Identification of infrared absorption spectral characteristics of synovial fluid of horses with osteochondrosis of the tarsocrural joint

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Objective—To determine the feasibility of the use of Fourier-transform infrared (FTIR) spectroscopy within the midinfrared range to differentiate synovial fluid samples of joints with osteochondrosis from those of control samples.

Animals—33 horses with osteochondrosis of the tarsocrural joint and 31 horses free of tarsocrural joint disease.

Procedures—FTIR spectroscopy of synovial fluid was used. Sixty-four synovial fluid samples from the tarsocrural joint were collected. Of these, 33 samples were from horses with radiographic evidence of osteochondrosis of the tarsocrural joint and 31 from control joints. Disease-associated features within infrared spectra of synovial fluid were statistically selected for spectral classification, and the variables identified were used in a classification model. Linear discriminant analysis and leave-one-out cross-validation were used to develop a classifier to identify joints with osteochondrosis.

Results—12 significant subregions were identified that met the selection criteria. The stepwise discriminant procedure resulted in the final selection of 6 optimal regions that most contributed to the discriminatory power of the classification algorithm. Infrared spectra derived from synovial fluid of joints with osteochondrosis were differentiated from the control samples with accuracy of 77% (81% specificity and 73% sensitivity).

Conclusions and Clinical Relevance—The disease-associated characteristics of infrared spectra of synovial fluid from joints with osteochondrosis may be exploited via appropriate feature selection and classification algorithms to differentiate joints with osteochondrosis from those of control joints. Further study with larger sample size including age-, breed-, and sex-matched control horses would further validate the clinical value of infrared spectroscopy for the diagnosis of osteochondrosis in horses. (Am J Vet Res 2007;68:517–523)
modality is generally useful, diagnoses of osteochondrosis have been made during arthroscopy in horses for which lesions were neither clinically nor radiographically apparent. Interventions to prevent osteochondrosis, such as medical management, physical therapy, and early detection of at-risk horses, are critical. Typically, high-risk horses are identified through the use of a combination of physical examination, diagnostic imaging, and serum and synovial fluid profiling. The identification of biomarkers that can be used to detect osteochondrosis early in its development is crucial for developing effective interventions and improving outcomes.

Infrared spectroscopy is a non-invasive technique that can be used to analyze biological samples, such as synovial fluid and serum, to identify chemical changes associated with osteochondrosis. Fourier-transform infrared spectroscopy (FTIR) is a powerful tool for analyzing the molecular composition of biological samples by measuring the absorbance of infrared radiation through the sample. This technique provides a fingerprint of the chemical composition of the sample, which can be used to identify changes associated with osteochondrosis.

A synovial fluid sample (n = 64) was collected from 33 horses admitted to the veterinary teaching hospital with tarsocrural joint effusion and radiographic evidence of osteochondrosis affecting the distal intermediate ridge of the tibia only (n = 23), the distal intermediate ridge of the tibia and lateral trochlear ridge of the talus (6), or the distal intermediate ridge of the tibia and medial malleolus of the tibia (4). All lesions were confirmed during arthroscopy and consisted of attached osteochondral defects that required loosening with a periosteal elevator prior to removal. No free fragments were found in any of the operated joints, and lesions were ≤ 1 cm in size. No new lesions were found that had not been identified radiographically. Following the donation of horses for reasons not related to joint disease (n = 3) or the signing of a consent form for participation in the study (28), 31 synovial fluid samples were collected as control samples from horses without effusion in either tarso-
crural joint or lameness and with no evidence of osteochondrosis or other disease based on radiographic examination (client horses) or necropsy findings (donated horses). One control synovial fluid sample was collected arthroscopically during laboratory exercises from a donated horse with a radiographically normal tarsocrural joint immediately prior to euthanasia. All 3 donated horses were euthanized for reasons unrelated to this study with pentobarbital sodium (125 mg/kg, IV).

FTIR—Synovial fluid samples were thawed at room temperature (20°C) and centrifuged at 2,700 x g for 10 minutes; supernatants were used for analyses. Samples were prepared as described previously with the following modification. For each sample, an aliquot was drawn and diluted in an aqueous potassium thiocyanate solution (4 g/L) in a 3:1 ratio of synovial fluid-to-aqueous potassium thiocyanate solution. Triplicate dry films were made for each sample by applying 8 µL of the diluted synovial fluid, spread evenly in a circular motion, onto 5-mm-diameter circular islands within a custom-made, adhesive-masked, 96-well silicon microplate (the adhesive mask served to spatially define and systematically separate the 5-mm-diameter islands on the microplate so that sample islands were correctly aligned with the FTIR spectroscopic radiation source and detector). Synovial fluid samples from all study horses were randomly assigned to well positions on the microplate. Synovial films were left to dry at 20°C for 12 hours. After the films were thoroughly dried, the microplate was mounted within a multisampler device interfaced with an FTIR spectrometer equipped with a deuterium tryglycine sulfate detector to allow for the acquisition of infrared spectra. Infrared spectroscopic measurements of samples were performed during the same period. Absorbance spectra in the midinfrared range (400 to 4,000 cm⁻¹) were recorded. For each acquisition, 512 interferograms were signal averaged and Fourier transformed to generate a spectrum with a nominal resolution of 4 cm⁻¹.

Data preprocessing—Triplicate spectra of each sample were averaged, and then differentiation and smoothing procedures (Savitsky Golay second-order derivatives with second-degree polynomial functions; 19-point smoothing) were performed on all spectra to resolve and enhance weak spectral features and to remove baseline variation. Spectra were then standardized with a wave number range of 800 to 1,450 cm⁻¹ as a basis of vector normalization by use of numeric computing language. Vector normalization was performed for each second-derivative spectrum by first summing the squares of absorption intensities for all datum points (1 datum point corresponded to approx 1 wave number) within the spectral basis range of 800 to 1,450 cm⁻¹. The square root of this sum of squares calculated from each spectrum was used as the normalization factor for that same spectrum. Intensities of the entire range within each spectrum were divided by this vector normalization factor prior to statistical analysis.

Figure 1—Graphic representation of standardized second-derivative infrared spectra (second-order derivative intensity value vs wave number). Notice the significant (P < 0.01) wave number regions identified by use of an ANOVA (shaded bands). *Regions selected by use of the stepwise discriminant procedure for inclusion in the final classification model.
Statistical analysis and selection of significant subregions—Spectral subregions, wherein the significant effect of group (osteochondrosis vs control) was determined in preprocessed infrared spectra, were identified as follows. The relative intensity of standardized spectra at each datum point (wave number) was used as a dependent variable. Statistical analysis was performed on the basis of each wave number for the entire midinfrared range of 400 to 4,000 cm\(^{-1}\). The set of independent variables included group (fixed effect), age (covariate), microplate (random effect), within-microplate row (random effect), and within-microplate column (random effect). Analyses of covariance were used to detect sets of wave numbers that had a significant (P < 0.01) effect of group, accounting for the age variable. Significant subregions were defined as a set of at least 4 consecutive wave numbers that had a significant effect of group at a value of P < 0.01. Spectral intensities within each subregion were then averaged, and this value was then considered as a variable for inclusion within a classification model.\(^{34}\)

Classification model development and validation—By use of the set of averaged subregional intensities as input variables for each horse, stepwise discriminant analysis was performed to select the subset of variables that most contributed to the power of the discriminatory function.\(^{35}\) These subsets of variables (optimal regions) were subjected to linear discriminant analysis to find the discriminatory function and rule that best separated the osteochondrosis and control groups. An equal prior probability was set. The performance of classification models, as indicated by accuracy, specificity, and sensitivity, was estimated on the basis of the leave-one-out cross-validation method.\(^{31}\)

Results

Analysis of covariance revealed 12 significant subregions that met the selection criteria (Figure 1; Table 1). From this set, 6 optimal subregions were identified by the discriminant procedure that most contributed to the discriminatory power of the classification algorithm. Linear discriminant analysis resulted in classification results with 73% sensitivity, 81% specificity, and 77% overall accuracy as estimated by the cross-validation method (Table 2). Age, accounted for in the final model, significantly influenced results of the analysis. On the basis of the identification of age as a significant determinant of outcome, the distributions of horses correctly classified and misclassified in each of 3 age categories were determined (Table 3).

Table 1—Significant infrared absorption spectrum subregions found to discriminate between synovial fluid samples from horses with a clinical diagnosis of osteochondrosis of the tarsocrural joint (n = 33) and from horses with clinically unaffected (control) tarsocrural joints (31).

<table>
<thead>
<tr>
<th>Wave numbers of infrared regions (cm(^{-1}))</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>536–542*</td>
<td>0.002–0.006</td>
</tr>
<tr>
<td>762–766</td>
<td>0.002–0.006</td>
</tr>
<tr>
<td>851–855*</td>
<td>0.004–0.008</td>
</tr>
<tr>
<td>906–912*</td>
<td>&lt; 0.001–0.007</td>
</tr>
<tr>
<td>923–927*</td>
<td>0.005–0.009</td>
</tr>
<tr>
<td>958–968</td>
<td>&lt; 0.001–0.009</td>
</tr>
<tr>
<td>985–991</td>
<td>0.001–0.002</td>
</tr>
<tr>
<td>996–1,019*</td>
<td>&lt; 0.001–0.006</td>
</tr>
<tr>
<td>1,027–1,036</td>
<td>&lt; 0.001–0.007</td>
</tr>
<tr>
<td>1,073–1,083</td>
<td>0.001–0.007</td>
</tr>
<tr>
<td>1,093–1,100</td>
<td>0.001–0.008</td>
</tr>
<tr>
<td>1,263–1,772*</td>
<td>&lt; 0.001–0.006</td>
</tr>
</tbody>
</table>

*Indicates the regions selected by the stepwise discriminant procedure for inclusion in the final classification model.

Table 2—Comparison of clinical diagnosis with infrared absorption spectral characteristic–based diagnosis of osteochondrosis by use of tarsocrural synovial fluid samples of 64 horses.

<table>
<thead>
<tr>
<th>Infrared-based diagnosis</th>
<th>Control</th>
<th>Osteochondrosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correct classification</td>
<td>25(^{*})</td>
<td>8</td>
<td>33</td>
</tr>
<tr>
<td>Missclassification</td>
<td>9</td>
<td>24(^{*})</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>30</td>
<td>64</td>
</tr>
</tbody>
</table>

Table 3—Distribution of 64 horses in 3 age categories correctly classified (and misclassified) as having osteochondrosis of the tarsocrural joint on the basis of infrared absorption spectral characteristics of synovial fluid samples.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Misclassification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Correct classification</td>
<td>0</td>
</tr>
<tr>
<td>Missclassification</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Osteochondrosis</th>
<th>Misclassification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correct classification</td>
<td>14</td>
</tr>
<tr>
<td>Missclassification</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
</tr>
</tbody>
</table>

*Indicates the number of samples that were correctly classified.
of a larger number of synovial fluid samples from each disease class may be crucial for optimizing the extraction of the disease-relevant information from infrared spectra, thus permitting the meaningful detection of a number of significant discriminatory infrared regions. Our classification success rate, although encouraging at 77%, may improve further in future FTIR-based studies of osteochondrosis in horses by use of a larger sample size. Such a larger-scale study would improve not only the accuracy of this diagnostic test but also the range of applicability to a larger and more diverse diseased (clinical and subclinical) population.

The central concept underpinning our study is that characteristic alterations in molecular synovial fluid constituents associated with joint disease lead to characteristic changes in infrared absorption patterns. Although the specific molecular changes and species contributing to features distinguishing infrared spectra of osteochondrosis-affected joints from control joints have not been identified to date, spectra of biological samples reflect the structure of the individual infrared active constituents (including known and unknown biomarkers) and their relative abundance. Alterations in known biomarker concentrations in synovial fluid have been reported in studies of osteochondrosis in horses by use of other methods. Authors of these studies have proposed that alterations in biomarker concentrations are associated with either growth or pathogenesis of joint disease in horses. The differences attributable to disease and age in our study support this contention. To our knowledge, none of the assays for known markers are developed for routine diagnostic screening of horses for osteochondrosis, and substantial intra- and interassay variation may limit their accuracy in this role. Results of radiographic screening also vary depending upon the age of the horse when examined and the joint affected and may fail to identify affected horses with nonradiographic signs of disease. In our study, 5 of 6 control horses that were misclassified were included in the control group on the basis of normal findings on physical examination and radiographic evaluation alone and may have had occult lesions of osteochondrosis. Consideration should be given to the arthroscopic evaluation of control horses in studies when possible. The variation in correct classification rates in different age groups in our study by use of FTIR spectroscopy will need to be addressed before the test may be applied to screening of the general equine population for osteochondrosis of the tarsocrural joint.

Alterations in chondroitin sulfate epitope 846 epitope and carboxy propeptide of type II procollagen in association with osteochondrosis are observed in young horses during musculoskeletal development, but not in mature horses. The presence of proteoglycan components in synovial fluid and serum may reflect physiologic and pathologic cartilage extracellular matrix turnover. In osteochondrosis-free equine joints, the highest concentration of glycosaminoglycans in synovial fluid is detected in neonates. This variable decreases with increasing age, with the effect of aging disappearing at 4 years. In our study, the FTIR-based approach allowed identification of several spectral subregions within which the absorptions of proteoglycans would be expected to contribute prominently, in particular the 996- to 1,019-cm⁻¹ region that lies within the range characteristic of carbohydrate carbon-to-oxygen bond stretching vibrations. Investigating synovial fluid markers of osteochondrosis, the progressive nature of the disease process and physiologic factors such as age, breed, and sex will have to be considered in the search for the optimal infrared signature within spectra. In our study, the wide age range of horses was controlled for in the analysis to minimize bias. However, it is clear from the rates of misclassification in the different age groups that this variable remains a possible confounder. Age influences the expression of known biomarkers for osteochondrosis. In addition, lesions in young horses may spontaneously resolve without apparent development of secondary osteoarthritis. Further investigations of young horses with osteochondrosis may determine whether infrared spectral characteristics exist that are associated with spontaneous resolution of osteochondrosis lesions and changes in the metabolic activity of cartilage. In our study, the misclassification rate within the osteochondrosis group aged < 2 years was lower (18%) than the misclassification rate for horses > 2 years of age (37%). As already alluded to, it is possible that the important features associated with osteochondrosis in FTIR spectra may be less prominent in older horses. In future studies it may be possible to identify different age-dependent spectral features that will allow the development of classifiers for osteochondrosis that encompass variations attributable to growth as well as pathologic progression. A combination of these attributes may provide a so-called real signature within infrared spectra that is highly specific to the presence or absence of osteochondrosis for all ages. Taking the age factor into account and quantifying group effects when adjusted for age are logical next steps toward the refinement and implementation of this diagnostic test. Although our synovial fluid samples were collected from clinically affected horses admitted for evaluation, future studies designed to develop the FTIR-based test for application to the equine population at large should consider age, sex, and perhaps breed-matched selection of control horses.

Conditions of prior probability may be adjusted on the basis of criteria such as the cost of misclassification and the proportion of sample size. Adjustment of this ratio may influence the sensitivity and specificity estimation. For our study, an equal prior probability was set assuming no prior knowledge on how spectra should be classified or preference for any group. The leave-one-out cross-validation method is a model validation method that requires that each discriminatory function be constructed by taking 1 spectrum out of the data set. Then that spectrum is used to validate the discriminatory model. This process is repeated for all spectra in the data set until every single spectrum takes its turn to validate the model. This method was used to enhance robustness of the linear discriminant analysis classifier and was proposed to be useful in classification of arthritic disorders in humans. With a lim-
ised number of horses, the total error rate of the data set on the basis of the cross-validation method was 23%. A more accurate rate of classification of osteochondrosis and control spectra may be achieved through a larger number of classification attributes (or spectral subregions) derived from larger sample sizes.

In conclusion, results of our study reveal significant features in the FTIR absorption pattern that are associated with osteochondrosis. The differentiation of infrared spectra obtained from osteochondrosis and control synovial fluid samples is feasible. The ultimate goal of this type of research is to develop novel tests that aid clinical and preclinical diagnosis of joint disease in horses. Further study with a larger sample size, including horses with occult osteochondrosis and matched control horses, would further validate the clinical value of FTIR-based diagnosis of osteochondrosis in horses, improve accuracy, and complete the transition to clinical utility.

References


Correction: Effects of buprenorphine on nociception and locomotor activity in horses

In the report, “Effects of buprenorphine on nociception and locomotor activity in horses” (AJVR 2007;68:246–250), information on page 248 in the Results section is incorrect. In the subheading, Antinociception evaluation, the third sentence in the first paragraph should appear as follows: The HWRL was significantly longer at all time points after administration of 10 µg of buprenorphine/kg, compared with results after administration of 5 µg of buprenorphine/kg or saline solution.