1H NMR investigation of normal and osteoarthritic synovial fluid in the horse

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Summary
Proton magnetic resonance spectroscopy (1H NMR) has been successfully used in the study of many biological fluids. The data presented here report on the metabolic profiles of normal equine synovial fluids compared with osteoarthritis (OA) fluids. Twenty-five OA synovial fluid samples and eight normal ones were collected from the forelimb fetlock joint in 22 horses, aged between five and 24 years. 1H NMR spectroscopy was carried out with a Bruker Avance DRX 500 equipped with a cryomagnet working at 1.1 Tesla, and ‘Mestre-C 4.9.9.6’ software was used to analyze the spectra. The study assessed the increase of lactate, alanine, acetate, N-acetylg glucosamine, pyruvate, citrate, creatine/creatinine, glycerol, HDL choline, and α-glucose in OA synovial fluid. The variations observed in samples from horses with OA compared to those in the control group, and similar data found in other studies, confirm that this technique may be useful in the study of joint metabolism. Its practical application may be in the evaluation of the treatment of OA in athletic horses.

Keywords
Osteoarthritis, synovial fluid, nuclear magnetic resonance spectroscopy, equine

Prepublished online November 2, 2007
doi:10.3415/VCOT-06–12–0101

Introduction
High resolution 1H nuclear magnetic resonance (NMR) has previously been used to study a number of body fluids, such as urine, plasma, synovial and cerebrospinal fluid (1–3), and it has been shown that a large number of low molecular weight components can be observed and quantified simultaneously. The use of 1H NMR for the quantitative evaluation is considered to be accurate and repeatable with a margin of error between ± 5% (4). The major advantages of high field NMR analysis of biological fluids, as compared to previously established techniques, are that it generally does not require any knowledge of sample composition prior to analysis and allows the rapid assessment of the nature and levels of a very wide range of endogenous (and, where appropriate, exogenous) components simultaneously (5).

In humans, 1H NMR spectroscopy allows early differential diagnosis between rheumatoid arthritis and osteoarthritis (OA) (6–9) and it has recently been employed to detect many ions complexed-molecules in human synovial fluid (10–13). In veterinary science, most studies have been carried out on dogs (14–17).

This study reports data on the metabolic profiles of normal synovial fluids compared with OA fluids in horses.

Materials and method
Case selection
Twenty-two horses, not in training, aged between five and 24 years, were selected for the study. Inclusion criteria in the OA group were: lameness of at least 1/5 degree in forelimbs and positive to intrarticular block of MCP joint, and at least one radiological sign of pathology, such as osteophytes, subchondral sclerosis, radiolucent areas in the subchondral bone, narrowing of the intrarticular space. On the contrary, MCP joints, that did not show any pathological clinical and radiological signs, have been included in Normal (control) group. In particular, we selected 11 horses with bilateral lesions and three unilateral. The other eight horses were considered not to be affected by OA and were used for normal synovial fluid (control) analysis. The synovial fluid samples were collected from a total of 25 OA and eight normal joints.

Once the horses had been assigned to the respective groups, and before the beginning of the study, they were all_stabled in box for three weeks, in the same farm under the same controlled diet, but with free access to water.

Synovial fluid sample preparation
Four millilitre samples of synovial fluid were collected from each selected fetlock joint from lateral pouch. The samples were filtered using membranes of 0.8 μm porosity (Albet-Jacs, Spain) and then centrifuged at approximately at 17000 g (14000 rpm) for 15 minutes in order to separate the cells. Samples were then stored at –80°C.

Before undergoing the 1H NMR spectroscopy study, 420 μl of each synovial fluid sample were mixed with 280 μl of a 3-(trimethylsilyl) 2,2,3,3-tetra deuter propionic acid sodium salt (TSP) solution in deuterium oxide (0.1 mgTSP/ml D2O) and then poured into 5 mm diameter 1H NMR tubes.
TSP was used as a standard reference, both in the measurement of the chemical shift scale (d=0), and in the quantitative analysis of metabolites in synovial fluid (TSP=2.31x10^{-4} M in each sample).

**Proton NMR spectroscopy**

\(^1\)H NMR Spectroscopy was carried out using a Bruker Avance DRX 500 equipped with a cryomagnet working at 11.7 Tesla, and 'Mestre-C 4.9.9.6' software was used to analyze the spectra. Spectra were recorded in proton NMR spectroscopy lock mode, by using the solvent deuterium resonance frequency as internal lock. Two hundred fifty-six scans were recorded on each sample using the standard presaturation sequence for the water signal suppression. For each scan, 4.0 μsec excitation pulse equivalent to a flip angle of 45°, 2.3 sec acquisition time, and a further 2 sec of relaxation delay were used. Such conditions allowed a quantitative analysis of the resulting spectra (4). The spectral zone studied in all spectra was between 0 and 6 ppm.

The peaks were attributed on the basis of the previously reported chemical shift analysis of metabolites in biological fluids. The qualitative and quantitative analysis of spectra was limited to those components which were most easily detectable because of their high concentration, or due to their resonance occurring in areas of the spectrum free of other signals.

**Statistical analysis**

The results were analyzed using ANOVA test with a confidence level of \( p<0.05 \).

**Results**

Selected chemical shifts of the metabolites signals used as inflammatory markers were as listed below: lactate, whose CH\(_3\) and CH signals were found at 1.33 and 4.12 ppm, respectively, alanine at 1.45 ppm, acetate at 1.60 ppm, N-acetylg glucosamine at 2.04 ppm, pyruvate at 2.39 ppm, citrate at 2.62 ppm, creatine/creatinine at 3.05 ppm, glycerol at 3.25 ppm, HDL choline at 3.65 and α-glucose at 5.23 ppm. The peak integral value of lactate recorded at 4.12 ppm (CH) was used in the quantitative analyses.

All data relating to normal and diseased joints are shown in Table 1 (values are expressed in M/L x 10^{-4}).

Fig. 1 shows a comparison of metabolite profiles between normal and affected joints. Comparing the values obtained from normal and OA joints, we were able to show that the range of metabolites in synovial fluids of subjects suffering from OA had increased dramatically, with statistically significant variations. Fig. 2 shows the spectra of samples obtained from an OA joint and from a normal one.

**Discussion**

Equine synovial fluid can be successfully analyzed with \(^1\)H NMR spectroscopy and characteristic spectra of normal equine synovial fluid can be described. \(^1\)H NMR analy-
sis provided signals arising from the metabolites acetate, alanine, N-acetylglucosamine, choline, creatinine, lactate and glucose.

This study also compared normal and OA synovial fluids. Basically, OA synovial fluids showed higher values ($p<0.05$) of all metabolites than in normal fluids.

Increased lactate levels are an initial indication of inflammatory disorder in the synovial fluid, both in acute and chronic joint disease (7, 8, 14–16, 18, 19). This metabolite is produced in greater quantities when joint metabolism is anaerobic as a result of inflammation, causing a decrease in pH. This metabolite increases proportionally to the glucose level, due to the utilization of carbohydrate during metabolic activity. Conditions of hypoxia, hypercapnia, and acidity, associated with increased anaerobic metabolism and lactic acid production, have been detected in both the synovial fluid and the subchondral bone venous outflow (20). Because the synovial fluid is the primary supplier of articular cartilage oxygen, glucose and other small nutrients and the remover of metabolic waste products, effusion creates a local ischaemia in the synovial membrane and an ischaemic-like condition in the articular cartilage (20). It has been reported that in the presence of an effusion an increase of intra-articular pressure of as little as 20 mmHg decreased synovial blood flow significantly exacerbating hypoxia probably contributes to an accumulation of lactate and other metabolites, due to diminished joint clearance (21).

Levels of substances with high molecular weights, such as unsaturated fatty acids, high density lipoprotein (HDL) and choline (lipoprotein), are higher in diseased joints. This could be the result of increased synovial permeability due to inflammation (8) and could also be the result of increased lipid detrithus from cartilage matrix breakdown (15).

An increased glycerol concentration suggests that fat metabolism plays an important role as an energy source in OA joints and is linked to the enhanced use or metabolic regulation of fat in OA fluids (15). The oxidation of unsaturated fatty acids produces acetylcoenzyme A, which inhibits pyruvate dehydrogenase. Glycerol production can be correlated with triglyceride hydrolysis, particularly if glucose is lacking. In diseased joints there is a marked increase in lipoprotein metabolism because of hypoxia (15, 16, 22).

Acetate is one of the bioproducts of cartilage and synovial fluid degradation (17). It has been described as the final product of polymeric carbohydrates breakdown contained in cartilage and synovial fluid; in particular after cleaving of N-acetylglucosamine (9). Studies have shown that enzymes cause synovial fluid modification (9). In particular, macrophages and PMNs, attracted into the joint by inflammatory cytokines, generate oxygen-derived free radicals (superoxide anion, hydroxyl radicals, hydrogen peroxide) and enzymes (xanthine oxidases and myeloperoxidase, MPO). In particular, MPO that regulate the production of hypochlorous acid (HOCl), and other reactive oxygen free radicals cleave the hyaluronic acid (HA) molecule producing oligomers (23). Acetate increased level, when correlated to an increase in the N-acetyl group, is the quantifiable molecular demonstration of poor quality HA in OA joints. Synovial HA is fragmented and depolymerized, with a corresponding reduction in synovial fluid viscosity and an increase in the synovial concentration of diffusible HA saccharide species, such as the N-acetyl group (6). Although this group is present in many molecules, (i.e. glycoproteins containing N-acetylmuraminic acid, that it is not NMR detectable) (5), in synovial fluid the only detectable N-acetyl group is the one that is contained in the cleaved N-acetylglucosamine polysaccharide (mainly represented in HA). Many studies have shown that when HA is experimentally cleaved, it produces an increase of the NMR signal of N-acetyl group ($\delta=2.04$ ppm) (24). Increases in the synovial fluid spectral resonance intensity of this region are due to the fragmentation of large polymer units into smaller ones (15). Many studies have shown that in OA, a loss of rheological quality in HA is due to the modified molecular structure of that sub-

![Fig. 2](image-url)
stance (5, 9, 25). Moreover, the levels of acetate and N-acetyl groups of mobile carbohydrate side chains of glycoproteins were also significantly elevated which suggests a further degradation of the polymeric components of synovial fluid with increasing OA severity (9, 26, 27).

Creatinine is the excreted by-product of creatine metabolism which occurs during muscle breakdown. Whether the observed increases in synovial fluid concentrations of this substance are the result of increased synovial membrane permeability consequent to inflammation, or they reflect the breakdown of the muscles proximal to arthritic joints, or they are the result of intra-articular metabolic activity, requires further investigation (15).

Finally, alanine was found to be significantly elevated in OA synovial fluid with respect to normal fluid. The significance of this is not clear although it may be related to the formation of free radicals, previously identified in rheumatoid synovial fluid and known to be active in glycoprotein degradation (15). Other authors have come to the hypothesis that after the evaluation of alanine levels in serum and synovial fluid, a possible explanation is its diffusion through the synovial membrane (5). Its exact role in the OA process has so far not been determined.

According to previous studies, glucose levels increase with the severity of osteoarthritis, in both human and canine synovial fluids (14, 17, 18). Furthermore, in the dog, this metabolite decreases after viscosupplementation with intra-articular administration of exogenous HA (17) and to normal group, and the similarity of data found in other studies in other species, suggests that this technique can be useful in the study of joint metabolism and that it may be possible to assess the therapeutic activity of anti-inflammatory drugs at the molecular level.

References