EQUINE VETERINARY JOURNAL

Equine Veterinary Journal ISSN 0425-1644
DOI: 10.1111/j.2042-3306.2011.00539.x

Synovial fluid chondroitin sulphate indicates abnormal joint metabolism in asymptomatic osteochondritic horses

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Summary

Reasons for performing study: Alternative methods to evaluate the joint condition in asymptomatic osteochondrosis dissecans (OCD) and other joint diseases may be useful.
Objectives: To investigate possible changes in synovial fluid composition that may lead to joint conditions in asymptomatic OCD, in mature horses.
Methods: Animals aged >2 years, of different breeds, with OCD in the intermediate ridge of distal tibia, symptomatic or not, were studied. Synovial fluid samples (10 healthy; 11 asymptomatic OCD; 25 symptomatic OCD) were collected by arthroscopy from 29 horses. Glycosaminoglycans (GAGs) were analysed by a combination of agarose gel electrophoresis and enzymatic degradation with specific GAG lyases. The viscosity, white blood cell (WBC) count, protein concentration and hyaluronic acid (HA) molecular weight were also determined.
Results: The method used here to analyse synovial fluid GAGs is reliable, reproducible and specific. The main synovial fluid GAGs are HA and chondroitin sulphate (CS), 93% and 7% respectively in normal horses. In symptomatic OCD, the concentrations of both increased (expressed as GAG/urea ratios), but CS increased more. The CS increased also in asymptomatic OCD. An inflammatory reaction was suggested by the increased WBC counts in OCD. The molecular weight of the synovial fluid HA was reduced in OCD, explaining the lower viscosity observed.
Conclusions: The increased CS in synovial fluid of OCD joints in mature horses suggests that the synovial fluid CS and the WBC count are good markers of the joint conditions, allowing the identification of pathological phase in joint diseases.
Potential relevance: The analysis of synovial fluid GAGs shows that cartilage damage occurs even in asymptomatic OCD, implying that arthroscopic removal of osteochondral fragments should be performed even in asymptomatic OCD.

Keywords: horse; chondroitin sulphate; glycosaminoglycan; hyaluronic acid; osteochondrosis dissecans; synovial fluid

Introduction

Osteochondrosis is a family of joint diseases that occurs in horses, as well as in other domestic animals and man. It is characterised by interruption of blood supply to the bone epiphysis, followed by necrosis and, later, regrowth. The exact aetiology of the disorder is unknown, but rapid growth, heredity, repetitive trauma, vascular abnormalities, mechanical factors, and hormonal and dietary imbalances may all play a role [1].

Osteochondrosis dissecans (OCD) is characterised by dissection of both cartilage and bone, resulting in an osteochondral fragment, which may be completely segregated from the original bone. Its free movement within the joint space may cause pain and further damage. In horses, the tarsocrural is the most frequently affected joint [2,3]. The disease is a dynamic process, with radiographically visible abnormalities often present shortly after birth. A proportion of these lesions become clinically important in mature horses, while others resolve before foals reach age 5 months [4]. The main clinical signs are joint effusion and lameness in animals starting sport training [5,6], but some horses will remain asymptomatic. In these cases, OCD is diagnosed by radiographic examinations, performed either as routine or for commercial purposes. Since OCD occurs during the early growth phase, most of the studies on this disease are commonly performed in horses aged ≤24 months [7]. There are few data on OCD in mature horses [8], and even fewer on asymptomatic ones.

The usual treatment is the removal of osteochondral fragment by arthroscopy. As some animals remain asymptomatic it is not generally accepted that arthroscopy should be indicated in all cases. Molecular biomarkers of cartilage and bone metabolism eventually present in synovial fluid could become useful tools to monitor the joint conditions, providing relevant data for the prognosis of each case.

Since cartilage is very rich in chondroitin sulphate (CS), and the viscosity of the synovial fluid depends largely on hyaluronic acid (HA), the analysis of synovial fluid and glycosaminoglycans (GAGs) remains promising, as long as adequate methods for their analysis are available.

We have previously shown that the dye-binding assay with 1,9-dimethylmethylene blue (DMMB), frequently used to quantify sulphated GAGs in biological fluids, is unreliable, since ions and other anionic macromolecules interfere [9]. To quantify HA, several methods have been described, including a sensitive and specific fluoroassay based on the interaction of HA with biotin-conjugated HA-binding protein and europium-labelled streptavidin [10]. Nevertheless, the concentration of HA in synovial fluid is too high for this method, which detects ng of HA.

The objectives of the present study were to establish a reliable methodology to identify and quantify synovial fluid GAGs, and to analyse these compounds in horses aged >2 years, with symptomatic or asymptomatic OCD. The aim was to investigate possible changes in synovial fluid composition in asymptomatic OCD, which could aid diagnosis of joint conditions.

Materials and methods

Materials

The standard GAGs chondroitin 4-sulphate (from whale cartilage), dermatan sulphate (from hog skin) and HA (from umbilical cord), and *Streptomyces hyalurolyticus* hyaluronate lyase (EC 4.2.2.1, HA lyase or hyaluronidase) were purchased from Sigma[a]. Heparan sulphate (from bovine pancreas) and chondroitinase AC (from *Flavobacterium heparinum*) were prepared by methods previously described [11,12]. Agarose (standard, low M_r) was purchased from Bio-Rad[b].

Animals and sample collection

The present work was approved by the Ethical Committees of Universidade Federal de São Paulo - UNIFESP (CEP 0800/07) and Universidade de São Paulo - USP (1238/2007), and was carried out in accordance with UNIFESP and USP guidelines, and also in accordance with EC Directive 86/609/EEC for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm

TABLE 1: Viscosity, protein concentration and cell count in synovial fluid (SF) of normal and OCD horses

Animal No.	Group	Age (years)	Gender	Joint	Bodyweight (kg)	Clinics*	Viscosity**	WBC count (x 10^9 cells/l)	Protein (g/l)	Serum urea (g/l)	SF urea (g/l)
1	Symptomatic OCD	3	Male	Right	420	L/E	Low	400	16	0.30	0.25
1	Symptomatic OCD	3	Male	Left	420	L/E	Low	300	16	0.30	0.21
2	Symptomatic OCD	3	Male	Right	450	L/E	Low	300	10	0.21	0.21
2	Symptomatic OCD	3	Male	Left	450	L/E	Intermediate	500	6	0.21	0.15
3	Symptomatic OCD	4	Female	Right	400	E	Normal	300	12	0.27	0.24
3	Symptomatic OCD	4	Female	Left	400	E	Intermediate	400	12	0.27	0.16
4	Symptomatic OCD	2	Male	Right	436	L/E	Intermediate	nd	20	nd	0.28
4	Symptomatic OCD	2	Male	Left	436	E	Low	nd	14	nd	0.29
5	Symptomatic OCD	11	Male	Right	285	L/E	Intermediate	300	20	0.32	0.27
6	Symptomatic OCD	3	Male	Right	406	L/E	Low	300	14	0.40	0.35
6	Symptomatic OCD	3	Male	Left	406	L/E	Low	200	24	0.40	0.33
7	Symptomatic OCD	7	Female	Right	470	L/E	Intermediate	100	10	0.46	0.40
7	Symptomatic OCD	7	Female	Left	470	L/E	Normal	200	4	0.46	0.38
8	Symptomatic OCD	2	Female	Right	331	L/E	Intermediate	1300	16	0.28	0.23
8	Symptomatic OCD	2	Female	Left	331	L/E	Intermediate	1400	16	0.28	0.30
9	Symptomatic OCD	8	Female	Right	520	L/E	Low	100	10	0.23	0.23
9	Symptomatic OCD	8	Female	Left	520	L/E	Low	100	12	0.23	0.17
10	Symptomatic OCD	8	Male	Right	619	L/E	Intermediate	400	16	nd	0.36
11	Symptomatic OCD	2	Male	Right	500	E	Intermediate	300	8	0.39	0.33
11	Symptomatic OCD	2	Male	Left	500	E	Intermediate	300	6	0.39	0.32
12	Symptomatic OCD	13	Male	Left	395	L/E	Intermediate	500	14	0.43	0.37
13	Symptomatic OCD	4	Male	Right	516	L	Intermediate	700	20	nd	0.32
14	Symptomatic OCD	3	Male	Left	450	L/E	Normal	300	10	nd	0.32
15	Symptomatic OCD	2	Female	Right	460	E	Intermediate	300	16	0.47	0.47
16	Symptomatic OCD	4	Male	Left	430	E	Intermediate	nd	11	0.35	0.23
15	Asymptomatic OCD	2	Female	Left	460	-	Intermediate	400	24	0.47	0.44
16	Asymptomatic OCD	4	Male	Right	430	-	Normal	nd	15	0.35	0.27
17	Asymptomatic OCD	2	Male	Left	470	-	Normal	nd	11	nd	0.26
18	Asymptomatic OCD	3	Male	Right	300	-	Normal	170	18	0.34	0.24
18	Asymptomatic OCD	3	Male	Left	300	-	Normal	290	16	0.34	0.21
19	Asymptomatic OCD	2	Male	Right	435	-	Low	260	11	0.35	0.26
19	Asymptomatic OCD	2	Male	Left	435	-	Intermediate	400	19	0.35	0.24
20	Asymptomatic OCD	6	Male	Right	460	-	Normal	200	14	nd	0.37
20	Asymptomatic OCD	6	Male	Left	460	-	Normal	300	14	nd	0.42
21	Asymptomatic OCD	5	Male	Left	517	-	Normal	100	14	nd	0.37
22	Asymptomatic OCD	4	Male	Right	480	-	Intermediate	200	16	0.37	0.33
23	Control	3	Male	Right	270	-	Normal	40	12	nd	0.27
23	Control	3	Male	Left	270	-	Normal	60	10	nd	0.28
24	Control	3	Male	Right	300	-	Normal	20	8	0.32	0.24
24	Control	3	Male	Left	300	-	Intermediate	20	10	0.26	0.24
25	Control	3	Male	Right	250	-	Intermediate	110	8	0.39	0.29
25	Control	3	Male	Left	250	-	Intermediate	100	9	0.39	0.31
26	Control	3	Male	Right	300	-	Normal	60	16	0.41	0.33
26	Control	3	Male	Left	300	-	Normal	110	16	0.41	0.32
27	Control	3	Male	Right	306	-	Normal	170	10	0.27	0.17
27	Control	3	Male	Left	306	-	Normal	140	14	0.27	0.24

*L, lameness; E, effusion; nd, not determined. ** Viscosity was estimated by the string of fluid test: <2 cm, Low; 2–5 cm, Intermediate; >5 cm, Normal.

The diagnosis of OCD was based on radiographic examinations performed either for commercial purposes or in animals with clinical signs of joint disease. Twenty-four animals were selected. These animals presented at the Veterinary Hospital, Large Animal Surgery Session, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (FMVZ-USP) for arthroscopy. Before the procedure, they were submitted to a standard lameness examination [13] that revealed the presence or absence of clinical signs (lameness and/or joint effusion). The control group was composed of 5 male sedentary healthy horses, of different breeds, age 3 years, free of any orthopaedic diseases on the basis of clinical, radiographic, and ultrasound examinations. These animals were submitted to experimental arthroscopy, and sham operated.

Synovial fluid samples were aspirated directly (neat) and without lavage from tarsocrural joints, at the beginning of arthroscopy. These samples were classified in 3 groups: Control – synovial fluid samples from 10 healthy joints of 5 control horses; asymptomatic OCD – synovial fluid samples from 11 joints with OCD in the intermediate ridge of distal tibia, but without any clinical sign, from 8 horses of different breeds, aged 2–6 years, 7 male and one female; and symptomatic OCD – synovial fluid samples from 25 joints with OCD in the intermediate ridge of distal tibia, and clinical signs of

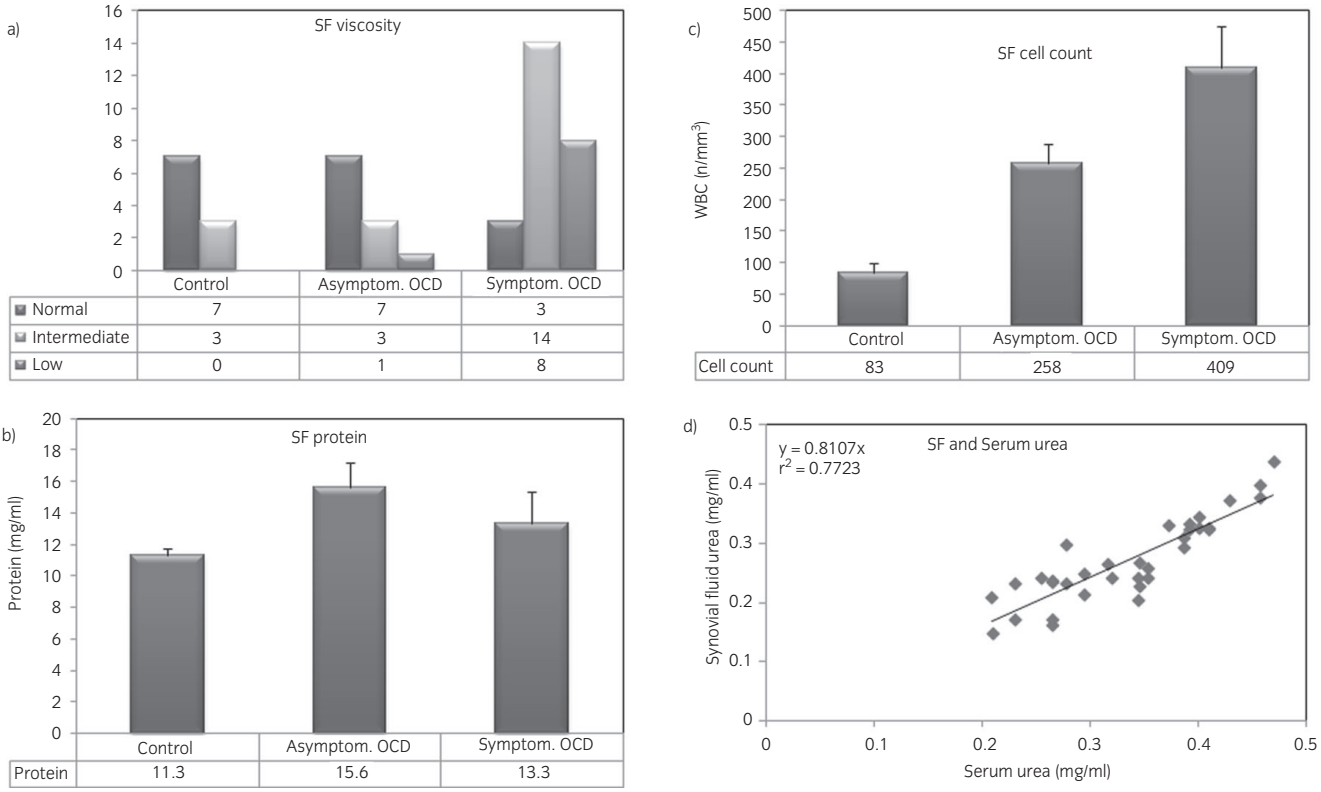

Fig 1: Synovial fluid (SF) viscosity (a), protein concentration (b), white blood cell (WBC) count (c), and SF and serum urea concentration from normal (Control) and both asymptomatic and symptomatic OCD horses. Synovial fluid samples were aspirated without lavage at the beginning of arthroscopic procedures. The viscosity was estimated by the string test, and protein concentrations, WBC counts and urea were measured. Urea was also measured in serum samples collected from the same animals, before arthroscopy. The figure shows the means for protein concentration and WBC counts, and the error bars show standard errors. Individual data are given for SF and serum urea concentrations. Brackets indicate statistically significant differences.

disease (effusion and/or lameness), from 16 horses of different breeds, 2–13 years, 11 males and 5 females.

Synovial fluid viscosity, protein and urea concentrations, and cell count

The synovial fluid viscosity was evaluated immediately after sample collection by the 'string test', which is performed by placing a drop of fluid between the gloved thumb and finger [14]. 'Normal viscosity' synovial fluid produced a string of fluid approximately 5–6 cm long, whereas 'low viscosity' synovial fluid produced a string of <2 cm, and 'intermediate viscosity' synovial fluid gave a string of fluid 2–5 cm long.

For white blood cell (WBC) count, 1 ml of each synovial fluid sample was centrifuged at 2000 ***g*** for 10 min, the supernatant removed and the pellet resuspended in 1 ml of phosphate buffered saline (PBS). The cells were counted in a Neubauer counting chamber.

Aliquots of the supernatant (4 μl) were used to measure soluble proteins by the biuret reaction in an automated biochemical analyser (Labmax 240)[c].

Urea was determined in synovial fluid (SF) and serum samples (5 μl) by completely automated enzymatic reactions (urease-glutamate dehydrogenase) (Randox RX Daytona)[d]. Urea concentration was used to correct the GAG concentration for fluid volume [15].

Glycosaminoglycans: hyaluronic acid and chondroitin sulphate

Synovial fluid samples were centrifuged at 10,000 ***g*** for 30 min to remove cells and debris, and the supernatants were stored at -80°C until used for analysis of GAGs.

For the analysis of GAGs, synovial fluid samples (100 μl) were submitted to proteolysis (4 g/l Maxatase [Detergent alkaline family of serine endopeptidases isolated from *Bacillus subtilis*. EC 3.4.21] in 0.05 mol/l Tris-HCl, pH 8.0, 200 μl). After overnight incubation at 50°C, Maxatase was heat inactivated (15 min, 100°C), and debris were removed by centrifugation (3000 ***g***, 10 min, room temperature). The supernatant was freeze dried, and resuspended in 50 μl of pure water.

Aliquots (5 μl) were submitted to 0.5% agarose gel electrophoresis in 0.05 mol/l 1,3-diaminopropane-acetate buffer, pH 9 (PDA), as previously described [16]. This is the optimum condition to separate HA from sulphated GAGs. After fixation with cetyltrimethylammonium bromide, sulphated GAGs were stained by 0.1% toluidine blue in 1% acetic acid and 50% ethanol (15 min, room temperature). The excess of dye was removed with 1% acetic acid in 50% ethanol solution. The gel slab was dried at room temperature, and then nonsulphated GAGs were stained in the same slabs by 0.1% toluidine blue in 0.025 mol/l sodium acetate buffer, pH 5.0. The excess dye was removed using the same buffer. The synovial fluid GAGs were quantified by densitometry of the gel slabs in a CS-9000 Shimadzu densitometer[e].

These compounds were further characterised by enzymatic degradation with specific GAG lyases, as previously described: *F. heparinum* chondroitinase AC [17] and *Streptomyces hyalurolyticus* HA lyase [18]. Briefly, aliquots (5 μl) of synovial fluid samples previously submitted to proteolysis were incubated with either 3×10^{-3} units of chondroitinase AC in 10 μl 0.05 mol/l ethylenediamine-acetate buffer, pH 8.0, or 1 unit of HA lyase in 10 μl of 0.1 mol/l sodium acetate buffer, pH 5.0, in final volumes of 15 μl. After 6 h incubation at 37°C, aliquots of the incubation mixtures were vacuum dried, resuspended in pure water, and submitted to agarose gel electrophoresis in PDA buffer, as above described. The degradation products do not precipitate with cetyltrimethylammonium bromide, and disappear from the gel. Controls were incubated with heat inactivated enzymes. Chondroitinase AC digests both CS and HA, while *Streptomyces hyalurolyticus* HA lyase is specific for HA.

The HA molecular weight was estimated by agarose gel electrophoresis in 0.04 mol/l Tris-acetate buffer containing 1 mmol/l EDTA (TAE buffer), as previously described by Lee and Cowman [19], except that 0.4% agarose was used and the electrophoresis run in the horizontal system described by Jaques *et al*. [20], using HA preparations of known molecular weight as standard (Sigma[a]).

The dye binding assay with 1,9-dimethylmethylene blue (DMMB) was according to Farndale *et al*. [21].

Data analysis

Data were evaluated for normality by the Kolmogorov-Smirnov test, which revealed that the sample distributions were parametric. Afterwards, the unpaired *t* test was used to compare the OCD groups with the control group. The software GraphPad Instat 3 was used to perform the statistical analysis.

Results

Synovial fluid viscosity, protein and cell count

Table 1 shows the age, sex, affected joint (for OCD), and bodyweight of control and OCD horses as well as the synovial fluid viscosity, WBC count, and protein and urea concentrations. Serum urea concentration is also given. The viscosity, estimated by the 'string test', was 'normal' in 7 of the control synovial fluid samples (strings >5 cm, 70% of samples) and 'intermediate' in 3 samples (strings of fluid 2–5 cm long, 30% of the samples). None of the samples had 'low viscosity'. In the asymptomatic OCD joints, similar percentages of 'normal' and 'intermediate' synovial fluid viscosity were observed (7 samples with 'normal viscosity' and 3 samples with 'intermediate viscosity', 64% and 27%, respectively), whereas one sample had 'low viscosity' (string of fluid <2 cm). In contrast, the percentages of 'intermediate' and 'low viscosity' were increased in the synovial fluid samples from joints with symptomatic OCD (14 and 8 samples, respectively, corresponding to 56% and 32% of total), and the percentage of 'normal viscosity' was much lower (3 samples, 12%).

The concentrations of synovial fluid protein and urea, as well as the serum urea, did not vary significantly among the samples (Table 1). In contrast, the WBC count was increased in OCD, whether symptomatic or not ($P<0.05$) (Table 1). The mean values and standard errors are shown in Figure 1, which also shows that there was a good correlation between synovial fluid and serum urea ($r^2 = 0.7723$, Fig 1d), with a ratio of 0.8107 (synovial fluid/serum).

Glycosaminoglycan identification and quantification

A method commonly used to quantify sulphated GAGs is the dye-binding assay with DMMB. Nevertheless, Figure 2a shows that HA also reacts, and Figure 2b shows that the CS concentration curves are affected by the presence of HA (synovial fluid concentration range).

Synovial fluid GAGs were identified by a combination of agarose gel electrophoresis and enzymatic degradation with specific GAG lyases, as described in above. Figure 3 shows representative agarose gel slabs stained with toluidine blue for both sulphated and nonsulphated GAGs. One discrete band migrating as CS was detected in all samples. This band was totally digested by chondroitinase AC, and was resistant to the action of *Streptomyces hyalurolyticus* HA lyase (Fig 4). A second broad band, migrating as a smear, was totally digested by *Streptomyces hyalurolyticus* HA lyase and also by chondroitinase AC, and was identified as HA (Figs 3, 4).

These GAGs were quantified by densitometry of the gel slabs, and Table 2 shows that the error of the method never exceeds 10% (independent analyses for only a few examples are shown; similar results were obtained for all samples).

The mean concentration of HA was increased by 30% in symptomatic OCD in comparison with controls (Fig 5a, $P<0.05$). Nevertheless, when expressed as HA/urea (to correct for fluid volume), the differences were not significant (Fig 5b). Agarose gel electrophoresis (0.4% in TAE buffer, Fig 5c), in which the HA migration depends on the chain molecular weight, also revealed that the HA modal molecular weight was reduced in joints with 'low' and 'intermediate' viscosity, in comparison to those with 'normal viscosity'.

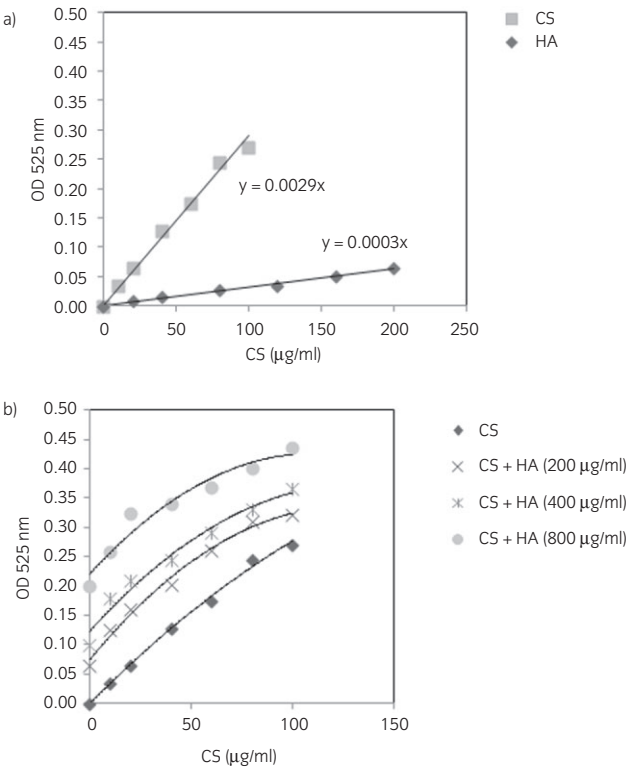

Fig 2: Calibration curves of chondroitin sulphate (CS) and hyaluronic acid (HA) with 1,9-dimethymethylene blue dye binding assay (DMMB). The assay was performed as described by Farndale *et al*. [37]. a) Calibration curves for CS and HA. b) CS curves (5–100 mg/l) in presence of different concentrations of HA (200, 400 or 800 mg/l). These concentrations include the range of HA concentration in synovial fluid.

Figure 6 shows that the concentration of CS was increased in all OCD synovial fluid samples. This increase was also observed in CS/HA and CS/urea ratios, indicating that the CS concentration increased more than urea and HA in all OCD samples.

Taken together, these data indicate that the increase in CS concentration in OCD occurred even in joints that did not present any clinical signs (effusion and lameness).

Discussion

Osteochondritis dissecans is a term introduced in 1888 by the medical surgeon Franz König to describe a condition that causes a predisposition to the formation of loose bodies in the joints of young individuals without the contribution of primary arthritis or trauma to the joint. Although considered inappropriate by many authors, it is still in use (see Nagura [22] for a historic review). The term 'osteochondrosis' has replaced 'osteochondritis' because it is generally agreed that inflammation is not a characteristic feature of the primary lesions.

The main lesion of osteochondrosis is an area of growth cartilage that fails to undergo matrix calcification and vascular invasion, and therefore is not converted to bone [23]. A focal area of cartilage necrosis that is confined to the epiphyseal cartilage is usually referred to as *osteochondrosis latens*, whereas the presence of a failure of endochondral ossification that is visible on macroscopic and radiographic examination is designated as *osteochondrosis manifesta*; after a fissure forms in the area of necrotic cartilage and extends through the articular cartilage (cartilage flap or loose body), the designation for the lesion is OCD [24].

Although the matrix of the epiphyseal growth cartilage is macroscopically indistinguishable from the overlying articular cartilage, it is characterised by the presence of vessels that invade the cartilage from

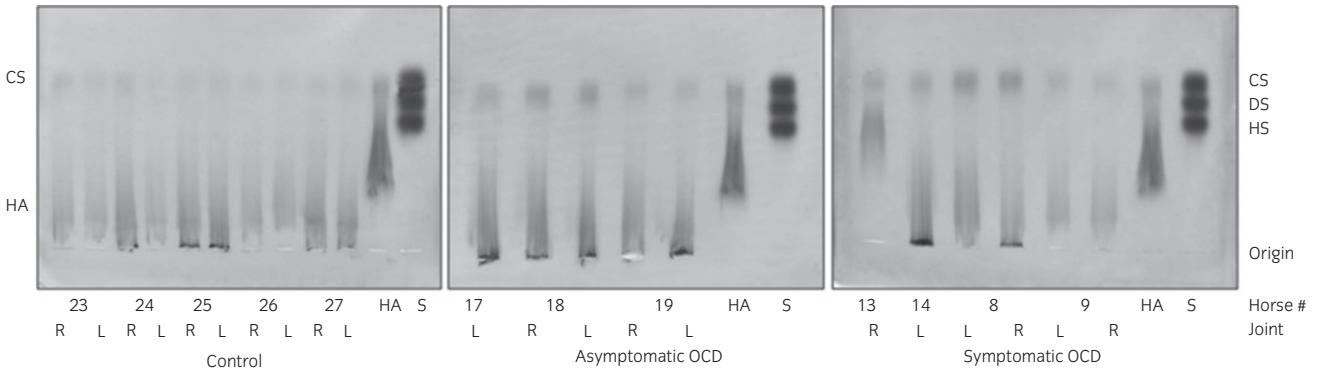

Fig 3: Agarose gel electrophoresis of horse synovial fluid glycosaminoglycans (GAGs). Synovial fluid samples were submitted to proteolysis, freeze dried and resuspended in water; 5 μl aliquots were applied to agarose gel in PDA buffer. Gel slabs were stained with toluidine blue. For horse numbers, see Table 1. L = left; R = right; CS = chondroitin sulphate; DS = dermatan sulphate; HS = heparan sulphate; HA = hyaluronic acid; OCD = osteochondrosis dissecans; S = mixture of standard GAGs.

the surrounding perichondrium and course through cartilage channels, whereas the articular cartilage is avascular [25]. It is generally believed that the primary event in OCD is cessation of blood supply in growth cartilage, which occurs as a result of damage to the newly formed vessels. This theory explains the rather specific location of lesions and the existence of a defined time window of susceptibility for the disease. In horses, osteochondrosis lesions appear during the first 5 months *post partum*, and often revert thereafter, although some will remain and may become clinically important in the mature animal [26].

Following ischaemic necrosis of growth cartilage, the cartilage matrix of the necrotic area degenerates, and a response occurs in the surrounding tissues, presumably initiated by the release of enzymes, signalling molecules and extracellular matrix components [24,27], a process likely to be accompanied by enzymatic degradation of cartilage matrix. That is why, in the search of molecular markers for joint conditions, the focus on the presence of cartilage matrix degradation products in the synovial fluid is a reasonable choice. Our findings seem to corroborate this concept.

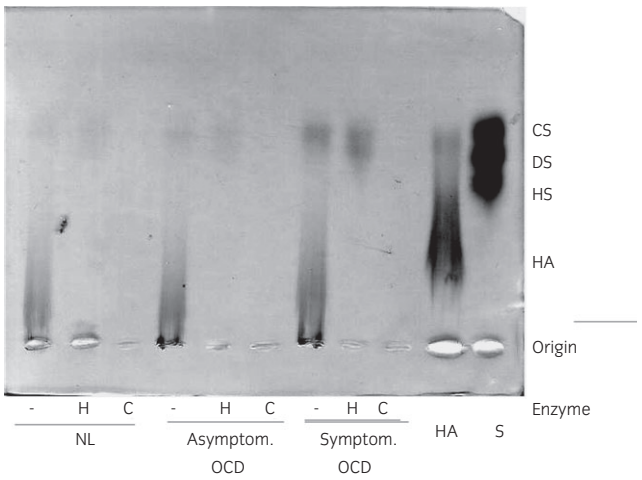

Fig 4: Agarose gel electrophoresis of horse synovial fluid glycosaminoglycans (GAGs) digested with either *Streptomyces hyalurolyticus* hyaluronic acid (HA) lyase (H) or *Flavobacterium heparinum* chondroitin sulphate (CS) lyase (C). Aliquots of horse synovial fluid from normal (NL), asymptomatic OCD (Asymptom. OCD) and symptomatic OCD (Symptom. OCD) were incubated with either *Streptomyces hyalurolyticus* HA lyase (H) or *F. heparinum* CS lyase (C). After overnight incubation at 37°C, the mixtures were freeze dried, resuspended in water and subjected to agarose gel electrophoresis, as described in Figure 3. Controls (-) were incubated with heat inactivated enzymes. DS = dermatan sulphate; HS = heparan sulphate; OCD = osteochondrosis dissecans; S = mixture of standard GAGs.

To avoid the early stage lesions, only mature horses were investigated in the present study (age 2–13 years). Synovial fluid from both symptomatic and asymptomatic joints was analysed in comparison with healthy controls.

We established a simple and reliable method for the identification and quantification of synovial fluid GAGs. This procedure is based on: 1) migration of GAGs in agarose gel by electrophoresis in an special buffer system (PDA) [16] that allows the complete segregation of CS and HA; 2) differential staining of sulphated and nonsulphated GAGs by toluidine blue in different pHs; and 3) susceptibility to specific GAG lyases (*Flavobacterium heparinum* chondroitinase AC, which degrades both CS and HA, but not heparan sulphate or dermatan sulphate, and *Streptomyces*

TABLE 2: Error of the method for quantification of synovial fluid glycosaminoglycans (GAGs)

Animal	Group	Joint	Quantification*	CS** (mg/l)	HA** (mg/l)
6	Symptomatic OCD	Right	1	271.4	845.1
			2	249.3	698.2
			Mean	**260.4**	**771.6**
			Error (%)	*4*	*9*
14	Symptomatic OCD	Left	1	99.5	1155.4
			2	80.6	1343.2
			Mean	**90.0**	**1249.3**
			Error (%)	*10*	*7*
19	Asymptomatic OCD	Left	1	111.1	534.9
			2	102.8	608.1
			Mean	**106.9**	**571.5**
			Error (%)	*4*	*6*
21	Asymptomatic OCD	Left	1	122.3	966.7
			2	101.7	1132.8
			Mean	**112.0**	**1049.8**
			Error (%)	*9*	*8*
23	Control	Right	1	44.0	931.3
			2	36.6	921.5
			Mean	**40.3**	**926.4**
			Error (%)	*9*	*1*
26	Control	Right	1	35.6	700.6
			2	31.0	656.6
			Mean	**33.3**	**678.6**
			Error (%)	*7*	*3*

*Quantitative results obtained in 2 independent preparations (see Methods) from each sample. ** CS = chondroitin sulphate; HA = hyaluronic acid; OCD = osteochondrosis dissecans.

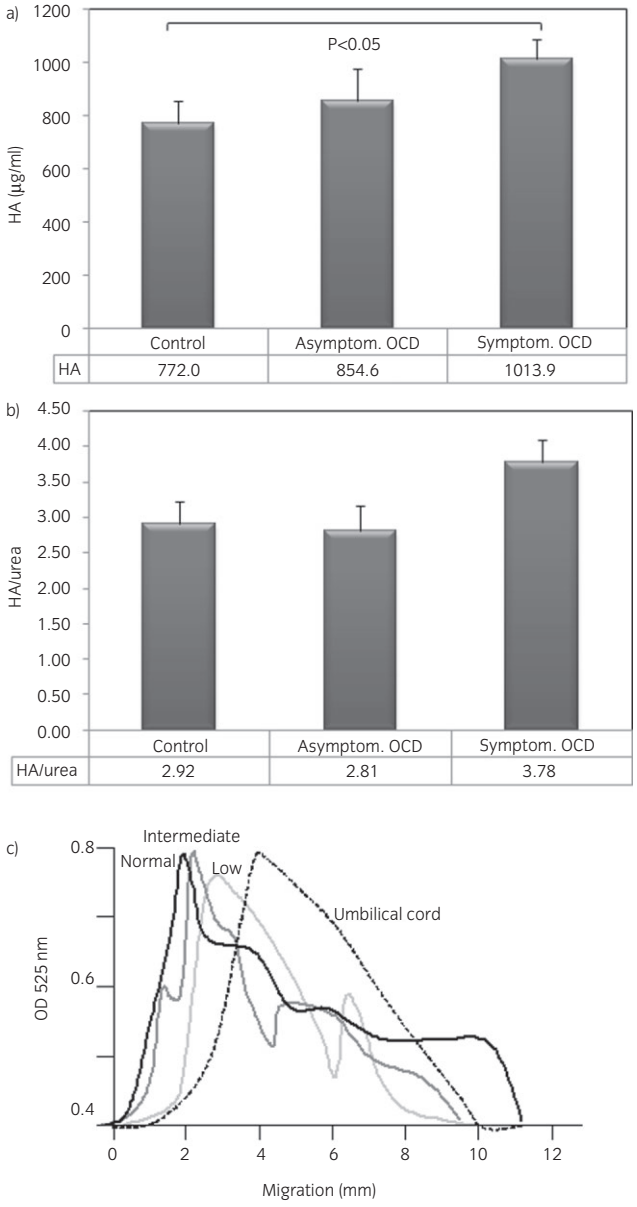

Fig 5: Synovial fluid hyaluronic acid (HA) was identified by agarose gel electrophoresis and enzymatic degradation with specific glycosaminoglycan (GAG) lyases, and quantified by densitometry of the agarose gel slabs. Quantitative data are means and are expressed as (a) HA concentration (mg/l) and as (b) HA/urea ratios (mg/g) to compensate for fluid volume. Error bars indicate standard error. *Differences statistically significant in comparison to control (P<0.05). (c) Densitometric profile of HA migration in 0.4% agarose gel electrophoresis in TAE buffer. Normal, synovial fluid of normal viscosity; 'Intermediate', synovial fluid of intermediate viscosity; 'Low', synovial fluid of low viscosity; 'Umbilical cord', commercial HA from umbilical cord. Brackets indicate statistically significant differences. OCD = osteochondrosis dissecans.

hyalurolyticus HA lyase, which degrades only HA). This procedure also avoided the interference of other macromolecular and ionic components.

The concentration of CS in synovial fluid was considerably increased in all mature OCD joints, symptomatic or not, in comparison with healthy controls. Increase in synovial fluid CS has been previously reported in chronic and acute joint diseases [28], but CS was measured by a DMMB dye binding assay, which is not specific and does not permit the identification of CS in a mixture of GAGs [9]. In the present paper we have shown that DMMB assay is inappropriate to quantify the synovial fluid GAGs because HA interferes with CS quantification. Aggrecan degradation products, detected by the monoclonal antibody 846, have also been shown to be increased in osteochondral fragmentation [29], but CS was not identified since this antibody recognises protein-related epitopes [30]. Thus, the present report seems to be the first clearly to demonstrate an increase in synovial fluid CS in OCD joints of horses aged >2 years. This finding indicates that changes occurred in the synovial fluid composition of OCD joints, even in the asymptomatic ones. The analysis of aggrecan biomarkers, such as alanine-arginine-glycine-serine (ARGS)-aggrecan [31], could give important clues concerning the origin of synovial fluid CS. Increases were also observed in the CS/HA and CS/urea ratios.

It is believed that most of the synovial fluid HA is synthesised by the fibroblast-like synoviocytes (also named type B-synoviocytes). HA, because

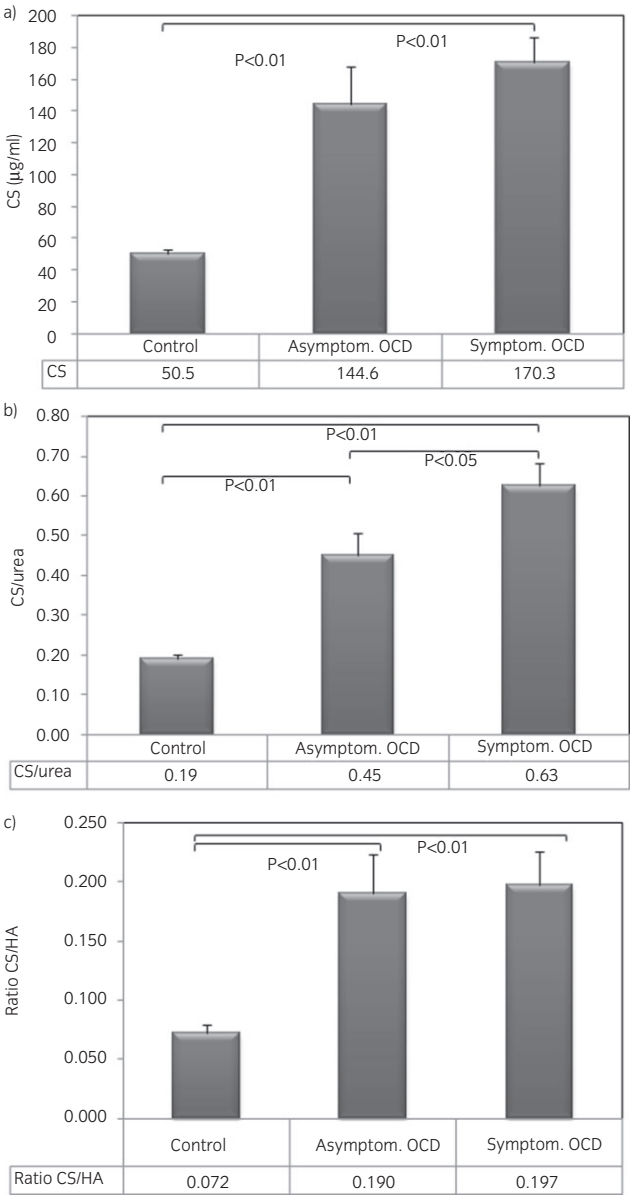

Fig 6: Synovial fluid chondroitin sulphate (CS) was identified by a combination of agarose gel electrophoresis and enzymatic degradation with specific glycosaminoglycan (GAG) lyases, and quantified by densitometry of the gel slabs. Quantitative data are means and are expressed as (a) CS concentration (mg/l), (b) CS/urea ratios (mg/g) to compensate for fluid volume, and (c) CS/HA ratios. Error bars indicate standard error. Brackets indicate statistically significant differences. HA = hyaluronic acid; OCD = osteochondrosis dissecans.

of its high molecular weight, forms viscous solutions, and therefore is largely responsible for the viscosity of the synovial fluid (review in Laurent *et al*. [32]). In the present study, an increased HA concentration and a decreased synovial fluid viscosity were observed in symptomatic OCD. This apparent paradox can be explained by the lower molecular weight of the HA in symptomatic OCD (Fig 5c). This decrease in HA molecular weight could be a consequence of either partial depolymerisation by synovial fluid hyaluronidase, or synthesis of smaller chains.

It is well known that the hyaluronic acid synthases HAS1 and HAS2 synthesise high molecular weight chains, while HAS3 synthesises smaller molecules [33]. It was recently shown that the synthesis of HA is up-regulated in fibroblast-like synoviocytes exposed *in vitro* to CS and interleukine 1-β, due to increased expression of HAS1 and HAS2, but not HAS3 [34]. If the same happens *in vivo*, it is unlikely that smaller HA chains are synthesised in OCD. Furthermore, it has been shown that the expression of hyaluronidase is increased in inflammatory reactions [35]. In the present study, inflammatory reaction was suggested by the increased WBC count in OCD synovial fluid, especially in symptomatic OCD. In fact, the synovial fluid WBC count is the most reliable test to classify an articular process as inflammatory or noninflammatory [36]. This inflammatory reaction could also induce the expression of hyaluronidase, leading to the smaller HA chains and decreased viscosity of synovial fluid.

In conclusion, the quantification of synovial fluid CS seems to be very useful in the evaluation and monitoring of joint conditions, as long as trustworthy methods are available. The procedure described for identification and quantification of synovial fluid GAGs is very simple and reliable, and in conjunction with protein and WBC quantifications gives useful information concerning the joint conditions, which could support therapeutic decisions.

Authors' declaration of interests

No conflicts of interest have been declared.

Source of funding

The research was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasília, DF, Brazil; Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasília, SP, Brazil; Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), São Paulo, SP, Brazil; and Sociedade Paulista para o Desenvolvimento da Medicina (SPDM - FADA), São Paulo, SP, Brazil. These funding sources had no involvement in the collection, analysis or interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

Acknowledgements

The authors want to express their gratitude to CNPq, FAPESP, CAPES and SPDM for funding, and to M.Sc. Aline Mendes and to Dr. Joäo Roberto M. Martins for technical assistance and helpful discussions on many experiments.

Manufacturers' addresses

[a]Sigma-Aldrich, St Louis, Missouri, USA.
[b]Bio-Rad Laboratories, Hercules, California, USA.
[c]Labmax 240, Tokyo Boeki Machinery Ltda., Tokyo, Japan.
[d]Randox, Crumlin, County Antrim, UK.
[e]Shimadzu, Kyoto, Japan.

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