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### Βιβλιογραφική αναφορά:

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## Analysis of factors influencing the qualitative characteristics of equine synovial fluid

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**ABSTRACT:** The objective of the present study was to investigate the viscoelastic properties in synovial fluid between normal horses and horses with naturally occurring OA and to detect factors affecting synovial fluid viscosity. In total, 105 horses were included in this study. Synovial fluid samples were obtained from 60 mature horses with mild to moderate osteoarthritis in the 2nd interphalangeal, the metacarpophalangeal or the intercarpal joint. Forty-five horses were used as controls. Full rheological sample characterization was performed in order to measure the elastic G' and viscous G'' moduli. For determining hyaluronic acid concentrations a commercially available ELISA kit was used. The results of the linear mixed effect (LME) model revealed statistically significant ( $p < 0.001$ ) effect of HA concentration, on the mean values of  $\log G'$  and  $\log G''$  measurements. The ANOVA findings of the final model revealed statistically significant effect of joint type ( $p < 0.001$ ) on the mean values of viscoelastic measurements. Interpreting

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the coefficients of the covariates osteoarthritis ( $p < 0.001$ ) and age ( $p = 0.013$ ), a negative correlation was detected on the response  $\log G'$  and  $\log G''$  measurements. Geldings seemed to present lower viscous properties compared to mares. To the authors' knowledge this is the first multivariate study to quantitatively evaluate the several factors that affect the viscoelastic properties of equine synovial fluid. Horses with osteoarthritis seemed to present lower viscoelastic properties compared to the healthy subjects that are joint type dependant. Finally, considering the multifactorial nature of osteoarthritis, one should expect an emerging need of personalized disease-modifying treatments.

**Keywords:** horse, osteoarthritis, synovial fluid, rheological properties

## INTRODUCTION

Osteoarthritis (OA) is one of the most common challenges in equine orthopedics causing career-limiting or career-ending lameness (Ferris et al., 2009; Ross and Dyson, 2010). It is a chronic, progressive disease, characterized by cartilage degeneration, subchondral bone sclerosis and osteophyte formation (Trotter and McIlwraith 1996). Synovial fluid (SF) constituents, like hyaluronic acid (HA), contribute both at physiologic and pathophysiologic status, to the boundary lubrication of apposing articular cartilage surfaces (Schmidt et al., 2007).

In healthy individuals, articular cartilage and an easily sheared film of SF provide a protective barrier between joint surfaces in relative motion (Neu et al., 2008). There is evidence that the rheological properties of SF alter in OA, exhibiting decreased viscoelasticity in both human and canine SF (Goudoulas et al., 2010; Madkhali et al., 2018), eventually affecting joint homeostasis. A thorough elucidation of the viscoelastic properties of equine SF is necessary in order to better understand its role in joint lubrication.

Rheology, including the elastic modulus or  $G'$  (reflecting the energy stored in the elastic structure of the material) and the viscous modulus or  $G''$  (representing the amount of energy dissipated in the material) is a convenient method to evaluate the viscoelastic behavior of SF (Morrison, 2001). Ogston and Stanier (1953) first described normal viscosity in ox SF. The results indicated that hyaluronan molecules may primarily contribute to SF viscosity, and hence to the boundary lubrication of articular cartilage. The rheologic behavior of commercially available HA solutions is strictly related to the molar mass and HA concentration (Vitanzo and Sennett, 2006). In early studies, although an attempt was made to separate HA molecules from SF, obtaining samples of HA free of proteins proved impossible (Bingöl et al., 2010).

The objectives of the present study are i) to inves-

tigate the viscoelastic differences of SF between normal horses and horses with naturally occurring OA, and ii) to detect factors affecting SF viscosity between different breeds and joint types.

## MATERIAL AND METHODS

### Animals

A total of 105 horses were included. Synovial fluid samples were obtained from 60 mature Warmblood and English Thoroughbred horses, aged from 5 to 18 years (median 8 years). Horses had mild to moderate lameness (grade 1-3 on the AAEP lameness scale) attributable to OA in the 2<sup>nd</sup> interphalangeal (coffin), the metacarpophalangeal (fetlock) joint or the intercarpal (carpus) joint. Moreover, 45 healthy Warmblood and English Thoroughbred horses aged from 4 to 17 years (median 8 years) were used as controls. Diagnosis of OA was based on a comprehensive lameness examination and a positive response to intra-articular anaesthesia. For radiographic assessment standard views specific for each joint type were used. Radiographs were scored blindly by two experienced radiologists (board-certified, ECVDI). Radiographs were assessed for swelling, presence and size of osteophytes, narrowing of the joint space, sclerosis or lysis of the bone underlying the joint cartilage. Lameness was required to be present for at least 3 months prior to enrolment in order to exclude horses with synovitis. 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.

### Synovial fluid analysis

Synovial fluid was directly aspirated from the joints by use of a 21-gauge needle in a routine sterile manner, as previously reported (Moyer et al., 2007). Samples were placed in tubes containing ethylenediaminetetraacetic acid (EDTA) for routine SF analysis. None of the affected joints had undergone intra-articular analgesia or treatment in the month prior to SF aspiration.

## Biochemical analysis

Smears of SF that has been placed in EDTA immediately after collection were prepared for differential cell counts. The total nucleated cell count (NCC) of each aliquot was determined using an automated cell counter (scil Vet abc Plus(+)). Refractometry (ATA-GO T2-NE CLINICAL, Atago Ltd, Tokyo, Japan) was also used to measure total solids as an estimate of total protein (TP).

For determining HA concentrations in equine SF samples, a commercially available ELISA kit (TECO Hyaluronic Acid, TECO medical AG®) was used. The assay was based on a HA-specific binding protein. The susceptibility of errors related to high dilution and high viscosity of SF samples was determined by repeated dilution of three samples and calculation of variations. SF concentrations of HA are reported in µg/mL.

## Rheological analysis

The rheological behavior of most SF samples was evaluated within 6 hours after aspiration. For cases where the measurement could not be performed within 6 hours, aspirated SF was put in a refrigerator at approximately 4°C for testing within 24 hours

The viscoelastic properties of the samples were determined by via steady state and dynamic experiments in order to measure the shear viscosity  $\eta$  and the elastic  $G'$  and viscous  $G''$  moduli respectively, at horse's body (37.5 °C) temperature. The investigation of the rheological behavior was carried out on a TA Instruments AR-G2 controlled stress CMT Rheometer via dynamic and steady state experiments. Steady state flow steps were performed with a shear rate from 1 to 400 s<sup>-1</sup> at 25°C and 37.5 °C. Average viscosity values were calculated for shear rates from 18 - 75 s<sup>-1</sup>. Data for lower and higher shear rates were not included due to noise and viscous heating respectively.

## Statistical analysis

In order to study the effect of factors and covariates on the mean values of the elastic modulus ( $G'$ ), and the viscous modulus ( $G''$ ), the Linear Mixed Effects (LME) modeling were used. Graphical validation was used to assess the underlying assumptions of homoscedasticity and normality of residuals of the selected models. All statistical analyses were conducted using the statistical language *R* (Team RC, 2013) and the function *lmer* from package *lme4*. In addition, the function *step* from package *lmerTest* (Kuznetsova

et al., 2015) was used in order to perform backward elimination of all effects of the examined LME. The p-values for the fixed component of the model were calculated from F test based on Kenward-Roger approach in order to get approximate degrees of freedom. 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.

## RESULTS

### Descriptive Statistics

#### Biochemical analysis

The results of univariate analysis for TP, NCC,  $G'$ ,  $G''$  parameters were expressed as mean (M), standard deviation (SD), median (Mdn), minimum (min) and maximum (max). Mean NCC at normal and pathological joints was 111.40±40.66 cells/µl and 231.47±59.91 cells/µl respectively. Mean TP levels at normal and pathological joints were 1.26±0.21 g/dl and 1.74±0.28 (Table 1).

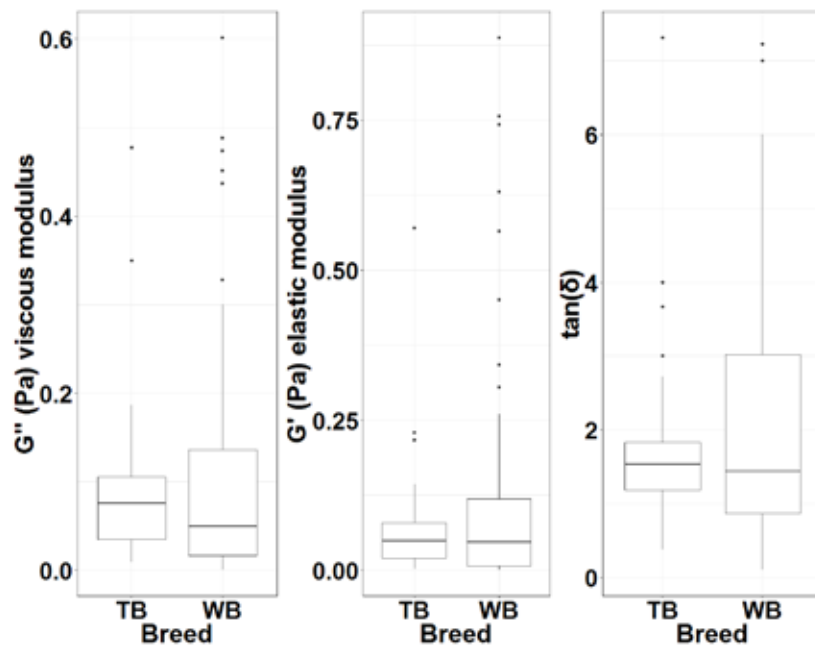
Mean HA concentration at normal and pathological joints was 939±188 µg/ml and 389±176 µg/ml respectively in the two groups. Horses with OA presented statistically significant ( $p < 0.05$ ) lower HA concentration, compared to the healthy subjects.

In order to graphically explore the distributions of  $G'$ ,  $G''$  parameters boxplots were constructed for each level of factor breed (Figure 1).

#### The effect of HA concentration

The simultaneous effect of several covariates and factors on  $G'$ ,  $G''$  values was tested for subjects that there were available measurements for HA parameter. Sex and age were inserted as control variables in the models, so as to examine their potential effects on the response variables.

The ANOVA findings revealed statistically significant ( $p < 0.001$ ) effect of HA concentration, on the mean values of  $\log G'$  measurements  $F(1, 58) = 38.162$  and on the mean values  $\log G''$  measurements,  $F(1, 58) = 71.676$ . More significantly, a positive effect of HA on the response  $\log G'$  measurements ( $b = 0.003$ ,  $SE = 0.0005$ ,  $p < 0.001$ ) and  $\log G''$  measurements ( $b = 0.003$ ,  $SE = 0.0003$ ,  $p < 0.001$ ) was detected.



**Figure 1.** Boxplots. Distributions of  $G''$ ,  $G'$  values for Thoroughbred and Warmblood breed

**Table 1.** Descriptive statistics for categorical variables of the study

Parameter	Breed	N	M	SD	Mdn	min	max
Total Protein (g/dl)	TB	15	1.37	0.15	1.40	1.20	1.60
	WB	20	1.20	0.21	1.20	0.80	1.50
	Total	35	1.26	0.21	1.25	0.80	1.60
	TB	30	1.82	0.28	1.80	1.60	2.60
	WB	40	1.63	0.24	1.60	1.30	2.20
	Total	70	1.74	0.28	1.65	1.30	2.60
Total nucleated cell count (cells/ $\mu$ l)	TB	15	130.00	54.41	125.00	70	200
	WB	20	102.08	30.44	100.00	55	153
	Total	35	111.40	40.66	100.00	55	200
	TB	30	256.84	52.71	250.00	180	370
	WB	40	199.33	53.91	180.00	120	320
	Total	70	231.47	59.91	210.00	120	370
$G'$ (Pa) elastic modulus	TB	45	0.069	0.092	0.049	0.001	0.570
	WB	60	0.122	0.204	0.047	0.001	0.887
	Total	105	0.099	0.167	0.047	0.001	0.887
$G''$ (Pa) viscous modulus	TB	45	0.085	0.085	0.076	0.010	0.477
	WB	60	0.109	0.139	0.050	0.001	0.601
	Total	105	0.099	0.119	0.055	0.001	0.601

Abbreviations: M, average; Mdn, median; min, minimum; max, maximum; N, number of samples; OA, osteoarthritis; SD, standard deviation; TB, Thoroughbred; WB, Warmblood

### The effects of Age, Breed, Sex, Joint Type and Joint status

In this section, the results derived from the analysis conducted in order to examine the simultaneous effect of several covariates and factors on  $G'$ ,  $G''$  parameters are presented.

### $G'$ (elastic modulus)

The ANOVA findings of the final model revealed statistically significant effect of Joint Type,  $F(2, 95) = 7.718$ ,  $p = 0.006$ , Age,  $F(1, 70) = 6.474$ ,  $p = 0.013$  and OA,  $F(1, 92) = 23.603$ ,  $p < 0.001$  on the mean values of  $\log G'$  measurements.



Interpreting the coefficients of the covariates OA, ( $b = -0.423$ ,  $SE = 0.085$ ,  $p < 0.001$ ) and Age ( $b = -0.110$ ,  $SE = 0.043$ ,  $p = 0.013$ ), a negative correlation was detected on the response  $\log G''$  measurements. Horses with OA seem to present lower elastic properties, gradually decreasing with age.

### $G''$ (viscous modulus)

The same analysis was used in order to fit a multivariate model for the response variable  $G''$ . The results did not reveal statistically significant interaction terms for Breed×Radiographic OA,  $F(1, 84) = 0.183$ ,  $p = 0.670$  and Breed×Joint Type,  $F(1, 90) = 1.322$ ,  $p = 0.253$  and they were removed from the final model. Moreover, marginally significant main effects of Age,  $F(1, 70) = 10.734$ ,  $p = 0.056$  and Sex,  $F(2, 65) = 3.022$ ,  $p = 0.069$  were noted and for this reason, we decided not to omit them from the final model.

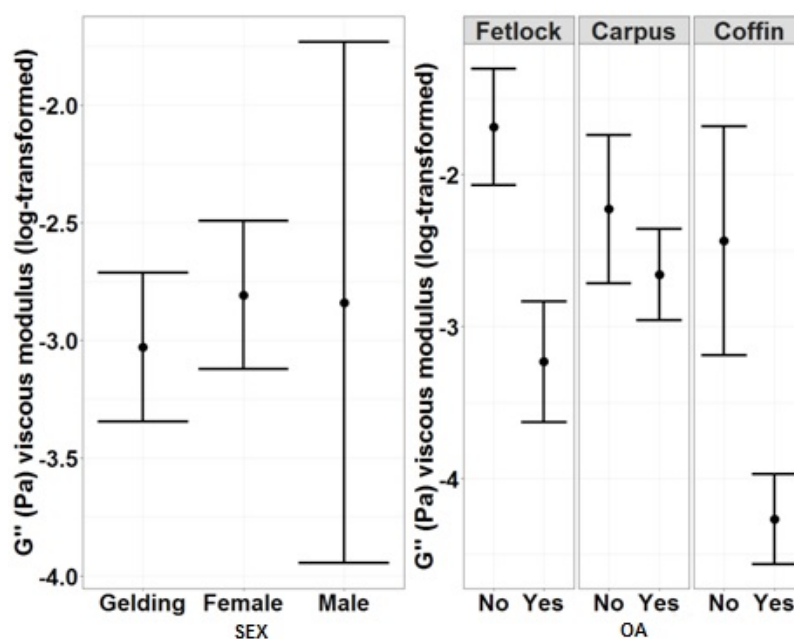
Once again, the ANOVA findings revealed that  $\log G''$  measurements vary depending on joint type,  $F(2, 86) = 5.374$ ,  $p = 0.006$  and Sex,  $F(2, 67) = 3.150$ ,  $p = 0.049$  while main effects of Age,  $F(1, 76) = 4.226$ ,  $p = 0.043$  and OA,  $F(1, 80) = 10.062$ ,  $p = 0.002$  were also detected.

Interpreting the coefficients of the covariates OA, ( $b = -0.350$ ,  $SE = 0.107$ ,  $p = 0.002$ ) and Age ( $b = -0.053$ ,  $SE = 0.025$ ,  $p = 0.043$ ), the negative sign indicates a negative correlation on the response  $\log G''$

values. Horses with OA seem to present lower viscous properties, gradually decreasing with age. Regarding the effect of Sex and osteoarthritis, depending on joint type on  $\log G''$  measurements the results are presented in Figure 2. Based on these results, it is concluded that geldings presented lower viscous properties (means of  $\log G''$ ) compared to mares. Moreover, it is noted that SF from coffin joints seems to present lower viscous properties compared to the one obtained from fetlock and intercarpal joints.

## DISCUSSION

In this study, a multivariate analysis was used in order to further investigate the several factors that can possibly affect the complex nature of equine SF. The clearly significant differences in the concentration of HA between normal joints and joints with OA indicate that there are differences in the metabolism of joint tissues. However, in a previous study by Venable et al. (2008) no differences were reported between concentrations of the experimentally induced-OA group and the control group. In our study, we detected significantly lower HA concentrations in osteoarthritic than in normal joints. These results are supported by other recent studies that investigated the HA concentration and molecular weight distribution with OA progression (Plickert et al., 2013; Band et al., 2015). A shift in the molecular weight distribution of SF HA toward lower values is associated with an increased risk for rapid OA progression (Band et al., 2015).



**Figure 2.** Means of  $G''$  measurement (log-transformed) with 95% CI for factors Sex and JointType×Radiographic. Geldings present lower viscous properties compared to mares. Synovial fluid from coffin joints presents lower viscous properties compared to the one obtained from fetlock and intercarpal joints

The concentration of HA has been reported to vary not only between animals but between joints within the same animal as well (Auer et al., 1980). Reduced levels of HA have been reported in joints following immobilisation, prompting the suggestion of mechanical control of SF HA concentration (Pitsillides et al., 1999). The decrease of HA concentrations in osteoarthritic SF has been attributed to degradation, fragmentation, alteration in joint metabolism and synthesis, and dilution by joint effusion (Henderson et al., 1991; Kuroki et al., 2002). However, HA concentrations in urea-adjusted and unadjusted SF samples were found to be similar, so that dilution effects were considered unlikely to explain the reduction of HA concentrations (Budsberg et al., 2006). In this report, the decreased HA concentration in diseased joints, in line with the fact that a significant effect was detected on the viscoelastic properties (in terms of  $\log G''$  and  $\log G'$  measurements), support this hypothesis. However, results from recent studies indicate that boundary lubrication is a result of interaction between HA and other macromolecules, such as lubricin (Bonnevie et al., 2015). Further research is required to investigate whether this interaction is purely a mechanical entanglement or it is dictated by the hydrophobic and hydrophilic nature of these molecules.

In a study of Temple-Wong and colleagues (Temple-Wong et al., 2016) the effect of age and stage of joint degeneration on the concentrations of protein and HA distribution were assessed. The concentration decrease of HA in SF with age, in the absence of OA, and the association of lower HA in SF with increased friction between cartilage surfaces, suggest that this relationship may be an important factor in the age-related deterioration of articular cartilage (Squires et al., 2003).

In this multivariate study a negative effect of age on the viscoelastic properties of equine SF was detected [mean values of  $\log G'$  measurements ( $p < 0.001$ ) and  $\log G''$  measurements ( $p = 0.013$ )]. In human patients, the most prominent hypothesis linking aging to OA is that chondrocytes undergo premature aging due to several factors, such as excessive mechanical load or oxidative stress (Squires et al., 2003; Courties et al., 2015). The risk factors for development of OA in humans has been classified into two fundamental mechanisms related either to the adverse effects of 'abnormal' loading on 'normal' cartilage or of 'normal' loading on 'abnormal' cartilage, and similar pathways have been described in horses (McWraith, 1996).

Considering the fact that horses included in this study were all active athletes, the repeated mechanical forces to the joints could explain the negative correlation between viscoelastic properties and aging.

Moreover, it was noted that sex presented a significant effect on the mean values of  $\log G''$ , with geldings presenting significantly lower viscous properties compared to the ones obtained from mares. Unlike humans, where women seem to be slightly more prone to OA than men (O'Connor, 2007; Hanna et al., 2009; Blagojevic et al., 2010) there is no evidence to support the argument of sex-related susceptibility to OA in horses. This result could be attributed to the fact that geldings are overrepresented in this study compared to mares. Still, more research is needed in order to extrapolate more accurate results, regarding sex predisposition to OA.

The investigation of SF properties has been of considerable interest, mainly due to its viscoelastic character. Viscosity measurements have been previously performed on SF and other polymer solutions (Yu et al., 2014) while it has been proposed that  $G'$  and  $G''$  values mainly depend on shear rate, temperature, pressure, and concentration (Giap, 2010). 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 behavior (Borzacchiello et al., 2010). In the study reported here an effort was made to detect the effect of several factors (breed, age, sex, joint type and joint status) in SF rheological properties with respect to the mean levels of  $G'$  and  $G''$  values. In our report a statistically significant effect of horses with OA was detected on the mean values of  $\log G'$  measurements ( $p < 0.001$ ) as well as on the mean values of  $\log G''$  measurements ( $p = 0.002$ ). Horses with OA seem to present lower  $\log G'$  measurements and  $\log G''$  measurements compared to the healthy subjects. Hence, it is concluded that, in OA, SF tends to lose both its elastic and viscous properties. It is hypothesized that compromise in viscoelasticity leads to the diminished rheological properties and to unmitigated forces transmitted to the cartilage and intercellular matrix.

This study was designed to investigate the several factors that contribute to changes in synovial fluid viscoelasticity. The statistical analysis results indicated that the mean values of rheological parameters are joint type dependent. This can be attributed to the

fact that the equine forelimb exhibits a wide variety in biokinematic variables in terms of ground force and stride adaptation (Back et al., 1996). Hence, depending on joint type, different adaptation to ground forces can lead to alterations in viscoelastic properties. An early report by Clayton and colleagues (Clayton et al., 2011) presented a full kinetic analysis of the relative motion of equine forelimb joints at the trot. It was reported that during stance phase, most of the energy absorbed was by fetlock joint, while only about 6% of the total energy absorbed was by coffin joint. Moreover it was concluded that during stance phase, coffin joints generate energy while the carpus and fetlock joints absorb energy. All Thoroughbred horses in our study were flat racehorses, trained to race over short or middle distances, while Warmblood horses were used for low-level show jumping. Considering that viscoelastic properties of SF alter, depending on joint type and ground forces, the different type of exercise may explain that fact.

## CONCLUSIONS

To the authors' knowledge this is the first multivariate study to quantitatively evaluate and compare the several factors that affect the viscoelastic properties of equine SF. Human OA is a multifactorial process in which systemic risk factors like age, sex, mechanical trauma, obesity and genes determine the susceptibility of an individual. In our study an approach was made to investigate the impact of some of these factors in an equine model. Based on our results, it can be summarized that HA concentrations are positively correlated with the viscoelastic properties of equine SF, while there seem to be significantly lower to OA horses compared to the normal ones. Moreover, viscoelastic properties of equine SF seem to reduce, during aging, and present variation depending on joint type. The present results can provide extent knowledge to further understand the complex role of SF, in order to maximize the potential to novel treatment strategies in OA.

## CONFLICT OF INTEREST

None declared by the authors.

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