Histochem Cell Biol (2007) 128:7–18
DOI 10.1007/s00418-007-0287-5

ORIGINAL PAPER

Distribution of lactate dehydrogenase in healthy and degenerative canine stifle joint cartilage

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Accepted: 16 April 2007 / Published online: 1 June 2007

Abstract In dogs, degenerative joint diseases (DJD) have been shown to be associated with increased lactate dehydrogenase (LDH) activity in the synovial fluid. The goal of this study was to examine healthy and degenerative stifle joints in order to clarify the origin of LDH in synovial fluid. In order to assess the distribution of LDH, cartilage samples from healthy and degenerative knee joints were investigated by means of light and transmission electron microscopy in conjunction with immunolabeling and enzyme cytochemistry. Morphological analysis confirmed DJD. All techniques used corroborated the presence of LDH in chondrocytes and in the interterritorial matrix of healthy and degenerative stifle joints. Although enzymatic activity of LDH was clearly demonstrated in the territorial matrix by means of the tetrazolium–formazan reaction, immunolabeling for LDH was missing in this region. With respect to the distribution of LDH in the interterritorial matrix, a striking decrease from superficial to deeper layers was present in healthy dogs but was missing in affected joints. These results support the contention that LDH in synovial fluid of degenerative joints originates from cartilage. Therefore, we suggest that (1) LDH is transferred from chondrocytes to ECM in both healthy dogs and dogs with degenerative joint disease and that (2) in degenerative joints, LDH is released from chondrocytes and the ECM into synovial fluid through abrasion of cartilage as well as through enhanced diffusion as a result of increased water content and degradation of collagen.

Keywords Dog · Degenerative joint disease · Immunolabeling · Electron microscopy · Enzyme cytochemistry

Abbreviations

AP	Alkaline phosphatase
ASAT	Aspartate amino transferase
CCB	0.1 M sodium cacodylate, 2 mM $CaCl_2$, pH 7.4
CCL	Cranial cruciate ligament
CFG	Cold fish gelatin
DJD	Degenerative joint disease(s)
ECM	Extracellular matrix
ICC	Immunocytochemistry
IHC	Immunohistochemistry
LDH	Lactate dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium chloride
NDS	Normal donkey serum
SF	Synovial fluid
TEM	Transmission electron microscopy
TNBT	Tetranitroblue tetrazolium chloride

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Introduction

Degenerative joint disease (DJD) is known as a slowly progressive degenerative condition that gradually leads to complete loss of joint function (Hayashi et al. 2003). This debilitating condition has been reported to affect as many as 20% of the canine population over 1 year of age (Johnston 1997). The most common cause of canine DJD in stifle joints is the rupture of the cranial cruciate ligament (CCL). This is usually the result of adaptive or degenerative changes within the ligament tissue and, thus, is influenced by age, body weight, and phenotype. Lactate dehydrogenase (LDH) is a ubiquitous cytosolic enzyme that catalyses a critical step in the glycolytic pathway, the reversible conversion of pyruvate to lactate. The enzyme is a tetramer, with either H or M subunits or a combination of both. Five different isoenzymes are known to date. In cartilage tissue, LDH4 and LDH5 are the predominant isoforms, as they play a pivotal role in anaerobic metabolic pathways (Yancik et al. 1987). Whereas the diagnosis of arthritis can fall back upon a number of reliable indicators present in synovial fluid (SF), convenient markers for DJD are still lacking in the dog. However, an increase in synovial LDH levels was recently associated with degenerative joint diseases (DJD) (Hurter et al. 2005; Schmöckel et al. 2001). The goal of the present study, therefore, was to elucidate the significant increase of LDH in SF from degenerative joints in the dog. Blood and SF samples were analyzed to monitor the inflammatory status, and articular cartilage from healthy and diseased joints were analyzed morphologically by light and electron microscopy. Furthermore, the presence and activity of LDH were determined by means of immunolabeling and enzyme cytochemistry. We herewith show that the presence of LDH is similar in chondrones of both healthy and degenerative cartilage. However, distribution of LDH within the extracellular matrix (ECM) differs, as the gradient from superficial to deeper layers observed in healthy tissue was lacking in diseased cartilage. These findings suggest that LDH is released from chondrocytes to ECM in both healthy dogs and dogs with DJD. Whereas, extracellular LDH remains constrained to ECM in sound cartilage, it is lost from damaged cartilage into the synovial fluid through abrasion and enhanced diffusion as a result of increased water content and degradation of collagen.

Materials and methods

Animals

Both stifle joints from three male and three female healthy Beagles and from one male Jack Russell Terrier (mean age 6.0 ± 4.1 years) were used as controls. These animals were euthanized for reasons not related to joint diseases or inflammatory processes. They had no history of lameness, nor did they show any macroscopic evidence of DJD.

Nine biopsies were from patients with degenerative stifle joints (one female and two neutered female Labrador Retrievers, two neutered female Bernese Mountain Dogs, one neutered male Australian Shepherd dog, one neutered male Boxer, one male English Bulldog, and one neutered female Hovawart, (mean age 5.3 ± 2.3 years). These animals had been diagnosed clinically with a rupture of the cranial cruciate ligament. Arthroscopic examination confirmed a partial rupture in five dogs and a total rupture in the remaining four individuals, with all patients showing signs of DJD. None of the dogs had received anti-inflammatory drugs within at least 2 weeks prior to surgery.

Materials and tissue processing

Synovial fluid

No SF was collected from control animals. From dogs with DJD, native and EDTA samples of SF were taken prior to surgery. Care was taken to avoid blood contamination. Samples were analyzed for color, clarity, and viscosity and were processed further within 30 min from sample collection. To reduce viscosity for further processing and analysis (Hurter et al. 2005; Schmöckel et al. 2001), SF was digested with hyaluronidase (final concentration of 0.1 mg hyaluronidase/ml, 20 min at 25°C). Aliquots for total cell counts and differential cell counts (EDTA samples) were removed and concentrated at 72×*g* (Cytospin 3, Shandon, Pittsburgh, USA). The remaining synovial fluid was centrifuged at 1,400×*g* for 15 min, and total protein content was determined with a Hitachi 912 laboratory analyzer (High Technology Inc, Walpole, USA). Enzymes assessed included LDH, alkaline phosphatase (AP), and aspartate amino transferase (ASAT); their activities were measured at 37°C with the Hitachi 912 laboratory analyzer. LDH activity was determined according to Schmöckel et al. (2001).

Blood

Blood was collected by venipuncture prior to anesthesia both from control animals and from dogs suffering from DJD. Parameters determined included cell count, differential blood count, total protein content, and albumin, lactate, glucose, ASAT (in EDTA sample) and AP (in heparinized blood) concentrations. One aliquot of every sample was centrifuged without delay in order to determine the concentration of LDH in plasma with a Hitachi 912 laboratory

analyzer (High Technology Inc, Walpole, USA) immediately after blood collection.

Cartilage

Thin cartilage slices were obtained from control animals immediately after euthanasia. In degenerative stifle joints, cartilage biopsies were collected from the region immediately lateral to the femoral notch during arthroscopy by means of a miniature double-spoon forceps. From dogs with DJD, specimens for morphology, enzyme histochemistry and cytochemistry, and immunolabeling were collected during arthroscopy.

For morphological examination of cartilage samples, 2.5% glutaraldehyde in CC-buffer (CCB 0.1 M sodium cacodylate, 2 mM $CaCl_2$, pH 7.4) was used as a fixative. Specimens were fixed for 5 h at room temperature, washed overnight in CCB, and post-fixed for 1 h with 1% osmium tetroxide in CCB. After dehydration through an increasing ethanol series, tissue was embedded in either paraffin wax or an Epon/Araldite mixture according to standard protocols. Ultrathin sections (70 nm) of Epon/Araldite-embedded material were mounted on collodion-coated copper grids and counterstained with uranyl acetate and lead citrate.

Tissue for immunolabeling was fixed with 0.5% glutaraldehyde, 4% paraformaldehyde, and 50 mM lysine–HCl in 0.1 M cacodylate buffer, pH 7.4. Samples were again fixed for 5 h at room temperature, dehydrated through an ascending ethanol series, and embedded in LR-White (British Biocell International, Brunschwig, Germany) by thermal curing in an incubator.

Immunohistochemistry and immunocytochemistry

For immunohistochemistry (IHC), semithin sections (0.5 µm) of LR White-embedded samples were transferred to glass slides and immunostained as shown in Table 1. A highly purified polyclonal goat anti-rabbit LDH antibody (L1011-09 from US Biological, Swampscott, Massachusetts, USA) that identifys all isoforms was used. After immunogold labeling and subsequent silver enhancement, sections were examined under epipolarization illumination.

For immunocytochemistry (ICC), ultrathin sections (80 nm) of LR White-embedded material were collected onto collodion-coated 200 mesh nickel grids and immunostained as described in Table 2. Control reactions for IHC and ICC included omission of both primary and secondary antibody, omission of primary antibody, and substitution of primary antibody with either normal goat IgG or goat anti-human choline acetyltransferase (Chemicon International, Inc., Lucerne) as an irrelevant primary antibody.

Enzyme histochemistry and cytochemistry

Enzymatic activity of LDH was assessed by histochemistry and cytochemistry in two healthy dogs and in two dogs with DJD.

Thick sections (200 µm) of unfixed joint cartilage were produced with a Vibratome (Oxford Laboratories, Foster City, USA). Incubation was performed with a reaction medium containing 150 mM sodium L-lactate (substrate), 3 mM NAD (co-enzyme), 0.32 mM 1-methoxyphenazine methosulphate, and 5 mM sodium azide in 100 mM phosphate buffer containing 18% polyvinyl alcohol, pH 7.45 (Van Noorden 1984). Finally, either 4 mg TNBT or 4 mg NBT chloride (Boehringer Mannheim GmbH) were dissolved in 20 µl of dimethylformamide and then added as a chromogen to 1 ml of the reaction medium. All chemicals were from SIGMA (Buchs, Switzerland) unless stated otherwise. Tissue slices were incubated for 2 or 3 h in a dark humid chamber at 37°C. The viscous incubation medium was rinsed off with warm 100 mM phosphate buffer (pH 5.3, approx. 50°C). Thereafter, slices were fixed in 10% formalin for 10 min, dehydrated in an increasing series of

Table 1 Incubation for immunohistochemistry (light microscopy)

Steps	Reagents	Duration	Temperature
Blocking step	5% NDS, 0.1% Tween 20, 1.35% CFG	30 min	RT
Primary antibody	Goat anti-rabbit LDH: 1:50 in blocking solution	5 h	RT
		overnight	4°C
Washing step	Blocking Solution	2 × 5 min	RT
	5% NDS, 0.1% Tween 20	3 × 5 min	RT
Secondary antibody	Donkey anti-goat IgG, 12 gold conjugate: 1:100 in 5% NDS, 0.1% Tween 20	90 min	RT
Washing step	5% NDS, 0.1% Tween 20	3 × 5 min	RT
	Distilled water	3 × 5 min	RT
Silver enhancement	Silver kit	20 min	RT (in the dark)
Washing steps	Tap water	3 × 5 min	RT
	Distilled water	5 min	RT

Primary antibody: Goat anti-rabbit lactate dehydrogenase (LDH) antibody (USBiological)

Secondary antibody: 12 nm colloidal gold-affinipure donkey anti-goat IgG (H+L) antibody (Jackson ImmunoResearch)

Table 2 Incubation for immunocytochemistry (transmission electron microscopy)

Steps	Reagents	Duration	Temperature
Blocking step	5% NDS, 0.1% Tween 20, 1.35% CFG	30 min	RT
First antibody	Goat anti-rabbit LDH: 1:50 in blocking solution	5 h	RT
		overnight	4°C
Washing step	Blocking solution	1 × 5 min	RT
	5% NDS, 0.1% Tween 20	2 × 5 min	RT
Second antibody	Donkey anti-goat IgG, 12 nm gold conjugate: 1:100 in 5% NDS, 0.1% Tween 20	90 min	RT
Washing step	5% NDS, 0.1% Tween 20	2 × 5 min	RT
	PBS	4 × 5 min	RT
Fixation	1% glutaraldehyde in PBS	10 min	RT
Washing step	PBS	3 × 5 min	RT
	Distilled water	3 × 5 min	RT
Silver enhancement	Silver kit	5 min	RT (in the dark)
Washing step	Tap water	3 × 5 min	RT
	Distilled water	5 min	RT

ethanol, and infiltrated and embedded with Unicryl (Polyscience Inc, BBI International, Brunschwig, Germany). In order to enhance tissue contrast for better orientation, a number of sections from one dog were osmicated following a cytochemical reaction. For control reactions, sodium L-lactate and NAD were replaced by distilled water. sections of 5–15 µm were used for light microscopy. For electron microscopic examination, 80 nm sections were mounted on collodion-coated 200 mesh copper grids.

A Zeiss Axioskop with an AxioCam HR (Feldbach, Switzerland) was used for light microscopic observation, whereas ultrathin sections were examined in a Zeiss transmission electron microscope 109 (Zeiss, Oberkochen, Germany) equipped with a GATAN wide angle slow scan CCD Camera 689 (GATAN GmbH, Munich, Germany).

Results

Distinction between intact and ruptured CCL in stifle joints was based on clinical examination of cruciate ligaments. Clinical diagnosis was extremely reliable, as arthroscopy and microscopical analysis of cartilage samples fully confirmed the presence of a ruptured CCL and of articular damage in the diseased animals as opposed to healthy dogs.

Morphology

Light microscopical analysis of specimens included assessment of overall tissue organization, state of cartilage surface, and cell characteristics. Cartilage samples from *healthy dogs* were smooth-surfaced and displayed the typical stratification (Fig. 1) and cell shapes (Arsenault and Hunziker 1988). Though usually present, the amorphous layer at the articular surface (Fig. 3a, inset) occasionally was discontinuous in *healthy dogs*. Underneath, the dense network of collagen fibers running in parallel with the articular surface was obvious (Fig. 3a, inset). The territorial and interterritorial ECMs were clearly distinct (Fig. 3b). These features applied to all cartilage strata. Most chondrocytes showed large nuclei and an abundance of organelles (Fig. 3a, b). Intracellular inclusions (Fig. 3a) were noted in all three zones, the largest ones occurring in the radial zone.

In contrast, biopsies from *degenerative stifle joints* revealed damage to the articular surface, formation of clefts, destruction of the tangential zone, and a disorganization of cartilage layering (Fig. 2a, b). The articular surface was rough and the amorphous layer was completely lacking (Fig. 4) except for some remnants in one single dog. Fibers were arranged at random with extensive amorphous foci between single fibers and fiber bundles (Fig. 4). Characteristic signs of cartilage degeneration, such as hypocellularity and hypercellularity, were obvious. Cell cloning was usually localized to transitional and tangential zones. As compared to healthy cartilage, chondrocytes from degenerative joints were smaller, cell nuclei were often big when present, and depletion of cytoplasmic organelles occurred. Instead, chondrocytes displayed intracellular vacuoles and pericellular blebs. In half the biopsies, small ovoid, amorphous, cell-shaped, translucent structures that contain a network of finer fibrils were observed underneath the surface (Fig. 4).

Immunolabeling

In *healthy dogs*, chondrocytes in all three zones were clearly immunopositive for LDH, with the strongest signal observed in the transitional and radial zones (Fig. 5). The

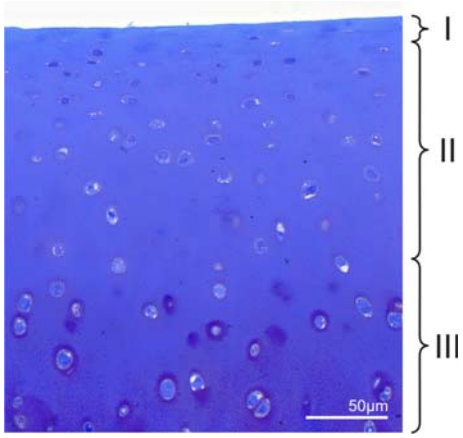

Fig. 1 Light micrograph of a Richardson-stained semithin section of healthy cartilage showing obvious stratification into tangential (*I*), transitional (*II*) and radial zones (*III*). The articular surface is smooth and even

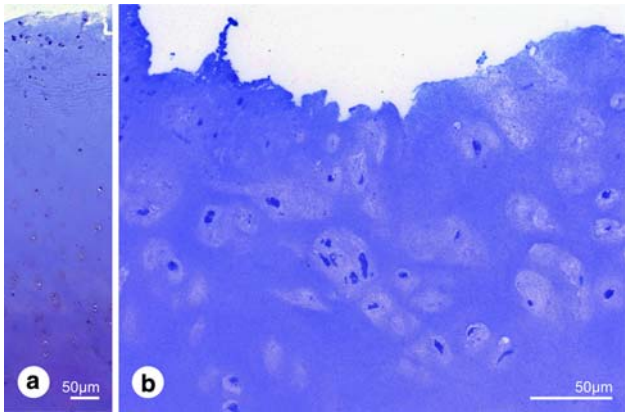

Fig. 2 Light micrographs of Richardson-stained semithin sections of degenerative cartilage. **a** Note loss of stratification as well as roughness and unevenness of articular surface. **b** Note damage to articular surface. Spindle-shaped chondrocytes, typical of the tangential zone, are almost completely missing. Articular surface and chondrones are damaged. The cytoplasm of chondrocytes contains cell debris and large vacuoles

signal was evenly distributed all over the cytoplasm and on mitochondria but was absent from vacuoles. The territorial matrix was basically devoid of labeling (Figs. 5, 8). With respect to the interterritorial ECM, labeling intensity decreased from the tangential zone to the radial zone. However, the amorphous layer and the most superficial layer of collagen fiber displayed a faint signal only (Fig. 6).

In *degenerative cartilage* (Fig. 6), overall labeling was faint. Chondrocytes in the tangential zone were virtually devoid of any signal, whereas cells in the transitional and radial zones displayed an increasing immunoreactivity. In contrast to healthy cartilage, the gradient in labeling intensity of the ECM was missing except for those areas where remnants of the tangential zone were still present. As in healthy dogs, signal was absent from the pericellular matrix (Fig. 6, inset).

All negative control samples were devoid of immunolabeling (Figs. 7, 9).

Tetrazolium formazan reaction

Enzyme histochemistry and cytochemistry provided a highly specific and conspicuous signal especially when using NBT chloride as a substrate. As tissue penetration is subject to limitations, observations were made in peripheral

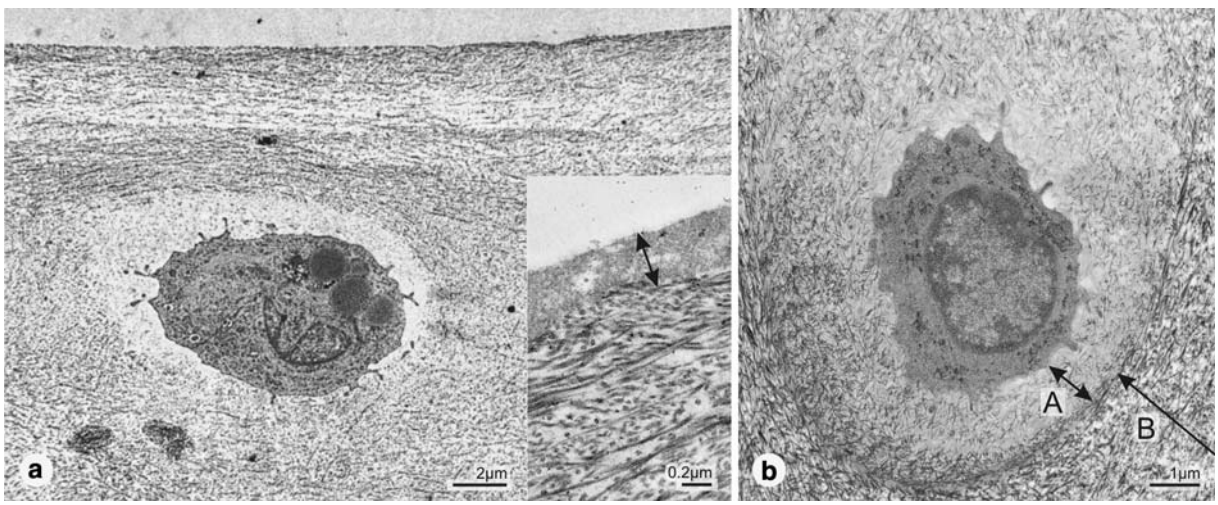

Fig. 3 Electron micrographs of healthy cartilage. **a** A dense superficial network of collagen fibers running in parallel with the articular surface is present. Chondrocytes contain numerous organelles and vesicles. *Inset* An amorphous layer (*double arrow*) extends at the surface. Underneath, densely packed collagen fibers run in parallel with the articular surface. **b** Territorial (*A*) and interterritorial (*B*) zones of extracellular matrix are clearly demarcated. Note the large nucleus and abundance of organelles in the chondrocyte

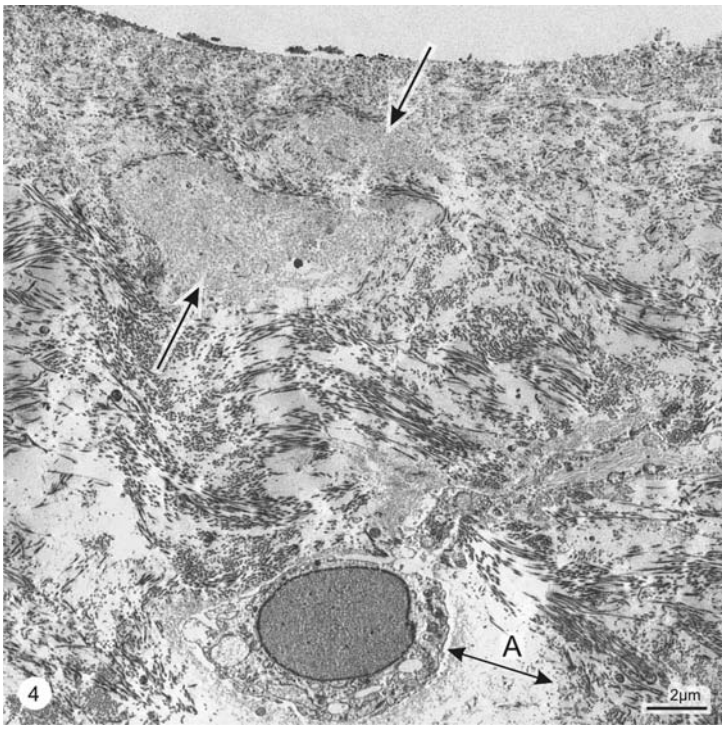

Fig. 4 Electron micrograph of degenerative cartilage. The amorphous layer is missing, and the articular surface is uneven. Collagen fibers are loosely packed and arranged at random. Note amorphous foci in the extracellular matrix (*arrows*). (*A*) Territorial extracellular matrix

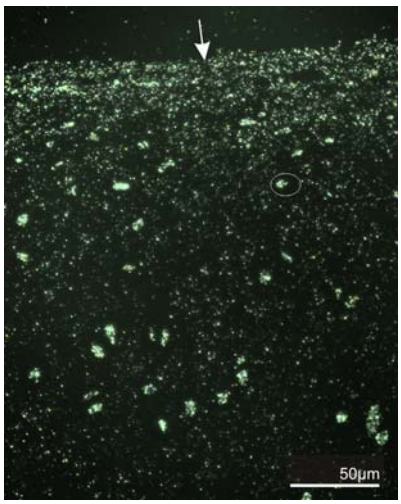

Fig. 5 Epipolarization micrograph of silver-enhanced immunogold-labeling of healthy cartilage with anti-LDH. Chondrocytes in all three zones are strongly immunopositive, with the strongest cellular signal being noted in the radial zone. The pericellular extracellular matrix is devoid of labeling (*encircled*). A decrease in extracellular labeling intensity from tangential to radial zones is conspicuous. Signal in the amorphous layer and in most superficial collagen fibers is faint. (*Arrow*) Cartilage surface

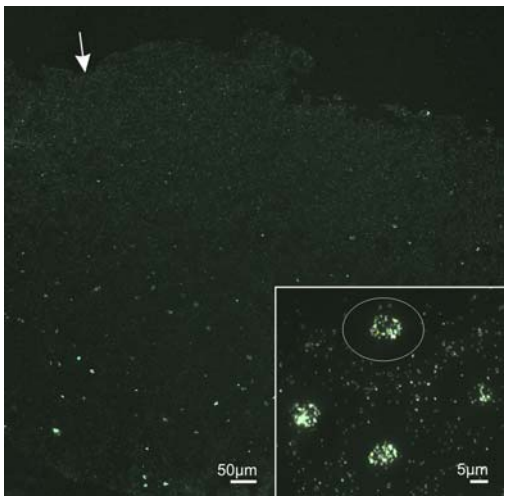

Fig. 6 Epipolarization micrograph of silver-enhanced immunogold-labeling of degenerative cartilage with anti-LDH. Labeling of chondrocytes is faint. Unlike in healthy cartilage, labeling intensity in the extracellular matrix does not increase from deeper to superficial layers. (*Arrow*) Cartilage surface. *Inset* Signal is absent from the pericellular zone of the extracellular matrix (*encircled*)

regions of tissue samples. Osmication following enzyme histochemistry did not affect specificity when exposure to OsO_4 was limited to 1 h but yielded false positive results when incubation was extended to 2 h.

Negative controls showed no or only a faint background staining underneath the cell membrane (Figs. 12, 14).

In *healthy cartilage*, nuclei of chondrocytes were free of reaction product, whereas the cytoplasm exhibited an intense staining strongest beneath the cell membrane (Figs. 10, 13). With regard to the ECM, signal decreased from the tangential to radial zones, with a narrow layer at the articular surface remaining devoid of reaction product (Fig. 10). Furthermore, a concentric gradient was observed within the territorial matrix, where the signal

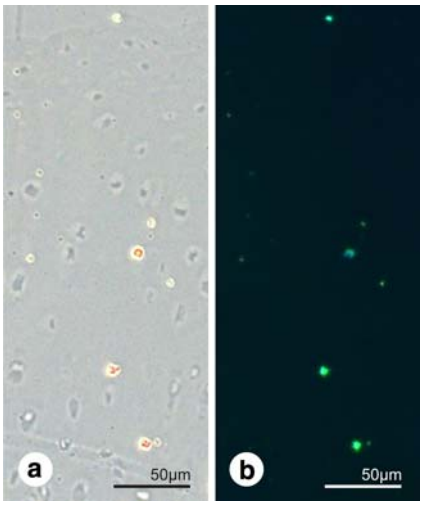

Fig. 7 Negative control for immunohistochemistry of degenerative cartilage. A goat anti-human choline acetyltransferase antibody was used as an irrelevant primary antibody for silver-enhanced immunogold-labeling. Note complete absence of signal except for a few solitary unspecific precipitates. **a** phase contrast, **b** epipolarization micrograph

decreasing from the cell border to the periphery (Fig. 10, inset).

In some respects, cellular staining in *degenerative cartilage* was similar to the signal observed in healthy animals. Thus, ECM of degenerative cartilage exhibited the concentric gradient in pericellular matrix as well, most prominently in the radial zone (Fig. 11, inset). The gradient from superficial to deeper layers, however, was absent in diseased animals (Fig. 11).

Synovial fluid

Synovial fluid (SF) was obtained from eight out of nine dogs with DJD, as aspiration of fluid failed in one dog. Volumes collected ranged from 0.5 to 1.9 ml. Results of laboratory analyses are summarized in Table 3.

Cytology was performed in seven cases. Mainly mononuclear cells (macrophages and synovial lining cells) with a moderately to markedly enlarged cytoplasm in combination with cytoplasmic vacuolization were observed. Polynucleated giant cells were present in one dog. Lymphocytes and polymorphonuclear granulocytes were within the normal range. Occasionally, a few osteoclasts (5 dogs), blood cells (4 dogs), and plasma cells (1 dog) were found.

Blood

Blood parameters from all *control animals* were within normal range except for a slight neutrophilia with left shift in one dog suffering from a protein-losing enteropathy (as based on gastro-intestinal endoscopic findings).

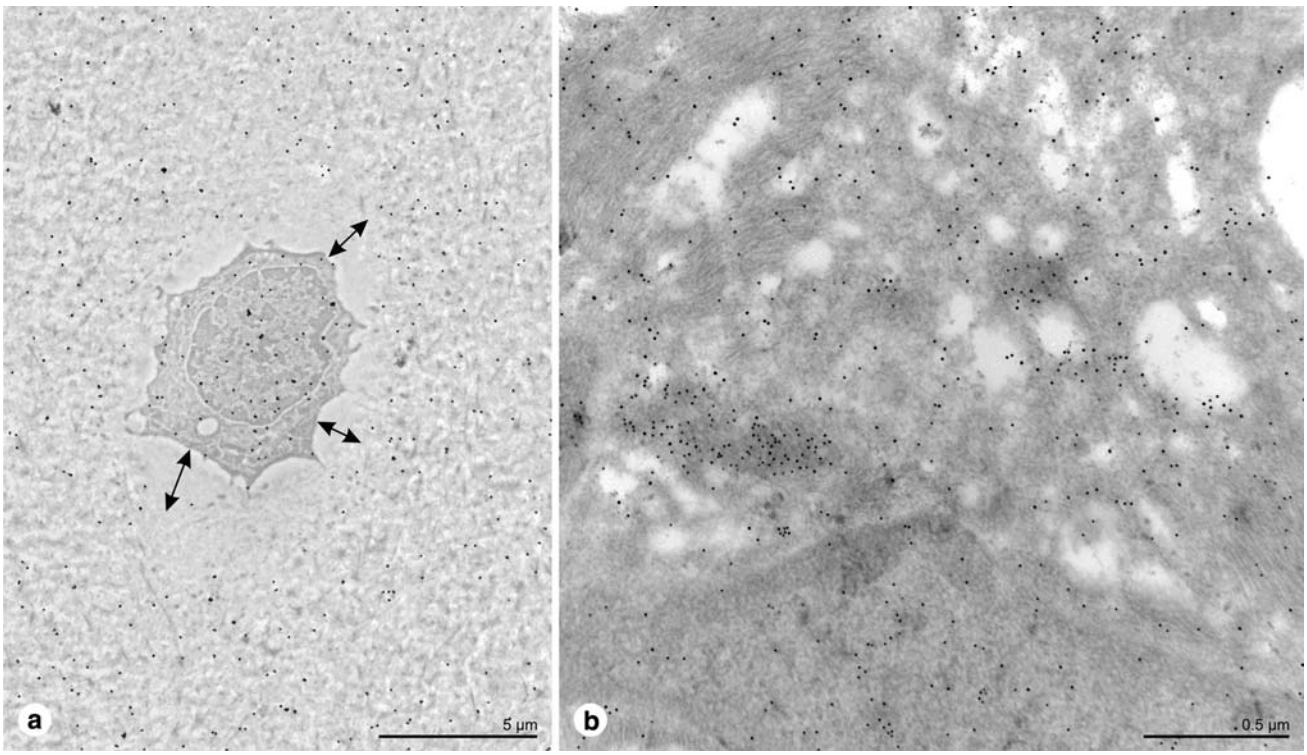

Fig. 8 Immunogold-labeling of healthy cartilage with anti-LDH. **a** Chondrocyte and interterritorial matrix are strongly immunopositive. The territorial extracellular matrix, however, is devoid of signal (*double arrows*) in this stained ultrathin section. **b** The dense labeling of the cytoplasm reveals regional differences in the abundance of LDH. The nucleus is also moderately labeled in this double stained ultrathin section

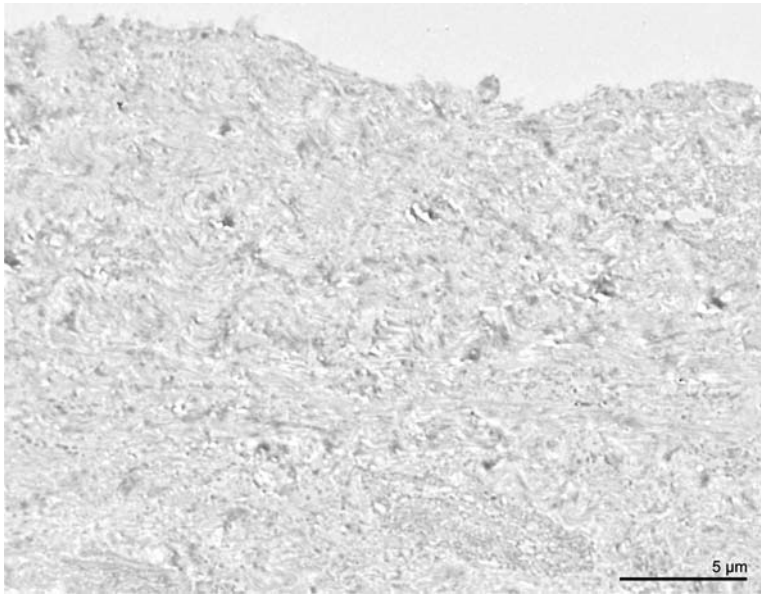

Fig. 9 Negative control for immunocytochemistry. A goat anti-human choline acetyltransferase antibody was used as an irrelevant primary antibody for immunogold-labeling. Note complete absence of any background labelling, unstained ultrathin section

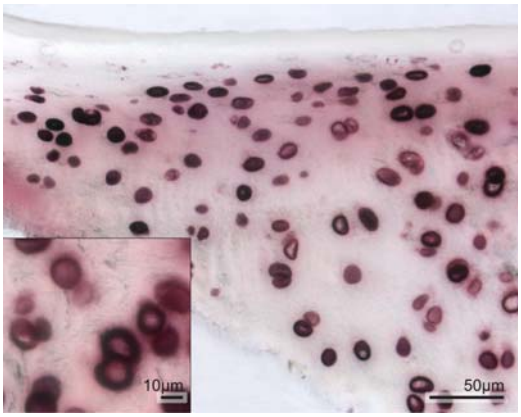

Fig. 10 Enzyme histochemical demonstration of LDH in healthy cartilage. The tetrazolium formazan reaction reveals an obvious decrease in LDH-concentration from tangential to radial zones. Furthermore, enzyme activity is completely absent from the most superficial layers at the articular surface. Inset: The territorial matrix displays a concentric gradient with decreasing enzyme activity from the cell border to the periphery

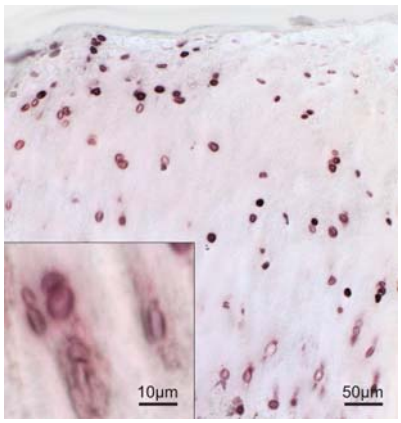

Fig. 11 Enzyme histochemical demonstration of LDH in degenerative cartilage. Unlike in healthy cartilage, no vertical gradient in enzyme activity is discernible. *Inset* The concentric gradient in the pericellular extracellular matrix is similar to its counterpart in healthy cartilage

Results of blood sample analyses from all the *dogs suffering from DJD* are summarized in Table 3.

Discussion

Chronic joint diseases are commonly characterized as being either inflammatory or degenerative. Whereas, the diagnosis of arthritis can fall back upon a number of reliable indicators present in synovial fluid (SF), convenient markers for degenerative joint diseases (DJD) are still lacking in the dog. Although a number of indicators of articular damage, such as collagen fragments, aggrecan fragments, metalloproteinases, and tissue inhibitor of metalloproteinase, have

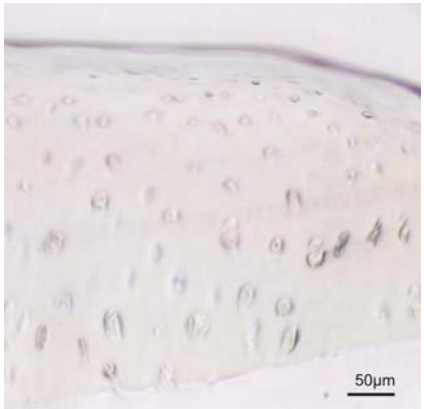

Fig. 12 Negative control for enzyme histochemistry. Light micrograph of healthy cartilage after incubation in the absence of substrate. Note complete absence of reaction product

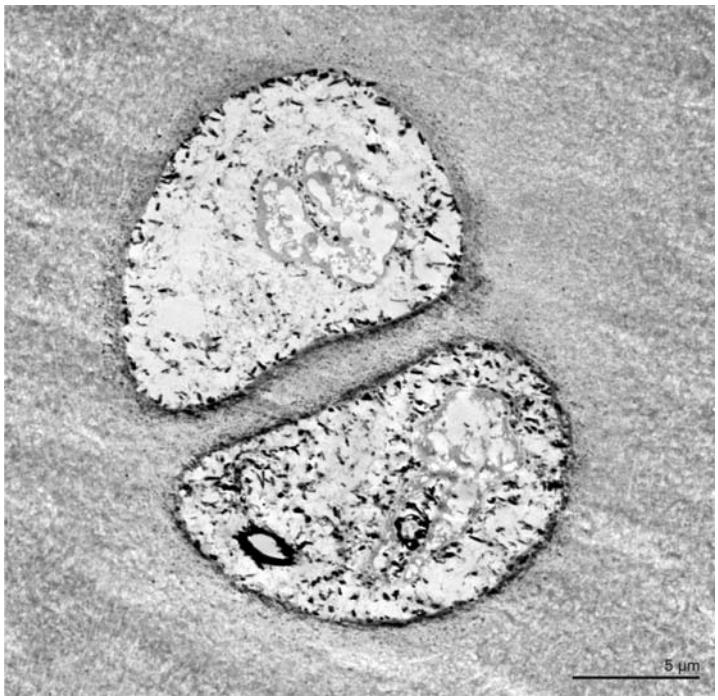

Fig. 13 Enzyme cytochemical demonstration of LDH in healthy cartilage. Conspicuous intracellular formazan deposits are present in the cytoplasm but are absent from vacuoles

Fig. 14 Control reaction in the absence of substrate. Note complete lack of reaction product

been determined in blood, urine, or SF, none of these parameters provides any specific clues as to the presence of DJD (Chu et al. 2002; Lohmander 1994; Lohmander et al. 1998; Slater et al. 1995; Thonar et al. 1992). Similarly, early reports implied that DJD might not alter enzyme activities in SF (Cohen 1964). However, later studies in horses and humans unanimously demonstrated an overall increase in synovial LDH activity in conjunction with a number of inflammatory and non-inflammatory joint diseases, such as infectious arthritis, rheumatoid arthritis, gout, and osteochondrosis dissecans (Lindy et al. 1971; Messieh 1996; Pejovic et al. 1992; Rejno 1976; Yancik et al. 1987). As for the dog, it has been shown recently that LDH activity in SF is likely to correlate with the extent of degenerative lesions, thus making LDH a potentially useful and specific marker for DJD (Hurter et al. 2005; Schmöckel et al. 2001).

The present study was designed to corroborate the correlation between increased LDH activities in SF and DJD in dogs and determine where the enzyme found in SF originates. Analyses of blood and SF samples were performed in order to eliminate dogs with immune mediated or acute inflammatory joint diseases from the study. Thus, only dogs with arthroscopically and microscopically diagnosed DJD but with blood and synovial leucocyte and neutrophil counts within normal range were included. Taking into account the diluting effect owing to the large sample volume in dog 4, the activity of LDH in SF was increased in six out of eight cases of DJD, thus supporting the contention of LDH as being a valuable indicator of DJD. Our results also confirm that LDH activity in SF from degenerative joints does not correlate with synovial cell counts (Hurter et al. 2005; Pejovic et al. 1992; Schmöckel et al. 2001). Thus, the enzyme cannot originate from blood cells.

The synovial membrane is another possible source of LDH that must be considered. However, permeability of synovial membranes is limited to molecules up to 100 kDa

Table 3 Results of SF and blood

Synovial fluid										Blood Plasma	
Patient	Color	Turbidity	Viscosity	Protein (g/l)	Cells ($\times 10^9$/l)	Volume (ml)	ASAT (IU/l)	AP (IU/l)	LDH (IU/l)	ASAT (IU/l)	LDH (IU/l)
Normal range	None to light yellow	Clear	+/++	20–25	0–3	−1	10–76	14–126	Healthy: 61 ± 9 DJD: 120 ± 9	20–73	66–319
1	Yellow	Turbid	+	29	0.9		50	46	**116**	**87**	187
2	Yellow	Clear	++	30	0.7		18	34	64	29	246
3	Yellow to reddish	Clear	++	20	0.7	0.5	36		**238**	34	156
4	Light yellow	Clear	++	32	0.8	1.9	26	40	64	23	79
5	Red			26	2.5	0.5			**346**	53	**868**
6	Light yellow	Turbid	++	26	0.8				**110**	30	174
7	Yellow	Light turbid	+	24	1.3	1.5	24	46	**181**	20	**660**
8	Yellow	Clear		13	0.9	1	19	58	87	39	310

(Lipowitz 1985; Lipowitz and Newton 1985; Maroudas and Schneiderman 1987). Molecular weight of LDH is 140 kD, and in addition, this enzyme carries a net negative charge. Bearing in mind that analysis of SF speaks against an increase in permeability, the possibility that LDH might originate from the synovial membrane can be ruled out.

The presence of LDH in joint cartilage has been documented in various species including the dog (Altman 1981; Dunham et al. 1986; Pelletier et al. 1985; Schiefke et al. 1998; Tushan et al. 1969; Weseloh and Fiesselmann 1975). In tibial plateau cartilage of normal dogs, LDH was reported to be homogeneously spread in superficial, upper transitional, and radial zones, with higher values noticed in the deeper transitional zone (Dunham et al. 1986). The present study now provides additional information as to the distribution of LDH in articular cartilage. Enzyme cytochemistry and immunolabeling clearly demonstrated high levels of cytosolic LDH activity in viable chondrocytes of both healthy dogs and dogs with DJD. Furthermore, some labeling was also seen in nuclei as shown by electron microscopy. LDH is known to be a soluble glycolytic enzyme. However, its intracellular distribution is not restricted to the cytoplasm. Rather, early reports on the presence of LDH in the nucleus date back to 1965 (Siebert and Humphrey 1965; Siebert et al. 1966). More specifically, LDH-5 has been located to the nucleus, and evidence has been provided that it may function in the regulation of gene transcription or DNA replication. It is considered to be a single-stranded DNA-binding protein that stimulates the activity of DNA poymerase α in vitro (Cattaneo et al. 1985; Grosse et al. 1986; Zhong and Howard 1990).

In addition, substantial amounts of LDH were present in the cartilage matrix. The enzyme has been suspected to be contained in matrix vesicles (Hosokawa et al. 1992, 1988; Maki et al. 2000; Ohashi-Takeuchi et al. 1990). In our material, such vesicles were commonly noticed by electron microscopy. However, neither enzyme cytochemistry nor immunolabeling were indicative of any LDH accumulation within vesicles.

In healthy dogs, a decrease in LDH activity in the ECM from superficial to deep cartilage layers was substantiated by both immunolabeling and enzyme cytochemistry, thus suggesting accumulation of LDH within the tangential zone as compared to the transitional and radial zones. We consider this to be a corollary of the higher metabolic activity in superficial cartilage layers (Dunham et al. 1986). Such a gradient, however, was absent in degenerative cartilage. The lack of a density gradient in degenerative joints was always associated with the erosion of the tangential layer as revealed by morphological analysis. Inversely, the gradient was still present in those areas in which the most superficial cartilage layer was preserved. Thus, an intact tangential zone, including a lamina splendens, obviously provides containment, and its absence predictably will allow leakage of LDH into SF. This contention is further supported by the observation that the exposed cartilage surface in degenerative joints was virtually devoid of LDH. Furthermore, homogeneous distribution of LDH in DJD obviously reflects differences between healthy and degenerative cartilage matrix with respect to permeability. This correlates with the observation of dissociated collagen fiber bundles and loss of fiber orientation in tangential and transitional zones, these characteristics being indicative of increased water content in the cartilaginous matrix (Adams and Billingham 1982; Roughley et al. 1992; Stockwell et al. 1983). Thus, whereas healthy cartilage is able to restrict the diffusion of molecules larger than albumin (Van Bree et al. 1994), differences in LDH distribution are leveled out in degenerative tissue due to increased permeability (Thonar et al. 1992).

In summary, we suggest that cytosolic LDH is released from chondrocytes into the cartilage matrix. Whereas LDH is retained in healthy cartilage due to permeability limitations, it is released into SF in degenerative joints through abrasion as well as through unrestricted diffusion as a result of increased water content and degradation of collagen.

Acknowledgments The outstanding technical assistance of Mrs C. Furer and Mrs C. Hug is gratefully acknowledged. We are also indebted to the laboratory of Prof. Tschudi for blood and SF analysis and to Mr. S. König for his excellent photographic work.

References

Adams ME, Billingham ME (1982) Animal models of degenerative joint disease. Curr Top Pathol 71:265–297

Altman FP (1981) A metabolic dysfunction in early murine osteoarthritis. Ann Rheum Dis 40(3):303–306

Arsenault AL, Hunziker EB (1988) Electron microscopic analysis of mineral deposits in the calcifying epiphyseal growth plate. Calcif Tissue Int 42(2):119–126

Cattaneo A, Biocca S, Corvaja N, Calissano P (1985) Nuclear localization of a lactic dehydrogenase with single-stranded DNA-binding properties. Exp Cell Res 161(1):130–140

Chu Q, Lopez M, Hayashi K, Ionescu M, Billinghurst RC, Johnson KA, Poole AR, Markel MD (2002) Elevation of a collagenase generated type II collagen neoepitope and proteoglycan epitopes in synovial fluid following induction of joint instability in the dog. Osteoarthr Cartil 10(8):662–669

Cohen AS (1964) Lactic dehydrogenase (LDH) and transaminase (GOT) activity of synovial fluid and serum in rheumatic disease states, with a note on synovial fluid LDH isozymes. Arthritis Rheum 19:490–501

Dunham J, Shackleton DR, Bitensky L, Chayen J, Billingham ME, Muir IH (1986) Enzymic heterogeneity of normal canine articular cartilage. Cell Biochem Funct 4(1):43–46

Grosse F, Nasheuer HP, Scholtissek S, Schomburg U (1986) Lactate dehydrogenase and glyceraldehyde-phosphate dehydrogenase are single-stranded DNA-binding proteins that affect the DNA-polymerase–alpha-primase complex. Eur J Biochem 160(3):459–467

Hayashi K, Frank JD, Hao Z, Schamberger GM, Markel MD, Manley PA, Muir P (2003) Evaluation of ligament fibroblast viability in ruptured cranial cruciate ligament of dogs. Am J Vet Res 64(8):1010–1016

Hosokawa R, Ohashi-Takeuchi H, Yamada N, Uchida Y, Fujiwara S, Noguchi T (1992) Lactate dehydrogenase isoenzymes in matrix vesicles (review). Bone Miner 17(2):177–181

Hosokawa R, Uchida Y, Fujiwara S, Noguchi T (1988) Lactate dehydrogenase isoenzymes are present in matrix vesicles. J Biol Chem 263(21):10045–10047

Hurter K, Spreng D, Rytz U, Schawalder P, Ott-Knusel F, Schmökel H (2005) Measurements of C-reactive protein in serum and lactate dehydrogenase in serum and synovial fluid of patients with osteoarthritis. Vet J 169(2):281–285

Johnston SA (1997) Osteoarthritis: joint anatomy, physiology and pathobiology. 27:699–723

Lindy S, Turto H, Uitto J, Vainio K (1971) Origin of synovial fluid lactate dehydrogenase in rheumatoid arthritis. Clin Chim Acta 35(2):377–382

Lipowitz AJ (1985) Synovial Fluid. In: Newton CD, Nunamaker DM (eds.) Textbook of small animal orthopaedics, International Veterinary Information Service, Ithaca, Chapter 86

Lipowitz AJ, Newton CD (1985) Degenerative Joint Disease and Traumatic Arthritis. In: Newton CD, Nunamaker DM (eds.) Textbook of small animal orthopaedics, International Veterinary Information Service, Ithaca, Chapter 87

Lohmander LS (1994) Articular cartilage and osteoarthrosis. The role of molecular markers to monitor breakdown, repair and disease. J Anat 184(Pt 3):477–492

Lohmander LS, Dahlberg L, Eyre D, Lark M, Thonar EJ, Ryd L (1998) Longitudinal and cross-sectional variability in markers of joint metabolism in patients with knee pain and articular cartilage abnormalities. Osteoarthr Cartil 6(5):351–361

Maki K, Hayashi S, Nishioka T, Kimura M, Noguch T (2000) A new type of matrix vesicles is found in fetal bovine tracheal cartilage. Connect Tissue Res 41(2):109–115

Maroudas A, Schneiderman R (1987) "Free" and "exchangeable" or "trapped" and "non-exchangeable" water in cartilage. J Orthop Res 5(1):133–138

Messieh M (1996) Levels of lactate dehydrogenase in osteoarthritic and failed total knee joints. J Arthroplasty 11(3):354–355

Ohashi-Takeuchi H, Yamada N, Hosokawa R, Noguchi T (1990) Vesicles with lactate dehydrogenase and without alkaline phosphatase present in the resting zone of epiphyseal cartilage. Biochem J 266(1):309–312

Pejovic M, Stankovic A, Mitrovic DR (1992) Lactate dehydrogenase activity and its isoenzymes in serum and synovial fluid of patients with rheumatoid arthritis and osteoarthritis. J Rheumatol 19(4):529–533

Pelletier JP, Martel-Pelletier J, Ghandur-Mnaymneh L, Howell DS, Woessner JF Jr (1985) Role of synovial membrane inflammation in cartilage matrix breakdown in the Pond-Nuki dog model of osteoarthritis. Arthritis Rheum 28(5):554–261

Rejno S (1976) LDH and LDH isoenzymes of synovial fluid in the horse. Acta Vet Scand 17(2):178–189

Roughley PJ, Nguyen Q, Mort JS (1992) The role of proteinases and oxygen radicals in the degradation of human articular cartilage. pp. 305–318

Schiefke I, Weiss J, Keller F, Leutert G (1998) Morphological and histochemical ageing changes in patellar articular cartilage of the rat. Anat Anz 180(6):495–500

Schmöckel H, Messmer M, Lutz H (2001) Messung der Aktivitäten der alkalischen Phosphatase, Aspartataminotransferase und Laktatdehydrogenase in der Synovialflüssigkeit gesunder und veränderter Gelenke beim Hund. Wiener Tierärztliche Monatsschrift (5):118–121

Siebert G, Humphrey GB (1965) Enzymology of the nucleus. Adv Enzymol Relat Areas Mol Biol 27:239–288

Siebert G, Villalobos J Jr, Ro TS, Steele WJ, Lindenmayer G, Adams H, Busch H (1966) Enzymatic studies on isolated nucleoli of rat liver. J Biol Chem 241(1):71–78

Slater RR Jr, Bayliss MT, Lachiewicz PF, Visco DM, Caterson B (1995) Monoclonal antibodies that detect biochemical markers of arthritis in humans. Arthritis Rheum 38(5):655–659

Stockwell RA, Billingham ME, Muir H (1983) Ultrastructural changes in articular cartilage after experimental section of the anterior cruciate ligament of the dog knee. J Anat 136(2):425–439

Thonar EJ-MA, Manicourt DH, Williams JM, Fukuda K, Campion GV, Sweet BME, Lenz ME, Schnitzer TJ, Kuettner KE (1992) Serum Keratan Sulfate: a measure of cartilage proteoglycan metabolism. In: Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds) Articular Cartilage and Osteoarthritis, Raven Press New York, pp 429–446

Tushan F, Rodnan GP, Altman M, Robin ED (1969) Anaerobic glycolysis and lactate dehydrogenase (LDH) isoenzymes in articular cartilage. J Lab Clin Med 73(4):649–656

Van Noorden CJ (1984) Histochemistry and cytochemistry of glucose-6-phosphate dehydrogenase. Prog Histochem Cytochem 15(4):1–85

Van Bree H, Justus C, Quirke JF (1994) Preliminary observations on the effects of meloxicam in a new model for acute intra-articular inflammation in dogs. Vet Res Commun 18(3):217–224

Weseloh G, Fiesselmann A (1975) [Distribution of the isoenzym lactatdehydrogenase in human cartilage (author's transl)]. Arch Orthop Unfallchir 83(3):345–351

Yancik SA, McIlwraith CW, Wagner AE, Trotter GW (1987) Evaluation of creatine kinase and lactate dehydrogenase activities in clinically normal and abnormal equine joints. Am J Vet Res 48(3):463–466

Zhong XH, Howard BD (1990) Phosphotyrosine-containing lactate dehydrogenase is restricted to the nuclei of PC12 pheochromocytoma cells. Mol Cell Biol 10(2):770–776