCTCACGTAGCCCACTTG | TACCGAGAGAAAGCTTACTT |
| MMP-13[b] | GAGCATCCTTCCAAAGACCTT ATCT | GGATAACCTTCCAGAATGTCATA ACCA | TTTAGAGGCCGAAAATT |
| TIMP-1[b] | CCGCAGCGAGGAGTTTCT | GTGATGTACAGCTTCTCGTCCAA | CATCGCCGGACAACTA |
| Versican[b] | CCTACGTGTGCACCTGTGT | GCACGTGGCTCCATTGC | CAGTGTGAGCTTGATTTT |

ADAMTS-5 = a disintegrin and metalloproteinase with thrombospondin motifs 5; Coll I = collagen type I; COMP = cartilage oligomeric matrix protein; GUSB = beta glucuronidase; MMP-13 = matrix metalloproteinase 13; MMP-9 = matrix metalloproteinase 9; TIMP-1 tissue inhibitor of metalloproteinase 1.
[a]Sequence Detection Primers and TaqMan® MGB Probe
[b]Custom TaqMan® Gene Expression Assays.



### Immunostaining for COMP

Immunostaining for COMP was performed with a rabbit anti-bovine antibody [35], recognizing equine COMP and previously used in several equine studies [29,36,37]. Deparaffinized and rehydrated sections were immersed in 3% $H_2O_2$ for 5 min. Sections were incubated with hyaluronidase (1 μg/ml in PBS; Sigma) and then nonspecific antibody binding was blocked using normal swine serum (diluted 1:20; Novocastra™, Novocastra Laboratories Ltd., Newcastle, UK). Drained sections were then incubated with the anti-COMP antibody diluted 1 : 2000, or normal rabbit immunoglobulin fraction (X0903, Dako, Glostrup, Denmark) overnight at 4°C. Following incubation with biotinylated swine anti-goat, mouse, rabbit immunoglobulin antibody (diluted 1 : 100; E0453, Dako), ABC reagent (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA, USA) was applied. Avidin–biotin–peroxidase complexes were visualized using diaminobenzidine (Sigma Fast™ 3.3′-diaminobenzidine tablet, Sigma-Aldrich). PBS (0.01 M, pH 7.4) was used for rinsing between the steps of the immunohistochemistry procedure, and blocking and incubations were performed for 30 min at room temperature unless stated otherwise. After a final rinse in tap water, sections were counterstained with Mayer's hematoxylin, and immunostaining was qualitatively assessed using light microscopy.

### Statistical analysis

Data were analyzed by analysis of variance (PROC GLM) in the SAS Software version 9 (SAS Institute Inc., Cary, NC, USA). The statistical model included the fixed effects of animal, site, treatment, and interactions between site and treatment. When the residual of analyzed parameters was not normally distributed, the tests of significance were not considered. Pairwise comparisons of least square means between cytokine-treated and untreated pellets, as well as between sites were tested for significance. $P$-values ≤ 0.05 were considered significant.

## RESULTS

### Light microscopy

The pellet cell population consisted of rounded, slightly polygonal, to spindle-shaped cells surrounded by extracellular matrix. A band of concentrically arranged spindle cells were typically present between centrally and peripherally located rounded cells. Untreated pellets showed a deep orange-red staining of the extracellular matrix with safranin O (Figure 2). The extracellular matrix of all IL-1β-treated pellets was lighter in color than untreated controls, whereas it was similar to controls in IL-6 and HMGB-1-treated pellets. Staining for collagens with Masson's trichrome was faint in all pellets, but often most clearly detected in the peripheral parts of the pellet (not shown).

### Gene expression

IL-1β-treated pellets showed significantly increased expression of versican ($P < 0.0001$), MMP-9 ($P = 0.0137$), and TIMP-1 ($P = 0.0059$); decreased expression of aggrecan ($P < 0.0001$), collagen type I ($P = 0.0077$), and collagen type II ($P = 0.0082$); and a trend toward decreased COMP expression ($P = 0.0543$) compared with untreated controls (Figure 3A and 3B). In addition, HMGB-1-treated pellets showed increased Sox9 expression ($P = 0.0120$) compared with untreated controls (Figure 3A). The residual was not normally distributed for MMP-13 and ADAMTS-5 expressions, thus test of significance was not considered for these parameters. However, both MMP-13 and ADAMTS-5 gene expressions were clearly increased in IL-1β-treated pellets (Figure 3B).

Across treatment, the expression of versican was higher in DRF compared with PC pellets ($P = 0.0020$),

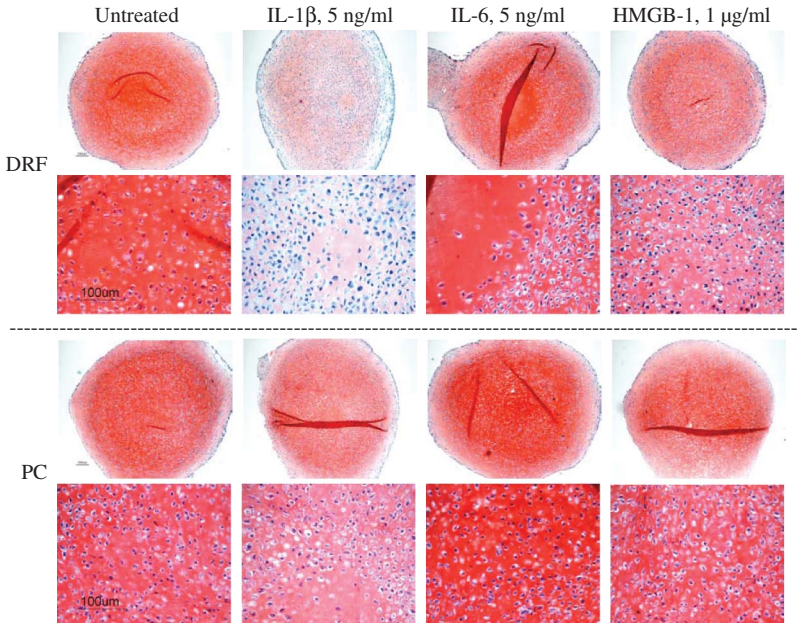


Figure 2. Safranin O-stained sections (4 μm thick) of pellets from horse aged 2 years (Horse 2). The proteoglycan content, as indicated by the orange–red color intensity of the extracellular matrix, was decreased in interleukin (IL)-1β-treated pellets, whereas IL-6 and high mobility group box protein (HMGB)-1-treated pellets showed similar proteoglycan staining to untreated controls.



and the opposite was found for aggrecan ($P = 0.0017$). Significant interaction of treatment and site was only demonstrated for aggrecan, with decreased expression in IL-1β-treated PC pellets ($P < 0.0001$) but not DRF pellets.

In untreated pellets of the three younger horses, differences in gene matrix profile could be discerned (most prominently in the two youngest horses), with higher expression of versican and collagen type I in DRF compared with PC pellets, and higher Sox9, aggrecan, and collagen type II in PC compared with DRF (Figure 4). Expression of MMP-9, ADAMTS-5, and TIMP-1 was present in all untreated pellets, whereas MMP-13 expression was not detected (results not shown).

To demonstrate the actual effect of cytokine treatment in each site, untreated DRF and PC pellets were set as reference points. In these comparisons, a notable site-related differences in response to cytokine stimulation were detected in pellets from the two youngest horses (1 and 2 years old), as the relative gene expression of Sox9, aggrecan, and collagen type II was less than half in the PC pellets compared with DRF pellets after IL-1β treatment, with similar findings for Sox9 and collagen type II after HMGB-1 treatment, and for collagen type II and MMP-13 after IL-6 treatment.

### Concentrations of CS 846 and active MMP-13

IL-1β-treated pellets showed the lowest mean concentrations of aggrecan epitope CS 846 (not statistically significant) in both DRF and PC pellet supernatants, and concentrations of active MMP-13 were markedly increased (tests of significance not considered due to the absence of normally distributed residual) (Table 2).

### Immunostaining for COMP

Immunostaining for COMP was performed to confirm synthesis of COMP in cell pellets. In most untreated pellets, slight to moderate extracellular immunostaining and some intracytoplasmic immunostaining for COMP were present. Although IL-1β-treated pellets most often showed decreased COMP gene expression, these pellets showed stronger extracellular immunostaining compared with untreated pellets (Figure 5). In IL-6- and HMGB-1-treated pellets, immunostaining was commonly similar to controls and clearly increased immunostaining was rarely detected with increased COMP gene expression.

## DISCUSSION

This study aimed to investigate the effects of IL-1β, IL-6, and HMGB-1 on equine articular cartilage metabolism using a 3D pellet culture system, and to detect possible differences in response to cytokine treatment between chondrocytes exposed to different mechanical loading in vivo.

IL-1β-treated pellets showed decreased gene expression of aggrecan, collagen type I and collagen type II, and increased expression of versican, MMP-9, MMP-13, and ADAMTS-5, indicating an overall catabolic effect on matrix metabolism induced by IL-1β. This was further demonstrated by low glycosaminoglycan content, as indicated by reduced safranin O staining.

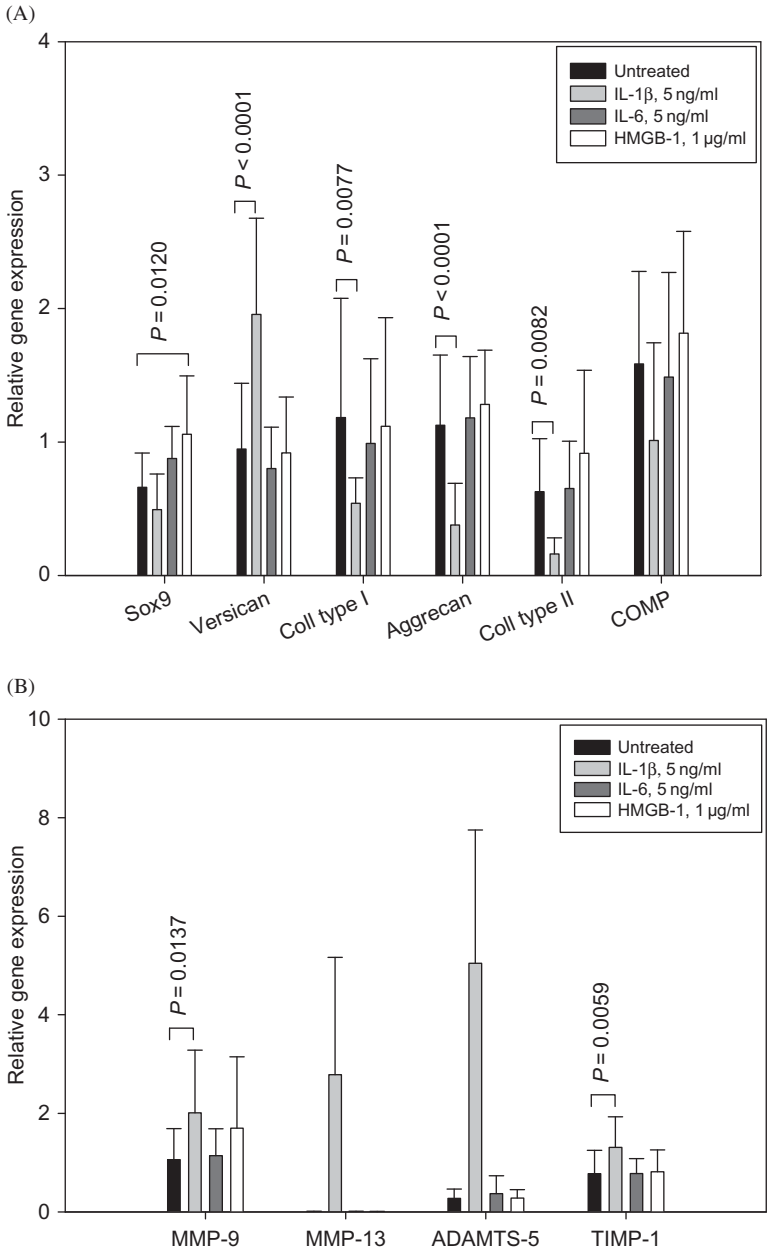


Figure 3. Mean values ($n$ = 8) and standard deviations (error bars) of relative gene expressions in untreated, interleukin (IL)-1β, IL-6, and high mobility group box (HMGB)-1-treated dorsal radial facet (DRF, $n$ = 4) and palmar condyle (PC, $n$ = 4) pellets. (A) Mean expression of Sox9 and structural matrix proteins. Compared with untreated controls, IL-1β-treated pellets showed decreased collagen type I and II and aggrecan expression and increased versican expression, and HMGB-1-treated pellets showed increased Sox9 expression. (B) Mean expression of matrix-degrading enzymes and tissue inhibitor of metalloproteinase (TIMP)-1 gene expression. Compared with controls, IL-1β-treated pellets showed statistically increased matrix metalloproteinase (MMP)-9 and a tissue inhibitor of metalloproteinase (TIMP)-1 expression, and clearly increased mean expression of MMP-13 and a disintegrin and matrix metalloproteinase with thrombospondin motifs (ADAMTS)-5.

Our results on gene expression of collagen type II and aggrecan agree with other equine IL-1β studies [11,21,22]. The effects on collagen type II and aggrecan expression could, at least partly, be the result of a primary antichondrogenic or dedifferentiating effect induced by IL-1β, as Sox9 expression was decreased and versican expression increased in most IL-1β-treated pellets. Sox9 acts as a transcriptional activator for several genes involved in the formation of extracellular cartilage matrix [38–42] and is needed for chondrocyte differentiation [43]. Suppression of Sox9 by IL-1β has previously been reported in murine chondrocytes [44]. In contrast to this study, an equine study reported a slight increase of Sox9 expression, and no significant changes in collagen type II and aggrecan expression in cartilage explant cultures stimulated with human IL-1β for 48 hr up to 8 days [45]. In the two youngest horses, in this study, a notably lower expression of Sox9, aggrecan,

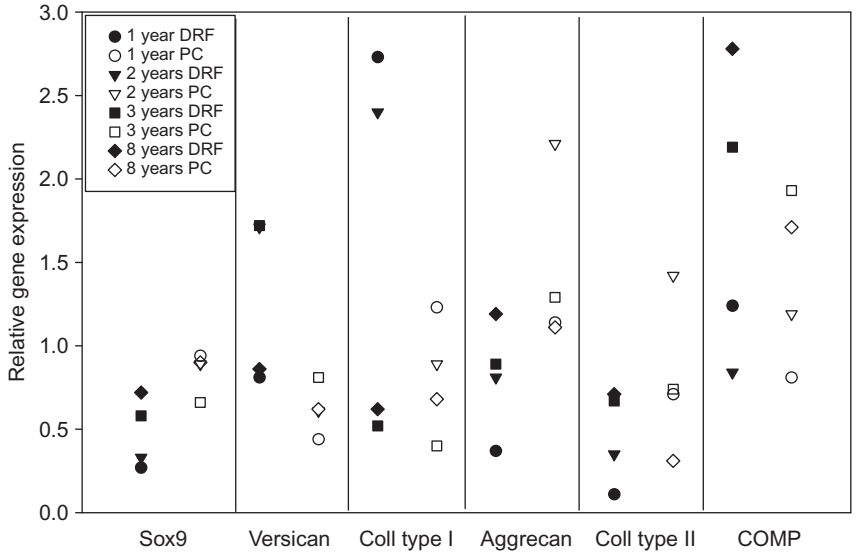


Figure 4. Relative gene expression of Sox9 and structural matrix proteins in untreated dorsal radial facet (DRF) and palmar condyle (PC) pellets. In the two youngest horses, versican and collagen type I expression was notably higher in DRF compared with PC pellets, whereas Sox9, aggrecan, and collagen type II expression was higher in PC compared with DRF pellets.

Table 2. Concentrations of the aggrecan epitope CS 846 and matrix metalloproteinase (MMP)-13 in culture supernatants of untreated, interleukin(IL)-1β, IL-6 and high mobility group box protein (HMGB)-1-treated pellets

| Treatment | CS 846 (μg/ml) Least square mean; range (maximum–minimum) | | Activated MMP-13 (ng/ml) Least square mean; range (maximum–minimum) | |
|---|---|---|---|---|
| | DRF ($n$ = 4) | PC ($n$ = 4) | DRF ($n$ = 4) | PC ($n$ = 4) |
| Untreated | 32.9 (53.6–20.0) | 35.3 (48.9–18.0) | 0.28 (1.12–0.00) | 0.03 (0.13–0.00) |
| IL-1β, 5 ng/ml | 25.8 (33.0–8.5) | 28.5 (34.8–20.3) | 62.5 (79.1–28.7) | 53.4 (98.2–17.7) |
| IL-6, 5 ng/ml | 31.7 (46.4–6.6) | 40.1 (53.8–30.9) | 0.21 (0.86–0.00) | 0.12 (0.49–0.00) |
| HMGB-1, 1 μg/ml | 27.1 (49.1–12.4) | 35.9 (55.7–21.0) | 0.25 (0.63–0.00) | 0.16 (0.63–0.00) |

Undetectable levels of MMP-13 are presented as zero values.

and collagen type II was present in PC compared with DRF pellets after IL-1β treatment. This may be due to a combination of increased responsiveness to cytokines in cells from young individuals [16,17,46] and the matrix gene expression profile of PC pellets being more hyaline cartilage-like than the corresponding DRF pellets.

Consistent upregulation of ADAMTS-5 expression in IL-1β-treated pellets in this study differs somewhat to the results by Little et al. [13], where IL-1β-induced ADAMTS-5 expression was detected in metacarpophalangeal but not in carpal cartilage explants. However, our finding of increased MMP-13 expression after IL-1β treatment is in agreement with other equine studies [10–15]. In accordance with Little et al. [13], who did not detect overall site-related differences in response to cytokine treatment (although individual variation was found), topographical differences in this study were only found for aggrecan expression after IL-1β treatment.

To our knowledge, the effects of IL-6 and HMGB-1 stimulation on gene expression of cartilage matrix proteins and matrix-degrading enzymes have previously not been reported in horses. Individual differences in gene expression in response to both cytokines created a more variable effect on matrix gene profiles compared with IL-1β-treated pellets. However, an overall upregulatory effect on Sox9 expression may indicate that IL-6 and HMGB-1 promoted chondrocyte differentiation. High collagen type II expression in DRF compared with PC pellets after HMGB-1 and IL-6 treatment in the two youngest horses supported increased chondrocyte differentiation, as these DRF pellets were found to be most dedifferentiated or fibrocartilage-like as untreated controls. Alternatively, increased Sox9 and collagen type II expression could purely relate to increased metabolic activity induced by IL-6 and HMGB-1 treatment. A regulatory role for HMGB-1 in chondrogenesis is possible considering the involvement of HMGB-1 in endochondral ossification [47], as well as in angio- and myogenesis [48,49]. Previous studies on IL-6 and soluble IL-6 receptor (sIL-6R) in human chondrocytes showed an increase in collagen type II, but not Sox9 and aggrecan core expression in long-term exposures [50]. On the contrary, IL-6 exposure

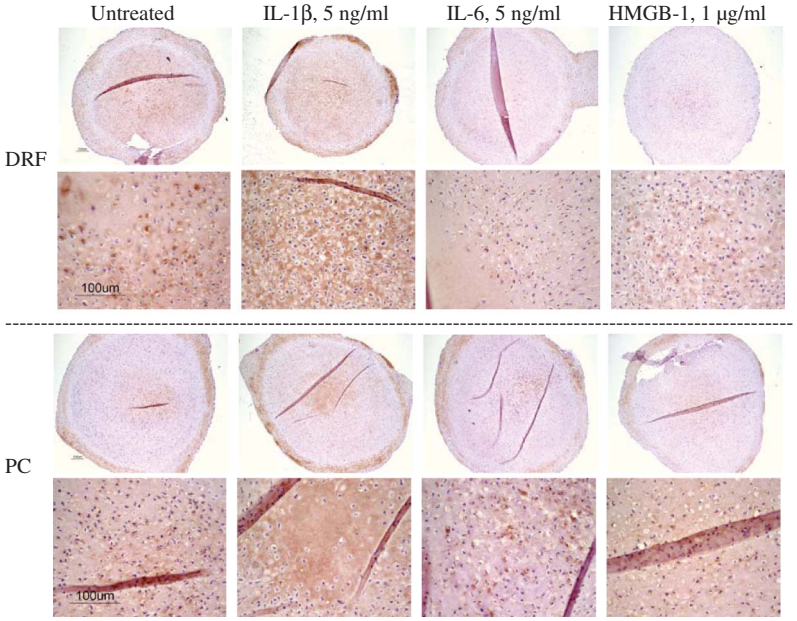


Figure 5. Immunostaining for cartilage oligomeric matrix protein (COMP) in untreated, interleukin (IL)-1β, IL-6, and high mobility group box protein (HMGB)-1-treated dorsal radial facet (DRF) and palmar condyle (PC) pellets from horse aged 2 years (Horse 2) confirmed synthesis of COMP in cell pellets. Although most IL-1β-treated pellets showed a decrease in COMP gene expression there was an increase in extracellular staining intensity.



reduced the collagen type II synthesis in rabbit chondrocytes [51], and at concentrations 10 times or higher than those used by us, IL-6 in combination with sIL-6R decreased collagen type II as well as aggrecan core mRNA levels [52]. Although a near 10-fold higher MMP-13 expression could be detected in occasional IL-6 and HMGB-1 pellets compared with untreated controls, this change was considerably less compared with IL-1β-treated pellets, and in several pellets down-regulation was seen. Our results are different compared with the results by Loeser et al. [53], who showed MMP-13 production in monolayer cultures of human chondrocytes after exposure to HMGB-1, and Legendre et al. [54], who demonstrated upregulation of both ADAMTS-5 and MMP-13 after combined IL-6 and sIL-6R stimulation of bovine chondrocytes. However, we confirmed synthesis of MMP-13 by measuring active MMP-13 in culture supernatants and only supernatants to IL-1β-treated pellets showed increased concentrations. The use of different species, culture methods, cytokine concentrations, and duration of stimulation may all be explanations for the differences in results between the experiments. Presence of membrane bound IL-6 receptor (IL-6R) or sIL-6R were not evaluated in this study, and it cannot be excluded that the relatively weak effects from IL-6 were related to low levels of IL-6R in the pellet cultures.

Although there was considerable individual variation in COMP and aggrecan synthesis in response to IL-6 and HMGB-1 treatment, neither treatment resulted in consistent upregulation of COMP and aggrecan gene expression, increased pellet immunostaining for COMP, nor significantly increased in concentrations of CS 846 in pellet supernatants. Thus, this study gave no support for direct links between exposure to IL-6 or HMGB-1 and COMP or aggrecan synthesis, postulated from previous studies [24,25,27,29]. Increased immunostaining for COMP in IL-1β-treated pellets may be related to increased matrix degradation, resulting in facilitated recognition of COMP epitopes by the polyclonal antibody. In IL-1α-stimulated bovine cartilage explants, COMP release is preceded by the release of proteoglycans [55].

The differences in gene expression of structural matrix proteins between DRF and PC in the three youngest horses (1–3 years old), suggested a more hyaline cartilage phenotype of cells originally harvested from the in vivo less loaded PC. This was indicated by relatively higher Sox9, aggrecan, and collagen type II expression compared with pellets from the in vivo highly loaded DRF. In contrast, cells from DRF showed a more fibrocartilaginous gene profile, indicated by higher versican and collagen type I gene expression. Macroscopic evaluation of sampled cartilage, absence of MMP-13 expression in untreated pellet cultures, and lack of differences in MMP-9, ADAMTS-5, and TIMP-1 gene expression between PC and DRF pellets suggests that the more dedifferentiated phenotype of cells in DRF pellets was not related to preexisting cartilage disease, but rather to the original anatomical location. The differences in gene matrix profiles between DRF and PC may relate to differences in in vivo mechanical loading of the two sites. Little et al. [7] showed that decorin synthesis was significantly

increased in the DRF compared with the PC in strenuously exercised horses, and decorin synthesis was increased but aggrecan synthesis decreased in the DRF in strenuously compared with moderately exercised horses. Influence from mechanical loading and exercise on either proteoglycan or collagen synthesis or content in articular cartilage has also been suggested from several other equine studies [4,5,56–60]. In this study, none of the horses were subjected to athletic training, and the differences in matrix gene expression profile were most noticeable in the youngest individuals. Therefore, it is possible that the maturity of the harvested chondrocytes is also important in determining the gene expression profiles and that with increasing age the chondrocyte population may become more uniform. It would have been preferable to apply mechanical loads to the chondrocyte cultures to improve their chondrogenic phenotype. However, phenotypic characteristics from previous in vivo loading may still have been present, as previous studies on equine cartilage explants have shown metabolic alterations to persist for as long as 16 weeks after the end of loading experiments [7], and chondrocytes placed in 3D culture after monolayer expansion redifferentiate and upregulate the synthesis of cartilage matrix protein such as collagen type II without additional loading in vitro [32]. To pinpoint influences from training and age, further studies on chondrocytes from untrained and trained young and old horses would be desirable. These studies should aim to determine whether the fibrocartilage-like gene profile of DRF reflects a predisposition for DRF chondrocytes to an in vivo synthesis of extracellular matrix with inferior biomechanical properties compared to PC.

In conclusion, this is the first study to investigate the effects of IL-6 and HMGB-1 on equine articular cartilage matrix metabolism. Neither IL-6 nor HMGB-1 treatment induced statistically significant changes in gene expression of matrix structural proteins, degrading enzymes, or TIMP-1 in 3D cultured chondrocytes after 48 hr of stimulation. However, their upregulation of Sox9 expression could indicate the promotion of chondrocyte differentiation or an increased metabolic state of the cultured chondrocytes. Future studies using different time points for evaluation of the metabolic response, and investigations focusing on regulatory pathways may help to clarify the possible involvement of these cytokines in chondrogenesis. Our results on IL-1β support previous findings regarding its catabolic effects on equine articular cartilage. Additionally, we observed a dedifferentiating effect of IL-1β, and identified IL-1β as an inducer of ADAMTS-5 expression, which may further contribute to the cartilage pathology seen in equine OA. We further conclude that the site-associated cartilage deterioration in equine carpal OA is not explained by topographical differences in the response to inflammatory mediators but are likely to involve other factors not examined in this study.

**Acknowledgments**

This study was supported by the Hippocampus program at SLU (Swedish University of Agricultural Sciences), FORMAS (Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning), and the Swedish–Norwegian Foundation for Equine Research. The authors are grateful to Nils Lundeheim, SLU, for performing the statistical analyses and thank Louise Danielsson and Åsa Gessbo, SLU, for their skillful laboratory assistance.

**Declaration of Interest:** The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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