Infrared spectroscopy of dystrophic mdx mouse muscle tissue distinguishes among treatment groups

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Shaw, R. A., H. H. Mantsch, and J. E. Anderson. Infrared spectroscopy of dystrophic mdx mouse muscle tissue distinguishes among treatment groups. J. Appl. Physiol. 81(5): 2328–2335, 1996.—Four groups of mdx mice (deflazacort, high dose of 1.5 mg/kg and low dose of 0.75 mg/kg; prednisone, 1.0 mg/kg; and a placebo) were examined in a double-blind protocol. The experiments tested the hypothesis that infrared spectroscopy can distinguish among gastrocnemius muscle tissues derived from dystrophic animals (n = 22) from different treatment groups and from control muscle tissue (n = 23). Results showed that muscle, inflamed muscle, and tendon can be distinguished on the basis of their infrared absorption patterns. Distinctions among the spectra of the four treatment groups were sought with automated pattern-recognition methods. These classification methods, based either on spectral regions (900–1,500 cm−1) or on principal-component analysis, were in close agreement, assigning 15 or 16, respectively, of 22 mdx spectra to the correct treatment group. Both trials cleanly separated the high-dose deflazacort from the placebo group of muscles, whereas the prednisone and low-dose deflazacort groups were persistently confused in these classifications. Changes in the histology of muscle inflammation paralleled the spectral-classification results. Thus the proposed method, combining infrared spectroscopy with pattern-recognition algorithms, can distinguish treatment effects on muscle tissue. Specific spectral features characteristic of tissue type, disease progression, and treatment effects are not yet elucidated.

muscular dystrophy; deflazacort; automated classification; linear discriminant analysis

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A spontaneous mutant of the C57BL/10ScSn control strain, the mdx mouse, is well characterized to demonstrate dystrophin-deficient myopathy in skeletal muscles. To date, the extent of limb muscle regeneration in mdx mice is known to largely compensate for dystrophy (2, 4), in contrast to the progressive nature of human Duchenne muscular dystrophy (DMD), which also results from defects in the dystrophin gene. The patterns of regeneration and dystrophy can be affected by endocrine treatments in mdx mice. However, so far, only clinical trials with prednisone and deflazacort on DMD (5, 13) have shown significant benefits to alleviate progressive muscle weakness. The manual strength-testing procedures that have been used to monitor treatment effects require the voluntary and consistent cooperation of young boys (who stand to benefit the most from treatment). It is therefore important to develop reliable measures of disease state that can distinguish and monitor treatment-induced changes in tissues. Although an ideal clinical procedure would detect such changes noninvasively, biopsy-based methods may be more sensitive in providing information concerning the biochemical modifications specific to a treatment.

Infrared spectroscopy of affected tissue can potentially reveal a wealth of information regarding the biochemical and biophysical bases of disease processes. Every pure compound gives rise to a unique pattern of infrared absorptions, essentially a distinctive “fingerprint.” For more complex mixtures, the spectrum is a combination of the component spectra weighted by the relative concentrations of those components.

For tissue, there may be hundreds of components contributing to the absorption profile, and, in general, the spectra are far too complex to permit assignment of specific absorptions to individual constituents. However, it is often unnecessary to resolve all of the individual components from one another; useful insights can be drawn from the spectra by focusing on classes of biological macromolecules rather than individual chemical species. For example, molecular structures such as long lipid CH2 chains or protein backbone N—H and C=O groups provide absorptions that are characteristic of these classes of compounds. This type of information has been able to distinguish white matter from gray matter and multiple sclerosis plaques in brain tissue (7).

Fundamental limitations can emerge when interpretation of very complex spectra on the basis of individual or group assignments is attempted. It is often the case that a potentially defining pattern that might distinguish among various groups of spectra is obscured by overlapping absorptions to such an extent that even the trained eye cannot discriminate the groupings. In this situation, automated pattern-recognition methods can be invaluable. Algorithms may be trained to recognize common patterns within subgroups of seemingly unrelated spectra and hence to distinguish the spectra within one group from those in a different group. Once trained, the same model may be used to classify the spectra of unknown “test” samples as belonging to specific groups, as was demonstrated in studies of...
Alzheimer’s disease (8) and in characterizing synovial fluid in subtypes of arthritis (9, 18).

To the best of our knowledge, the only reported infrared spectroscopic studies of muscle tissue are near-infrared studies that focus specifically on absorptions due to hemoglobin, myoglobin, and their deoxy counterparts (for example, Refs. 6, 12, 19). Those studies applied near-infrared spectroscopy to probe tissue oxygenation in various activation or disease states. In contrast, all tissue constituents in muscle are probed by the present infrared measurements.

The basic hypothesis of this study is that gross pathological changes and underlying biochemical changes that occur in muscle tissue during the progression of a well-characterized disease are reflected in the infrared spectra of the muscles. Three related questions are addressed here. The first is whether infrared spectroscopy can distinguish among various gross subtypes of muscle tissue, viz., “unaffected” muscle, “inflamed” muscle, and tendon. The second is to test whether spectra of skeletal muscle tissue from normal control mice differ from those measured for age-matched mdx dystrophic mouse muscle. Finally, we examined whether infrared spectroscopy can detect changes in dystrophic tissues during glucocorticoid treatment (prednisone or deflazacort) of mdx mice during the period of maximal dystrophic damage to the muscle in much the same double-blind manner as in an ongoing clinical trial in the treatment of DMD.

MATERIALS AND METHODS

Animal and tissue preparation. A total of 22 mdx dystrophic (mdx C57B1/10ScSn) and 23 age-matched control mice were used for this study in accordance with the guidelines of the Canadian Council on Animal Care, the University of Manitoba, and the National Research Council. At 3 wk of age, the mdx mice were randomly assigned to four treatment groups, designated as groups A, B, C, and D (n = 6, 5, 5, and 6, respectively). Starting at day 0, the mdx mice were injected (double blind) for 28 days with the same volume of vehicle (0.05 ml sc) containing either prednisone (1.0 mg/kg), deflazacort (high dose, 1.5 mg/kg; low dose, 0.75 mg/kg), or placebo only. The prednisone and deflazacort doses were chosen to provide equipotent anti-inflammatory effects. All doses were selected to match those in a current multicenter clinical trial of DMD (C. Greenberg, personal communication). On day 28, the mice were anesthetized and killed. The medial gastrocnemius muscle was rapidly removed, placed in a cryovial, sealed, and frozen in liquid nitrogen before storage at −80°C. Other tissues were sampled for additional studies (3), and the double-blind code was broken only after these studies were completed.

A horizontal cut was made across the midbelly of each muscle immediately on thawing. From the cut face of the proximal half of the muscle, four samples of muscle (~1 mm²) were removed and prepared for infrared spectroscopy. All four samples were measured within 20 min of thawing and before the next muscle was thawed. Immediately after spectra were collected, the tissues were placed in labeled (coded) vials of 10% buffered Formalin, fixed for 1 wk, rinsed overnight in phosphate buffer containing 5% sucrose, and prepared for frozen sectioning (6-μm thickness). Three to five sections per sample were collected on glass slides and stained with hematoxylin and eosin for routine morphological examination and tissue classification. Each sample was designated to contain intact unaffected muscle, inflamed muscle, tendon, adipose tissue, and/or nerve by one observer (J. E. Anderson), independent of knowledge of the source of the sample, with conventional light microscopy.

Infrared spectroscopy. Infrared spectra were collected at a resolution of 4 cm⁻¹ (512 scans, boxcar apodization) with a Digilab FTS-40A spectrometer equipped with a Split-Pea sampling accessory (Harrick Scientific, Ossining, NY). This accessory measures the attenuated total reflection spectrum of a portion of the sample, which is placed on the surface of a silicon optical element. The tissue volume probed by the infrared beam was a circle 300 μm in diameter penetrates to a depth of ~4 μm. A total of 88 mdx (4 × 22) and 92 control (4 × 23) spectra were recorded in this fashion. Band narrowing was applied to all spectra with a half bandwidth of 15 cm⁻¹ and a band-narrowing factor of 2. Spectral processing was carried out with both the Win-IR program package (Bio-Rad Industries, Cambridge, MA) and software developed in-house.

Classification methods. The deconvolved spectra were classified in three stages: spectral compression, linear discriminant analysis (LDA) (15), and validation.

Two alternate spectral-compression methods were used in this work. The “region-selection” algorithm chooses “N” spectral regions (typically 6–20 regions each 2–20 cm⁻¹ wide) and extracted from the spectrum the average absorption intensity within each of the N regions. Every spectrum was then reexpressed as a vector containing N average absorption intensities. These compressed representations of spectra were used as the basis for classification all spectra, and the accuracy of each classification was gauged as outlined below. The set of spectral regions that is optimal for separating the groups (e.g., muscle from tendon, treatment group A from B) was obtained through a genetic algorithm developed at the Institute for Biodiagnostics (Winnipeg, Manitoba) (16).

The second data-compression method made use of the principal components (11). By using this approach, each spectrum in a data set was reexpressed as a linear combination of those components (i.e., the ith spectrum is given by $A_{1i} \times PC_1 + A_{2i} \times PC_2 + \ldots + A_{Mi} \times PC_M$, where $A_i$ is a coefficient, $i$ is the spectrum number, $PC$ is principal component, and $M$ is the number of principal components). Typically for infrared spectra, 15 or fewer principal components were required to reproduce a given spectrum in the data set to within the noise level (i.e., $M \leq 15$). Each spectrum was thereby reduced to a column vector of the coefficients ($A_{1i}, A_{2i}, \ldots, A_{Mi}$). This new representation may then be used as the basis for classifying the spectra. The classification accuracy may be improved (and the data further compressed) by selecting an appropriate subset of these coefficients. The optimal choice was derived here by checking the classification accuracy for all possible subsets of the 15 coefficients.

For either region-selection or principal-component data compression, the ith spectrum was therefore represented by a column vector of N coefficients ($A_{1i}, A_{2i}, \ldots, A_{Mi}$), where N is either the number of principal components in the optimal subset or the number of regions obtained through the genetic algorithm. By viewing these as points in N-dimensional space, the LDA algorithm then creates planes in that space that optimally separate clouds of points that correspond to different classes. This separation is generally not complete, and further validation of the model is required to assess the accuracy expected in classifying spectra outside the calibration set.

The cross-validation method accomplishes this by first removing one of the (compressed) spectra from the set, recalculating a new LDA model for the remaining spectra,
Table 1. Classification table

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<th>Actual Group</th>
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Infrared Spectroscopy

Visual Examination | UM | IM | T |

Unaffected muscle | 6  | 3  | 1 |
Inflamed muscle   | 1  | 11 | 1 |
Tendon           | 1  | 2  | 10 |

UM, unaffected muscle; IM, inflamed muscle; T, tendon. Nos. in bold, no. of mice correct predicted for that group. Rest of mice were misclassified.

Table 2. Classification tables for mdx mouse treatment groups

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<th>Actual Group</th>
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Infrared Spectroscopy

**RESULTS**

Infrared spectra. The infrared spectrum of unaffected muscle tissue is displayed in Fig. 1. Variations among the spectra of unaffected muscle, inflamed muscle, and tendon illustrated by the difference spectra (tendon – unaffected muscle, inflamed – unaffected muscle) are included in this display. The low noise level over the spectral region from 1,800 to 2,000 cm⁻¹ confirms that those features below 1,800 cm⁻¹ are genuine differences between the absorption spectra of the three tissue constituents. These differences are discussed further below, but the richness and complexity of the difference spectra illustrate, first, the high information content and, second, the requirement for automated methods to determine the essential features and patterns of the infrared absorption spectra from tissues.

The overall average of the mdx spectra is shown in Fig. 2. Figure 2 also includes some general assignments, for example, the major absorptions above 1,500 cm⁻¹ due to protein and lipid components. Although the difference spectra show that distinctions exist among the four treatment groups, these signals are again too complex to allow for conventional peak assignments and interpretation.

Figure 3 shows infrared spectra for the mdx muscles from two of the four treatment groups, illustrating the shortcomings of purely visual inspection of the spectra. Although there are subtle variations among them, no evident patterns appear to distinguish between the infrared spectra of group B muscles and those from group D; the same observation is true for all comparisons among the four treatment groups. The displayed
The range of 900–1,500 cm\(^{-1}\) is commonly referred to as the fingerprint region of the infrared spectrum, i.e., that region that is generally considered to be optimal for discriminating among compounds. Consistent with this notion, the same spectral region was the optimal choice for automated classification of the spectra both by tissue type (see Classification trial by tissue type) and by treatment group (see Treatment groups: classification trials and decoded group designations).

Figure 4 shows the average of all infrared spectra collected for the normal control muscles, the corresponding average for the mdx muscle spectra, and the trace of the arithmetic difference between these spectra. There are clear features in this difference spectrum, such as positive bands at 1,550 and 1,650 cm\(^{-1}\) corresponding to protein, and a negative band at 1,740 cm\(^{-1}\) due to lipid, that indicate that the ratio of protein to lipid is lower in mdx than in control muscle.

Classification trial by tissue type. Table 1 summarizes the classification according to tissue type. To illustrate how Table 1 is interpreted, it can be seen, for example, that of the 13 samples determined visually to contain nearly pure tendon (>85% of the tissue sample), 10 of the corresponding spectra are classified as tendon. Of the remaining three spectra, two are classified as inflamed muscle and one as unaffected muscle tissue. The success rate of classification is similar for inflamed muscle, whereas that for unaffected muscle is somewhat lower. This tissue type classification is based on the optimal five spectral regions shown in Fig. 5.

Treatment groups: classification trials and decoded group designations. At the conclusion of the analyses, the groups were decoded as follows: group A, high-dose deflazacort; group B, prednisone; group C, low-dose deflazacort; and group D, placebo. For the average spectra of muscles in the four mdx treatment groups, two LDA classification results, Spectral regions and Principal components, are shown in Table 2. The two classification results show comparable levels of accuracy despite being based on quite different methods. Spectral regions is based on the 6 spectral regions displayed in Fig. 6, whereas Principal components is based on the optimal 6 of 15 principal components for the 900- to 1,500-cm\(^{-1}\) region. Of the 22 spectra, the pattern-recognition algorithm places 15 (principal-component trial) or 16 (region-selection trial) in the correct category. Both of these trials are presented here to illustrate their similarities; both the correct and incorrect classifications follow similar patterns for the two cases. For example, there is no overlap between groups A (high-dose deflazacort) and D (placebo) for either of the classifications. The two groups are sorted into completely different classes. The misclassifications are also consistent for the two methods, with at least one spectrum from group A predicted to fall in group C, one group B in group C, one group C in group B, and one group D in group B in each case.

![Fig. 1. Differences between unaffected muscle and average spectra for tendon (13 spectra; tendon – unaffected; A) and inflamed muscle tissue (13 spectra; inflamed – unaffected; B) and average of 10 infrared spectra for samples designated as unaffected muscle (C). Difference spectra (A and B) are plotted on magnified (×15) absorbance axis compared with the absorption spectrum (C).](image-url)

![Fig. 2. Overall average of 88 infrared spectra measured for mdx mouse tissue (bottom trace). Top 4 traces, difference spectra for groups D (placebo), C (low-dose deflazacort), B (prednisone), and A (high-dose deflazacort). Traces were calculated by subtracting overall average spectrum (88 mdx spectra) from average spectrum for samples in each group. Difference spectra are plotted on a magnified absorbance axis (×5) compared with overall average absorption spectrum.](image-url)
This consistency between the two trials suggests that the consensus of the two might represent the best estimate of the true classification accuracy. The consensus (Table 2, average) illustrates two patterns that are common to both trials. First, and most obvious, is that the four groups can be distinguished, albeit incompletely, on the basis of the infrared spectra. Second, the pattern of misclassifications in both cases includes a confusion between groups B (prednisone) and C (low-dose deflazacort), with B misclassified as C and vice versa in both trials, but with a clean separation of the high-dose deflazacort and placebo groups.

The microscopic assessment of coded tissue samples (the same samples used for spectroscopy) showed that the frequency distribution of samples containing a majority (by area) of inflamed muscle was as follows: high-dose deflazacort, 0.40; prednisone, 0.45; low-dose deflazacort, 0.50; and placebo, 0.58. Thus the present data are consistent with deflazacort reducing muscle inflammation. In agreement, a detailed histopathology study (4) showed that deflazacort treatment produced an increase in muscle fiber growth, an increase in formation of new muscle fibers during regeneration, and a marked decrease in inflammation of dystrophic muscle.

DISCUSSION

This study provides a number of insights into testing muscle status by infrared spectroscopy. The key to interpretation is that the pattern-recognition classification derives an optimal separation among the spectra presented to it. It is useful to view the classification tables from two points of view, with the following questions in mind: 1) can the groups be separated and 2) for those cases where the separation is <100%, can the “failure” be reasonably attributed to genuine similarities between the inseparable groups? For example, in separating the treatment groups, the objective was
to optimally separate the four groups from one another. In performing these classifications it was anticipated that complete separation might not be feasible; overlap would be expected between certain groups when certain treatments promote similar or congruent changes in the histopathology of the muscle tissue.

The success of the tissue type classification (see Table 1) confirms that unaffected muscle, inflamed muscle, and tendon may reasonably be distinguished on the basis of their infrared spectra. The fact that the success rate is not 100% is likely due in large part to the heterogeneity typical of the muscle samples. That variation creates difficulties in ensuring that the area characterized by conventional histology corresponds exactly and is restricted to only the small area sampled by the infrared beam in acquiring the spectrum. It appears likely that many of the “misclassifications” of Table 1 can be accounted for in this way; for example, the three unaffected muscle samples misclassified as inflamed muscle may, in fact, contain regions of genuine inflammation immediately adjacent to more unaffected tissue. Although omitted from the classification trials for reasons presented in Classification trials, it is reasonable to expect that nerve tissue, adipose tissue, and calcification also contribute distinctive signatures to the profile of infrared absorptions observed for muscle tissue containing them. For adipose tissue and calcification, this is undoubtedly true because their chemical composition is very distinctive. The infrared spectra therefore reflect, at a minimum, the presence and relative amounts of unaffected muscle, inflamed muscle, tendon, calcification, and adipose tissue; other components such as vascular and other tissues also undoubtedly contribute to the spectra.

The studies of mdx and control mouse muscles suggest that infrared spectroscopy combined with LDA in automated-classification routines might be a potential diagnostic tool. The infrared spectroscopy studies of mdx dystrophic (treated and untreated) and control muscle samples indicate that it is possible to clearly separate spectra of muscles from high-dose deflazacort and placebo treatment groups with automated-classification methods. The success in separating these two groups in particular may well be related to a significant change in the phenotypic expression of disease pathology, possibly the extent of inflammation, observed in the samples. Conversely, the consistent inability to separate spectra from two other groups (prednisone and low-dose deflazacort) by LDA suggests that the tissue pathology is very similar in the two instances. The spectra are therefore similar to such an extent that no distinguishing features could be isolated through the pattern-recognition and/or LDA algorithm. It should be emphasized that all classifications were carried out with the double-blind designations A, B, C, and D only. As such, the classifications did not make use of any a priori information that might skew the results, for example, favoring a separation of the placebo samples from the remainder.

The present approach to “interpretation” of the infrared spectra is very different from the classic approach to pure compound identification. The sample is a complex tissue involved in a complex disease process; therefore, detailed assignment of the various infrared spectral features proved fruitless as an initial strategy for their interpretation. On the other hand, it is not unreasonable to anticipate that information derived from the present classifications will prove useful in the future for deriving and/or confirming key assignments. For example, the region-selection algorithm isolates specific spectral regions that are optimal for discriminating among the classes (Figs. 5 and 6). Presumably, these regions correspond to absorptions of tissue components that vary in concert with treatment group. It is anticipated that ongoing parallel studies of the mdx tissue (4; L. M. McIntosh, and J. E. Anderson, unpublished data) will contribute further to our efforts to reach more detailed assignments of these spectra in particular, as well as promote our general understanding of the potential for infrared spectroscopy as an adjunct to conventional histology. Indeed, these results encourage further pursuits to expand the role of infrared spectroscopy in detecting the various chemical species involved in different steps of a pathophysiologic process. Similar conclusions were drawn from previous studies of brain gray, white, and multiple sclerosis plaque tissues (7), scar tissue, and the sequelae of cardiac muscle ischemic injury (14) as well as various cancers (e.g., Refs. 10, 17).

Although the pattern-recognition algorithms do not indicate explicitly the tissue properties that distinguish one class from another, some general conclusions can be drawn from the classification trials. As indicated previously, all classifications presented here are based on the spectral region of 900–1,500 cm⁻¹ or features within that range. Several additional trials were carried out, for example, including the 1,500–1,750-cm⁻¹ range where prominent protein and lipid absorptions are found (Fig. 2). Including this additional region in classification trials did not improve the discriminating power for the mdx treatment groups, and using this range alone gave markedly poorer results. The tissue protein content, lipid content, and lipid-to-protein ratio can therefore be ruled out as strict discrimifiers in separating the treatment groups. This is also consis-
tent with the comparison of class average spectra shown in Fig. 2; even though the protein bands in particular dominate the absorption spectra, they are relatively weak in the difference spectra.

This comparison between the infrared spectra of control and mdx muscles clearly suggests a relative decrease in overall protein levels for mdx compared with control muscle tissue, with a slight increase in the lipid (but not necessarily adipose tissue) content. Such a change could result, for instance, if membrane lipid differs between control and mdx muscles, as was reported in a tissue culture study (1).

Finally, it is useful to comment on the relative efficiency of the two classification types reported here. The three tissue types (unaffected muscle, inflamed muscle, and tendon) were separated perhaps as cleanly as might be expected given the challenges imposed by the tissue heterogeneity of ensuring that precisely the same areas are sampled by infrared and morphological examinations. This consideration is an issue in separating the spectra by treatment type because the gold standard in this case is simply the treatment group to which the tissue belongs. It is, therefore, our belief that although the success rate in classifying by tissue type might be improved by systematic improvements (ensuring that infrared and visual probes sample precisely the same tissue volume), this is not the case for the classification by treatment type. We have therefore been particularly thorough in exploring various spectral regions and alternatives in spectral data processing (e.g., various derivatives and deconvolutions) for this problem. The conclusion of these efforts is that the classification tables of Table 2 represent the optimum result achievable and that the classifications therefore denote genuine differences and similarities among the four treatment groups. The overall agreement between classifications based on quite different methods (principal components vs. region selection) provides strong support for the validity of both trials.

Concluding remarks. Many of the recent studies on the resolution of tissue pathology with infrared spectroscopy take advantage of the powerful algorithms of pattern recognition and computer discrimination of automated classifications. Clearly, the future potential of infrared spectroscopy for diagnosis and/or tracking disease progression and treatment will require those computer applications to be reliable, accurate, sensitive, specific, and reproducible among samples taken under systematic but separate circumstances. For example, all the samples in the present study were exactly age matched, so it is not known how the changes that accompany the progression of muscular dystrophy (or indeed normal aging processes) might affect the resolving power of infrared spectroscopy in assigning spectra to the appropriate group. The present data do not indicate the precise chemical nature underlying spectral changes that are the basis of the classifications, so further study of the origin of the misclassifications between the prednisone and low-dose deflazacort groups is as important as is understanding what species provide for the accurate separation of spectra from the high-dose deflazacort and placebo groups.

This work provides a further indication of the potential of infrared spectroscopy for characterizing disease states. The approach employed here is generally applicable as a powerful means for detecting the existence of characteristic signatures in the spectra; once the existence of these patterns is established, it may reasonably be anticipated that systematic studies will shed some light on their origin. Further work related to muscle disorders, and muscular dystrophy in particular, will therefore focus on better characterizing the individual spectra of the various tissue subcomponents, with both infrared microscopy and statistical methods under development in our laboratories.

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