Method to determine collagen density distributions in fibrous tissues

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Short Communication

Method to Determine Collagen Density Distributions in Fibrous Tissues

*†T. J. A. Mommersteeg, †J. M. G. Kauer, *R. Huiskes, and *L. Blankevoort

**Biomechanics Section, Institute for Orthopaedics, and †Department of Anatomy and Embryology, University of Nijmegen, Nijmegen, The Netherlands*

Summary: We present a method for the measurement of hydroxyproline density distributions, as an estimate for collagen density distributions, in fibrous tissues such as ligaments and tendons. To evaluate this method, a single flexor tendon of a human hand was divided into seven tissue locations. Triplicate determinations of the dry weight tissue mass, volume, and hydroxyproline mass were made at each location: two samples were analyzed at the same time (a and b) and one was analyzed later (c). The intralocation variation is an estimate for the measurement error variance, which indicates both the precision (a compared with b) and the repeatability (b compared with c) of the technique for determination of volume, dry weight tissue mass, hydroxyproline concentration, and hydroxyproline density. The precision was about 5% for all variables, and the repeatability ranged from 1.5-4.3%. In comparison with the interlocation variations, the error variances were small, except for collagen concentration. This indicates that despite the measurement errors, differences in hydroxyproline density can be detected within fibrous tissues with the proposed method. The use of only a single tendon is adequate to evaluate the measurement error of the method, but more tendons should be measured to generalize the absolute values of the variables.

The mechanical properties of connective tissues are controlled, to a large extent, by collagen in its polymeric form. Such properties depend on the number of fibers opposing the traction force and their mechanical properties and architectural organization (8). In most studies, the collagen mass per dry weight tissue mass (collagen concentration) has been used to relate the amount of collagen to the mechanical properties (2,9). Because mechanical properties, such as the tangent modulus and the strength, are independent of the length and the cross-sectional area, a correlation with the amount of collagen per unit volume of connective tissue might prove more useful (6). Furthermore, it has become clear that the biochemical and biomechanical properties of tendons (1) and ligaments (5,10)

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Address correspondence and reprint requests to Prof. Dr. R. Huiskes at Biomechanics Section, Institute for Orthopaedics, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

vary internally. This inhomogeneity indicates that the collagen density should be quantified at different locations within these structures.

The purpose of this investigation was to evaluate a method to determine the distribution of collagen mass per unit of volume in fibrous tissues by estimation of the measurement error variance, which refers to both the repeatability and the precision of the method. The question was whether variations in collagen density within these tissues can be detected despite the measurement error variance.

MATERIALS AND METHODS

We obtained the tendon of the musculus flexor digitorum superficialis of the third finger of a hand from the cadaver of an elderly person whose age and sex were unknown. The hand had been kept frozen at –20°C since immediately after death until the time of usage. A small segment, 3 cm long, was excised from the tendon, just proximal to the chiasma tendinum. The tendon was placed in a box filled with water containing 3% saccharose to facilitate division of the tendon longitudinally, with a freeze microtome, into 1 mm thick slices (Fig. 1). The box was surrounded with dry ice in acetone, so that the tendon was quickly embedded in ice. At each location, three successive samples (a, b, and c) were used for analysis. The remaining tissue between sample locations was not analyzed.

At each side of a sample, a 20 μm slice was cut from the ice-block while the slice remained fixed to a special adhesive tape, to prevent the thin slice from rolling up and distorting. Attached to the tape, the slices were colored with a Mallory/Cason stain. The areas of the slices were measured with a graphics-image analyzer (MOP Videoplan; Kontron Elektronik GmbH, Eching-München, Germany). The mean area of two slices adjacent to a sample was a measure for the volume of that particular sample.

The samples were washed to remove the saccharose and were freeze-dried overnight. The dry weights (1,000-2,000 μg) were determined with a balance (Mettler AE240; Mettler Instrumenten BV, Tiel, The Netherlands) (reproducibility 20 μg). Next, the samples were hydrolyzed in 6 N HCl for 16 h at 120°C. Aliquots of the amino-acid mixtures generated were processed, with use of the method of Kivirikko et al. (3,7), for the evaluation of the amino-acid 4-L-hydroxyproline mass as an index of the collagen mass. Because tendinous collagen contains mainly type-I collagen and hydroxyproline occupies 10-11% of the residues of type-I collagen (corresponding to a mass percentage of about 12.5% [4]), collagen mass can be approximated by the hydroxyproline mass. A conversion factor was not determined in this particular tendon, so the collagen content of a sample was expressed in terms of the hydroxyproline concentration (the ratio between the hydroxyproline mass and the total dry weight

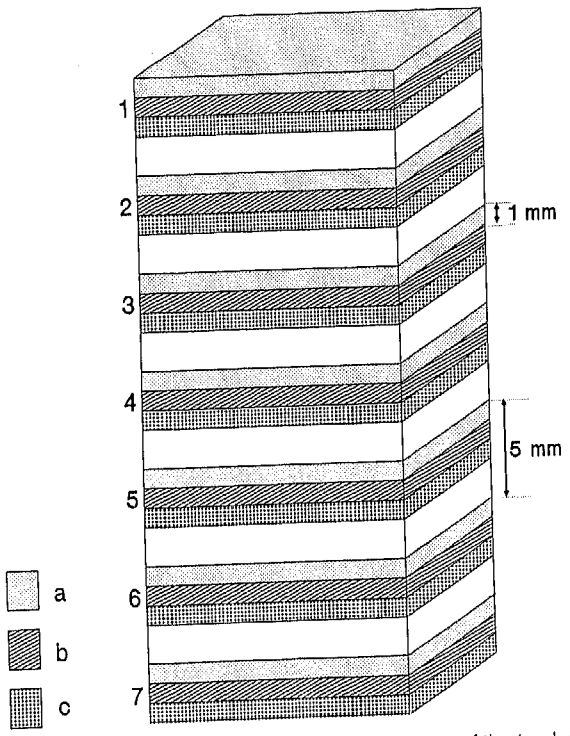

FIG. 1. Schematic representation of the division of the tendon piece in samples. The white samples were not analyzed. Samples a and b were analyzed on the same day, and c, at a later date.

tissue mass), as well as in terms of the hydroxyproline density (the ratio between the hydroxyproline mass and the volume). From each of the seven locations (Fig. 1), two samples (a and b) were analyzed on one day and one sample (c) was analyzed on another day in order to determine the temporal variation, or repeatability. This is an important aspect if many measurements are required and they cannot be made on the same day.

A one-way analysis of variance (ANOVA) was performed to compare the interlocation and intraloca-

TABLE 1. *The mean and intralocation and interlocation variation of the variables*

	Mean	$\hat{e}_1$	$\hat{e}_2$	$\hat{e}$	g^2
Hydroxyproline mass (μg)	417.7	20.4 (4.9%)	18.0 (4.3%)	21.4 (5.1%)	39.4 (9.4%)
Volume (mm^3)	10.6	0.6 (5.3%)	0.2 (1.5%)	0.5 (4.5%)	1.2 (11.1%)
Hydroxyproline concentration (%)	11.8	0.5 (4.2%)	0.4 (3.0%)	0.4 (3.1%)	0.4 (3.1%)
Hydroxyproline density ($\mu g/mm^3$)	39.5	1.9 (4.7%)	1.5 (3.8%)	1.5 (3.9%)	4.9 (12.3%)

The intralocation variation is an estimation of the measurement error variance, which is composed of variation concerned with precision ($\hat{e}$) and repeatability ($\hat{e}_2$). The interlocation variation (g^2) is an estimation of the variation among the tendons. The variations are expressed in absolute values, with the percentage of the mean value in parentheses.

tion variations in the hydroxyproline mass, volume, hydroxyproline concentration, and hydroxyproline density. Whether differences between locations are statistically significant depends on the ratio between the interlocation variation and the intralocation variation and on the sampling frequency. Additionally, a Kruskal-Wallis test was performed. The results of the two analyses were compared to determine if the intralocation variation was different among locations. If so, one cannot calculate an overall value for this parameter.

The interlocation variation or variation of interest (g^2) was estimated from the variation among the locations for each variable. The overall intralocation variability is directly related to the measurement error variance, which was estimated from the mean square error within each location. Two aspects of the measurement error variance, indicating precision ($\hat{e}_1$) and repeatability ($\hat{e}_2$), were expressed as the root mean square error of the sample values a and b and of the sample values b and c, respectively, for each parameter:

$$\hat{e}_1 = \left[\frac{\sum_{i=1}^{7} (V_a - V_b)_i^2}{n_a + n_b}\right]^{\frac{1}{2}} \qquad \hat{e}_2 = \left[\frac{\sum_{i=1}^{7} (V_b - V_c)_i^2}{n_b + n_c}\right]^{\frac{1}{2}}$$

where V_a, V_b, and V_c are the parameter values for samples a, b, and c at the seven locations and n_a, n_b, and n_c are the number of samples a, b, and c at the seven locations.

RESULTS

Values for hydroxyproline mass, volume, hydroxyproline concentration, and hydroxyproline density varied among the locations (Fig. 2). The one-way ANOVA revealed that the interlocation variation was significantly higher than the intralocation variation for hydroxyproline mass, volume, and hydroxyproline density ($p < 0.05$) but not for hydroxyproline concentration ($p > 0.05$) (Table 1 and Fig. 2). The Kruskal-Wallis test resulted in the same conclusions, indicating that the intralocation variation was not different among locations. Thus, it was possible to calculate an overall measurement error. The intralocation variation or measurement variance, expressed as a percentage of the mean parameter value, ranged from 3.1% (hydroxyproline concentration) to 5.1% (hydroxyproline mass). The precision ($\hat{e}_1$) was about 5% for all variables, and the repeat-

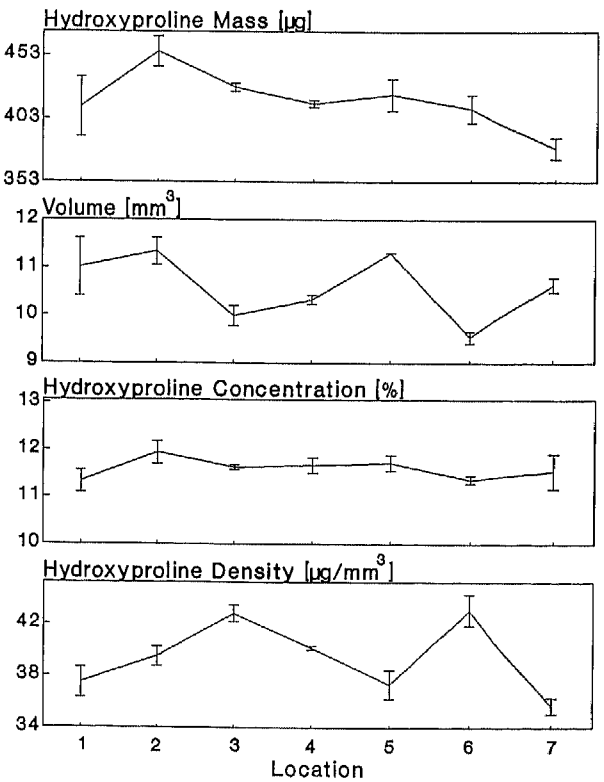

FIG. 2. The interlocation and intralocation variation for the four variables. The error bars represent SEM; n = 3 at each location.

ability ($\hat{e}_2$) ranged from 1.5% (volume) to 4.3% (hydroxyproline mass) (Table 1).

DISCUSSION

In order to assess a method for the determination of collagen density distributions in fibrous tissues, the measurement error variance, composed of a variation component indicating the precision and a variation component indicating the repeatability of the method, was determined in this small preliminary study. The precision and the repeatability were similar for all variables (<5%), indicating that no additional variance was introduced by measurements made at different times. Despite the measurement error variance and the small sample size, the prediction of the parameter values at each location was precise enough to detect variations among locations, except for variations in hydroxyproline concentration. For the hydroxyproline concentrations, the differences among locations were too small (compared with the measurement error) to be detected. Differences in local hydroxyproline density can, however, be determined with the applied sample frequency and a measurement error of 3.9%.

A limitation of this study is that it involved only one tendon of one human cadaver, for which the exact characteristics were not known. This makes generalization or external validation of the absolute parameters impossible. The external validity of the method could not be evaluated because of the absence of a defined standard for collagen density. However, it was not our aim to determine the collagen density distribution in a human tendon but rather to test a method to collect such data in future studies and to perform relative comparisons.

Additionally, the method has a number of limitations. First, the conversion of the amount of hydroxyproline in the amount of collagen varies in different tissues, and a parallel study on collagen typing in the particular tissue should be performed if this conversion factor is required. In fact, collagen density cannot be determined with the methodology described. With the assumption that the conversion factor is constant in the tissue studied, however, relative differences in hydroxyproline mass provide an estimate for relative differences in collagen mass. Furthermore, it must be taken into account that 4-L-hydroxyproline is not specific for collagen and also is identified in small proportions in other proteins, such as elastin. In addition, 1% of the amino acid residues in type-I collagen (3-L-hydroxyproline) cannot be measured with the described method. With the assumption that the amount of elastin and 3-L-hydroxyproline are constant throughout fibrous tissues, these points are not relevant in the consideration of differences in collagen density. Another limitation is the embedding of the specimen in ice water containing 3% saccharose, a step that can affect the volume of the fibrous tissue due to osmotic hypotonic effects. The osmotic gradient (0.09 *M* compared with 0.15 *M*) is negligible, and the number of cells for which osmosis can have destructive effects (lysis) is small in fibrous tissues.

As far as we know, there are no records of hydroxyproline or collagen mass per unit of volume in tendons and ligaments. Hence, a comparison with values from the literature is not possible. However, overall values for hydroxyproline and collagen concentrations have been obtained for tendons in other studies, with a method also based on the measurement technique described by Kivirikko et al. (7). For example, Amiel et al. (2) found hydroxyproline concentrations of 10.5 and 10.3% in the patellar tendon of the right and left knee of the rabbit, respectively. A possible explanation for the lower concentrations than ours (11.8 ± 0.4%) is that a different kind of tendon was analyzed or that the ages and histories of the donors differed. As mentioned before, more tendons must be measured to generalize the absolute measurement values.

The method can be applied to map collagen density distributions in human knee ligaments. The combination of these data with variations in mechanical properties described in the literature will make it possible to investigate whether there is a relationship between collagen density and mechanical properties.

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