Alterations in the Viscoelastic Properties of Equine Synovial Fluid from Fetlock Joints with Naturally Occurring Osteoarthritis

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Abstract

Objective: The viscoelastic properties of synovial fluid are crucial to joint performance. The objective of the present study was to evaluate qualitatively and quantitatively the viscoelastic properties of equine synovial fluid from normal joints and joints with osteoarthritis and to detect any possible differences.

Methodology: Synovial fluid was aspirated from 16 joints with osteoarthritis in a fetlock joint, obtained from 12 mature English Thoroughbred horses. Additionally, synovial fluid samples obtained from 6 normal joints were used as controls. Full rheological characterization was performed in order to measure the elastic G' and viscous G'' moduli. Hyaluronic acid (HA) concentration was determined using a commercially available ELISA kit.

Results: Viscoelastic properties of osteoarthritic joints were significantly lower compared to the ones obtained from normal joints. Joints with osteoarthritis presented lower HA concentration compared to normal joints (p < 0.001). In addition, a negative correlation between viscoelastic properties and osteoarthritis (lameness score and radiographic score) (p < 0.001) was detected.

Conclusion: Osteoarthritic joints present significantly lower viscoelastic properties and lower HA concentrations compared to normal ones. Despite considerable research, the complex role of synovial fluid as a lubricant is not fully understood. Confronting the need of developing new methods to control osteoarthritis, the horse provides an excellent animal model for tentative biomedical extrapolations.

Keywords: osteoarthritis; horse; rheology; hyaluronic acid

1. Introduction

Osteoarthritis (OA), the most common cause of wastage in equine industry, is a degenerative joint disease, characterized by the breakdown of articular cartilage, causing pain, swelling, and limited mobility of the joint [1]. In healthy subjects, articular cartilage and a slimy film of Synovial Fluid (SF) are closely linked to provide lubrication and further reduce friction between the bones of the joint [2]. In osteoarthritic joints, damage to articular cartilage may result in changes in the rheological properties of the SF, which becomes less viscous and,
therefore, less effective in joint lubrication [3, 4]. The goals of OA treatment are to minimize pain and maintain joint mobility. One of the common treatments for OA is the intra-articular injection of Hyaluronic Acid (HA) [5]. Endogenous HA is a natural polysaccharide, existing in the animal body [6, 7] and especially in joints, where it plays an important role in lubrication, shock absorption, and viscoelastic behaviour of SF [8, 9]. This viscoelastic behaviour involves entanglements in HA and/or protein-HA bonds, based mainly on electrostatic interactions [10].

Viscoelastic properties are related to the function of shock absorption during movement. The elastic modulus (G') is a measure of the energy stored in the elastic structure of the material. The viscous modulus (G'') is a measure of the amount of energy dissipated in the material [11]. Rheology, which includes intrinsic viscosity and dynamic viscoelastic measurements [i.e., the measurement of the elastic modulus (G') and the viscous modulus (G'')], is the most suitable approach to evaluate the viscoelastic properties of SF [12, 13]. The rheological behaviour of linear HA solutions is strictly related to the product of molar mass and HA concentration, which also controls HA's biological effects [9, 14, 15]. However, HA is an anionic polyelectrolyte able to interact with positive charges on proteins.

Presumably, the viscoelasticity of SF may be related to the formation of a complex of endogenous HA and proteins forming a physical three-dimensional network [16, 17]. Hence, a thorough elucidation of the rheological properties of SF is necessary to better understand its role in joint lubrication. Surveys estimate that equine OA is the most significant causing factor for loss of performance, making the horse the ideal animal model; it may provide extensive experience with clinical cases and serve as a consistently predictable model of OA [18, 19].

Treatment modalities in equine osteoarthritis are extrapolated from human data where viscosupplementation is based on the fact that human OA leads to decreased viscoelastic properties of the SF. Still, to the authors' knowledge, that fact has not been objectively proven for horses. The objectives of the present study were to i) detect the possible differences in the viscoelastic properties (G' and G'' values) between normal horses and horses with naturally occurring OA in a metacarpophalangeal (fetlock) joint and to ii) examine the possible role of HA concentration in horses with OA.

2. Materials and Methods

2.1 Animals and Samples

Synovial fluid was aspirated from a total of 16 joints with mild osteoarthritis obtained from mature English TB horses (n=12). Horses included in this study, presented with signs of mild to moderate lameness (grade 1–2 on the AAEP lameness scale). Additionally, 6 control samples were obtained from 6 normal TB horses. The diagnosis of OA was based on a comprehensive orthopaedic lameness examination and a positive response to intra-articular anaesthesia of the affected joint. For radiographic assessment of the joint a minimum of four views (lateromedial, dorso-palmar and two oblique views) were used. Radiographs were scored blindly by consensus opinion of two experienced radiologists (one board-certified, ECVDI). Scores were allocated to all radiographic findings recorded, using a severity grading system from I to V, based on radiographic lesions, adapted from other previously described systems [20–22]. All findings were categorized by the location and type of change and then graded depending on their severity. Radiographic osteoarthritis was defined as grade I, where anatomical variation was detected (e.g. subchondral bone sclerosis, joint space narrowing) with no soft tissue effusion. Osteoarthritis was defined as Grade II, where minimal radiographic change was detected, with 1–3 osteophytes at the articular margins on the medial or lateral aspects of the proximal phalanx or enthesophyte formation at the proximal dorsal attachments of the joint capsule close to the proximal end of the sagittal ridge. Mild osteophyte formation (> 3 small osteophytes) at the articular margins on the medial or lateral aspects of the proximal phalanx and evident soft tissue swelling was attributed to Grade III osteoarthritis. Moreover, osteoarthritis was defined as Grade IV where moderate to severe radiographic changes (large osteophytes at the articular margins on the medial or lateral aspects of the proximal phalanx and evident soft tissue swelling) was attributed to Grade III osteoarthritis. Moreover, osteoarthritis was defined as Grade IV where moderate to severe radiographic changes (large osteophytes at the articular margins on the medial or lateral aspects of the proximal phalanx and periarticular osteophytes on the proximal and distal margins of the proximal sesamoid bones). Finally, severe radiographic findings (large osteophytes, supracondylar lysis in the distal palmar aspect of the third metacarpal...
bone proximal to the condyles, sesamoiditis or presence of “chip” fractures) were categorized as Grade V. Only horses with grade I and grade II OA were included in the study, in order to avoid excessive variability of data. Radiographs were assessed for presence and size of osteophytes, narrowing of the joint space, sclerosis or lysis of the bone underlying the joint cartilage. The study protocol used in this project complied with the guidelines for the use of animals in research. Horses were included if the owner provided written consent and was deemed to be willing and capable of complying with the requirements of the study. All horses included in this study were residents at the same premises (Markopoulo racetrack).

2.2 Sampling

SF was directly aspirated from the joints by use of a 21-gauge needle in a routine sterile manner, as previously reported [23]. Samples were immediately placed in tubes containing EDTA for routine SF analysis (cytological evaluation, total protein concentration). None of the affected joints had undergone intra-articular analgesia or treatment in the month prior to SF aspiration.

2.3 Synovial Fluid Analysis

2.3.1 Cytological Examination, Cell Counting and Total Protein Measurement

Cytospin SF preparations were made for microscopical evaluation, using the cytocentrifuge function of an automated slide stainer (Aerospray® Hematology Pro Slide Stainer, Wescor Biomedical Systems, Logan, UT, USA). All prepared slides were stained with Giemsa and were subsequently examined by two experienced clinical pathologists, for the classification of nucleated cells, evaluation of their morphology and investigation of the possible presence of microorganisms.

The TNC of each sample was determined using an automated analyser (Scil Vet ABC™, Animal Blood Counter, Scil Animal Care Company, Holtzheim, France). Refractometry (Model T2-NE-Clinical, Atago Ltd, Tokyo, Japan) was employed to determine total solids as an estimate of TP.

2.3.2 Determination of HA Concentration

Quantification of HA in SF samples was performed using a commercially available ELISA kit (TECO® Hyaluronic Acid PLUS, TECOmedical AG Sissach, Switzerland). The assay is based on HA binding protein, enabling its application in multiple animal species. After dilution of SF samples at 1: 1,000 in sample diluent, further processing was carried out according to the manufacturer’s instructions. Absorbance values were recorded at 450 nm, with reference wavelength at 630 nm, using a microplate photometer (Stat Fax® 3200, Awareness Technology Inc., Palm City, FL, USA). Analysis of the absorbance values of samples and calibrators was performed using the GraphPad Prism software (version 7.03, GraphPad Software Inc., La Jolla, CA, USA). SF concentrations were expressed in μg/ml. SF samples with HA concentration exceeding the dynamic range of quantification of the method were re-analysed at a dilution 1: 2,000.

2.3.3 Rheological Analysis

The rheological behaviour for the majority of SF samples was evaluated within 6 hours after aspiration. For cases where the analysis could not be performed within the aforementioned timeframe, aspirated synovial fluid was kept in a refrigerator at approximately 4°C for testing within 24 hours after collection.

The viscoelastic properties of the samples were determined via dynamic experiments, in order to measure the elastic G’ and viscous G” moduli respectively. The investigation of the rheological behaviour was carried out on an AR-G2 controlled stress rheometer (Rheometer, TA instruments, UK) via dynamic oscillatory experiments. The properties of the samples were examined at the physiological horse’s temperature, ie at 37.5°C, with an accuracy of 0.1°C provided by the instrument’s peltier plate temperature control system. The rheometer’s accurate operation was verified using a Cannon certified viscosity reference standard oil S60 (Cannon Instrument Company, State College, PA, USA). The average measured values of the standard oils were found to be on average 4.5 % lower than their expected values.

2.4 Statistical Analysis

Descriptive statistics analysis was carried out and results were expressed as Mean (M), standard deviation (sd), Median (Mdn), minimum (min) and maximum (max) values, whereas for the categorical variables of the study the number of horses (n) and the number of joints (N) were presented. Moreover, the Kolmogorov-Smirnov test
(K-S test) for normality was used to examine, whether the continuous variables followed the normal distribution.

In addition, due to the ordinal nature of the radiographic and lameness severity of OA, the non-parametric Spearman’s coefficient was used to evaluate the correlation between the pairs for the set of the examined parameters. The non-parametric Mann-Whitney test was conducted for variables that did not satisfy the normality assumption. Statistical analyses were performed using the R environment (R Core Team, 2013, Foundation for Statistical Computing, Vienna, Austria). In all tests a difference was considered as statistically significant when $p$-value (significance) was less than 0.05. All the tests conducted were two-tailed (non-directional) in the sense that the alternative hypothesis is that the measures tested are not equal. For the case of the Mann-Whitney test, we utilized the formula proposed by Rosenthal, $r = z/\sqrt{N}$ ($z$ is the $z$-score provided by the test procedure and $N$ is the number of the total observations).

3. Results

The number of horses ($n$), joints ($N$), radiographic scores, lameness scores and the frequency distributions were presented in Table 1.

<table>
<thead>
<tr>
<th>Joint status</th>
<th>$n$ (horses)</th>
<th>$N$ (joints)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lameness score</td>
<td>Normal</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Radiographic score</td>
<td>Normal</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

Sixteen SF samples from osteoarthritic joints were obtained from 12 TB horses, aged from 4 to 8 years (Mdn = 7.5 years). Additionally, 6 control samples of healthy joints were obtained from 6 normal TB horses aged from 5 to 13 years (Mdn = 7 years).

Based on radiographic score, from a total of 22 joints, 6 were normal, 8 were categorized as Type I, and 8 were categorized as Type II OA (Table 1).

3.1 Biochemical and Cytological Analysis

The descriptive statistics for TP and TNC levels of SF samples obtained from both normal and OA groups were summarized in Table 2. HA concentrations obtained from normal joints and joints with OA were $M = 864.40$, $SD = 260.42 \mu g/ml$ and $M = 402.67$, $SD = 131.30 \mu g/ml$, respectively.

In order to examine whether there was a statistically significant difference between normal joints and OA joints the non-parametric Mann-Whitney test was used. Regarding the HA concentrations, the results of the test revealed a statistically significant difference between normal joints and joints with OA ($p=0.001$) (Table 3).

Finally, the evaluation of the non-parametric Spearman’s correlation coefficient revealed statistically significant strong and negative correlations between HA and lameness score measurements, $r(14) = -0.779$, $p < 0.001$ and between HA and radiographic score measurements, $r(14) = -0.784$, $p < 0.001$.

3.2 Rheological Analysis

The strain frequency at which the $G'$ and $G''$ intersect is referred to as the crossover point. Typical frequency sweeps and cross over points from normal and OA SF detected in our study can be seen in Figure 1.

3.2.1 $G'$ parameter

Due to the fact that the $G'$ parameter presented a highly skewed distribution, the normality assumption was not satisfied. For this reason, the non-parametric hypothesis Mann-Whitney test was utilized in order to derive meaningful conclusions. The results of the test revealed a statistically significant difference between normal joints and joints with OA ($p=0.003$) (Table 3).

Finally, the non-parametric correlation coefficients revealed statistically significant negative but moderate correlations between $G'$ and lameness score measurements, $r(22) = -0.567$, $p < 0.001$ and between $G'$ and radiographic score measurements, $r(22) = -0.572$, $p < 0.001$. 


Table 2: Descriptive statistics for TP, TNC and HA concentrations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Joint status</th>
<th>Number of Joints (N)</th>
<th>Mean (M)</th>
<th>Standard Deviation SD</th>
<th>Kolmogorov-Smirnov test (K-S test)</th>
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<tbody>
<tr>
<td>TP (g/dl)</td>
<td>Normal</td>
<td>6</td>
<td>1.37</td>
<td>0.15</td>
<td>1.128 (p = 0.157)</td>
</tr>
<tr>
<td></td>
<td>OA</td>
<td>16</td>
<td>1.74</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>TNC</td>
<td>Normal</td>
<td>6</td>
<td>130.00</td>
<td>54.41</td>
<td>0.648 (p = 0.795)</td>
</tr>
<tr>
<td></td>
<td>OA</td>
<td>16</td>
<td>243.13</td>
<td>41.10</td>
<td></td>
</tr>
<tr>
<td>HA (μg/ml)</td>
<td>Normal</td>
<td>5</td>
<td>864.40</td>
<td>260.42</td>
<td>0.690 (p = 0.728)</td>
</tr>
<tr>
<td></td>
<td>OA</td>
<td>9</td>
<td>402.67</td>
<td>131.30</td>
<td></td>
</tr>
</tbody>
</table>

(The K-S test for normality was used to examine whether the continuous variables followed the normal distribution)

Table 3: Comparison between normal and OA groups regarding the G', G'' values and HA concentration. A difference was detected between normal and OA joints for all the variables tested.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Joint status</th>
<th>Number of Joints (N)</th>
<th>Mann-Whitney U</th>
<th>Z</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>G'</td>
<td>Normal</td>
<td>6</td>
<td>10</td>
<td>-2.802</td>
<td>0.003*</td>
</tr>
<tr>
<td></td>
<td>OA</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G''</td>
<td>Normal</td>
<td>6</td>
<td>2.5</td>
<td>-3.356</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>OA</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>Normal</td>
<td>5</td>
<td>0</td>
<td>-3.000</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>OA</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(U = Mann-Whitney statistical tests; Z= score Z; *marked effects significant at p < 0.05; **marked effects significant at p < 0.01)

Figure 1: Frequency Sweep at 37.5°C of synovial fluid obtained from normal and osteoarthritic fetlock joint. The dotted lines indicate a typical cross-over point.
3.2.2 *G"* parameter

Regarding the *G"* parameter, the steps were similar to the analysis process followed for the case of the *G’* parameter. Again, the Mann-Whitney test revealed a statistically significant difference between normal joints and joints with OA (*p* < 0.001) (Table 3).

Finally, the correlation analysis revealed statistically significant negative and moderate correlations between *G"* values and lameness score measurements, *r*(22) = -0.674, *p* < 0.001 and between *G"* values and radiographic score measurements, *r*(22) = -0.684, *p* < 0.001.

4. Discussion

The objective of the present study was to evaluate qualitatively and quantitatively the viscoelastic properties of normal and pathological equine SF in TB horses with mild osteoarthritis. In this study, a conventional analysis of SF, including assessment of TP concentration and cytological evaluation, was used for descriptive purposes and in order to exclude other inflammatory articular diseases (ie, synovitis). Moreover, a determination of HA concentration, using a commercially available ELISA kit was performed. The HA concentrations in SFs obtained from diseased joints in our study were consistent with the results from other reports [23]. In addition, osteoarthritic joints presented statistically significant lower HA concentration compared to normal joints. Moreover, a negative correlation between HA and lameness score measurements, and between HA and radiographic score measurements was detected, indicating that HA plays a major role in SF composition in equine OA. The altered HA concentrations observed in previous studies [24, 25] indicate local changes afflicting a specific joint, rather than a systemic change.

HA plays an essential role in SF properties at the cartilage-cartilage interface. Although at high shear rates, the properties of commercially available HA solutions and SF are very similar, leading to the conclusion that the mechanical properties of SF are controlled exclusively by HA, one cannot presume this effect occurs in diseased joints *in vivo*. Presumably, lubrication is a result of synergistic action between HA and other molecules [17].

The viscoelastic properties of SF have been of considerable scientific interest, mainly due to its important role in joint lubrication. The viscoelasticity of normal SF was first investigated by Ogston and colleagues [26] reporting that human SF possesses elastic properties, hypothesis later supported by other studies [27, 28]. During human walking, the strain frequency on SF in the knees is in the range of 0.1–0.5 Hz, and during running and jumping, strain frequency increases. In normal human adult knees, the SF HA begins to change from a predominantly viscous solution to a predominantly elastic solution at a strain frequency of around 0.5 Hz [29]. Previous studies have suggested that the cross-over point in pathological SF may occur at higher strain frequencies than in normal joint fluid, or in some individuals the curves do not cross at all [30]. In this report, equine normal SF presented a cross-over point close to 4 Hz, while pathological SF presented lower values compared to the normal one and a cross-over point close to 9 Hz (Figure 1). While the behaviour of equine SF seems to be similar compared to human SF reported from other studies [31], the cross-over points in our report seemed to differ significantly. Moreover, based on this graphical representation, one would be inclined to suggest that the viscous character of both normal and OA joint SF (related to *G") prevailed over the elastic one (related to *G’*) at lower angular frequencies; the opposite response could be observed at higher frequencies. This could be attributed to the fact that at low frequencies (ie walk) equine SF maintains its viscous character, while at higher frequencies (trot and gallop) tends to maintain its elastic properties, potentially protecting the articular cartilage from unmitigated forces.

Moreover, equine SF in this report, presented very low viscoelastic properties both in normal and OA joints, compared to human SF [30], indicating a very thin nature [32] and its complex role in joint pathophysiology. These findings suggest that rheological properties of equine SF could serve as a useful tool in early detection of diminished viscoelasticity in normal horses, probably indicating susceptibility to joint injuries and OA.

Many studies have been carried out investigating alterations in viscoelastic properties in normal and osteoarthritic human SF [28, 33]. However, there is limited evidence investigating the possible variations in the viscoelastic properties of SF between equine normal joints and joints with naturally occurring OA, while most
of these studies focus on the *in vitro* rheological behaviour of SF after viscosupplementation [34]. In this study, an effort was made to detect the differences between normal horses and horses with naturally occurring OA with respect to the mean levels of $G'$ and $G''$ values. The results revealed a statistically significant difference between normal joints and joints with OA. The fact that the horses included in the study had mild OA (grade I and grade II) based on radiographic scores, underlines the importance of quantitative evaluation of viscoelastic properties of SF as a possible future grading system in line with radiographic abnormalities. In our report a statistically significant negative correlation between $G'/G''$ measurements and radiographic score/lameness score in horse’s body temperature was also detected. Hence, it is concluded that, in OA, SF tends to lose both its elastic and viscous properties. Despite the fact this was only a pilot study, performed on a limited number of horses, the results presented herein indicate a close relationship between a decrease in viscoelastic properties of SF and equine OA.

The equine fetlock joint was chosen in this report, as it has the largest number of degenerative lesions of all joints of the appendicular skeleton [35]. The high susceptibility to injury of the fetlock joint has been related to the relatively small surface area, large range of motion and impact of full bodyweight during the stance phase [36]. It is believed now that most equine joint lesions are probably induced by acute trauma, repetitive load or overload [37]. However, the ability of primary synovitis (without any instability or traumatic injury) to produce early degradation of articular cartilage in the horse was first demonstrated by McIlwraith and van Sickle [38]. Hence, an early detection of the diminished viscoelastic properties in horses with mild OA could be used as a prognostic indicator for future athletic performance. Moreover, direct comparison of the viscoelastic properties of pathological SF to normal SF is important for understanding the molecular basis for this altered lubricating function.

### 5. Conclusion

The objective of this study was to detect the possible differences in the viscoelastic properties of SF between normal horses and horses with OA. To the authors’ knowledge this is the first report to quantitatively evaluate and compare the viscoelastic properties ($G'$ and $G''$ values) of SF obtained from normal horses and horses with naturally occurring OA. Based on the results of our report, the rheological behaviour of equine SF in OA joints alters significantly. However, from a clinical point of view, translating basic knowledge from bench to bedside is still challenging. In order to maximize the potential of any therapeutic intervention in OA, it is essential to further investigate the role of SF viscoelastic properties, conducting large-scale research studies with different grades of OA.

### 6. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AAEP</td>
<td>American Association of Equine Practitioners</td>
</tr>
<tr>
<td>ECVDI</td>
<td>European College of Veterinary Diagnostic Imaging</td>
</tr>
<tr>
<td>g/dl</td>
<td>grams per decilitre</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>K-S</td>
<td>Kolmogorov-Smirnov</td>
</tr>
<tr>
<td>M</td>
<td>mean</td>
</tr>
<tr>
<td>max</td>
<td>maximum</td>
</tr>
<tr>
<td>Mdn</td>
<td>median</td>
</tr>
<tr>
<td>μg/ml</td>
<td>microgram/milliliter</td>
</tr>
<tr>
<td>min</td>
<td>minimum</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius degrees</td>
</tr>
<tr>
<td>SF</td>
<td>synovial fluid</td>
</tr>
<tr>
<td>TB</td>
<td>Thoroughbred</td>
</tr>
<tr>
<td>TNC</td>
<td>total nucleated cell count</td>
</tr>
<tr>
<td>TP</td>
<td>total protein</td>
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</table>

### References


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