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THE EFFECT OF IMMOBILIZATION ON COLLAGEN TURNOVER IN CONNECTIVE TISSUE: A BIOCHEMICAL-BIOMECHANICAL CORRELATION

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Immobilization of the knee joint for 9 weeks results in a reduction of the mechanical properties in the lateral collateral ligament. Specifically, ligament stiffness is reduced in this tissue. No statistical change in collagen mass was detected for the medial collateral ligament (MCL) or patellar tendon. An increase in collagen turnover (synthesis and degradation) was, however, found in the immobilized medial collateral ligament and patellar tendon. It is thus proposed that stiffness reduction is due to a change in the ligament substance itself, rather than a result of tissue atrophy.

Key words: collagen; collagen turnover; immobilization; ligament

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Changes in the biochemical composition of periarticular fibrous connective tissue (e.g. patellar tendon, ligament and joint capsule) have been demonstrated following immobilization of synovial joints (Akeson et al. 1968). These changes consist mainly in a reduction of water and glycosaminoglycans (Akeson et al. 1973) and an alteration in collagen cross-linking, specifically an increase in the concentrations of hydroxylysinonorleucine (HLNL), histidinohydroxymerodesmosine (HHMD), and dihydroxylysinonorleucine (DHLNL) (Akeson et al. 1977). No change in collagen type (Amiel et al. 1980) or collagen mass between control and immobilized fibrous connective tissue was detected (Akeson et al. 1968).

The biomechanical properties of these joint components may have also altered following a period of immobilization. Noyes et al. (1974b) demonstrated in primates that, after 8 weeks of immobilization, a 39 percent decrease in the maximum failure load in the anterior cruciate ligament-bone complex occurred. These authors also noted that the incidence of avulsion failure increased in the immobilized group (Noyes et al. 1974a, Noyes 1977). Furthermore, in a study on canine medial collateral ligament (MCL) repair, Tipton et al. (1970) demonstrated that immobilization reduced the strength of the "separation force" of the collateral ligament insertion to bone. Morphological studies of these specimens showed atrophy of cortical bone beneath the ligament insertions (Laros et al. 1971). However, the question of changes in the mechanical properties of the ligamentous substance has not arisen, as the stress and strain of ligament were not measured. Recently, our laboratory developed a video dimensional analyzer (VDA) system, permitting the strain of the ligament substance during stretch to be measured (Woo et al. 1979).

Since collagen provides a major contribution to tissue tensile strength, we were led to suggest that the dynamics of collagen turnover may in

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some way be altered under conditions of stress and motion deprivation. The present study reports the effect of immobilization on synthesis and degradation of collagen in patellar tendon and medial collateral ligament of the rabbit knee. Additionally, the lateral collateral ligaments from the same animals were isolated and subjected to biomechanical testing for the determination of the change in the mechanical properties of the ligament substance following immobilization.

MATERIALS AND METHODS

Animal model

Ten New Zealand white male rabbits, 6 weeks of age and weighing approximately 1.5 kg, were injected intraperitoneally three times a week with 200 μCi per kg body weight of L-5-^{3}H proline over a period of 8 weeks (Klein et al. 1977). At termination of the injections 4 weeks were allowed to pass in which nothing was administered to the animals except regular food and water. This was done to allow the animals enough time to incorporate the majority of the tritiated proline prior to immobilization. The left knee was then fixed in acute flexion as described previously (Akeson et al. 1973). The right knee remained free to serve as a control. All animals were maintained for 9 weeks in flexion and then sacrificed. At this time the animals weighed 3.5 ± 0.2 kg.

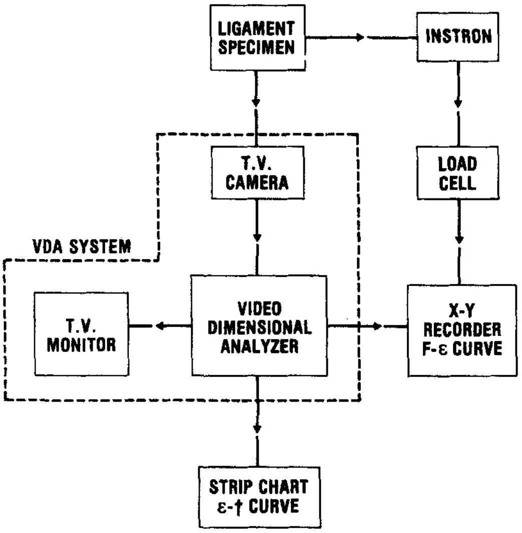

Figure 1. A block diagram showing the experimental apparatus for measuring the tensile load-deformation characteristics of ligaments. The VDA system is used to measured the strain.

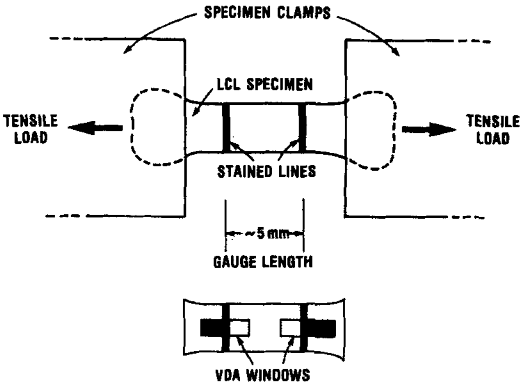

Figure 2. A schematic diagram of the TV monitor display showing the ligament specimen during tensile testing. The monitor display also shows two windows of the signal from the video dimensional analyzer following the stain lines on the specimen for strain measurement.

The lateral collateral ligament was promptly isolated from its femoral and tibial insertions for biomechanical property assessment. The length of the isolated ligament was approximately 18 mm. Using Verhoff stain for elastin, two parallel lines, approximately 5 mm apart, were drawn on the central ligament perpendicular to its long axis as gauge length marks for tensile strain determination. Subsequently, the width and thickness of the ligament with the marks were measured for cross-sectional area calculations. For thickness determinations, a specially designed micrometer instrument, as described previously by Woo et al. (1980), was used. The accuracy and reproducibility of the measurement was within 5 micrometers. For determination of the width of the ligament, a cathetometer* with an accuracy up to 10 micrometers was employed. Care was taken to keep the ligament moist at all times during these measurements. The ligament specimen was then clamped and mounted on an Instron apparatus for tensile testing (Figure 1). The load cell recorded the tensile load while the tensile strain values were measured by a video dimensional analyzer (VDA) system. The VDA system consisted of a video camera, a TV monitor, and an electronic dimensional analyzer. The video camera sends the test specimen information to the TV monitor through the electronic dimensional analyzer to determine the tensile strains.

The VDA system has two windows which can be placed on the video image of the test specimens. The windows were set to follow the two dark lines (Verhoff stained lines) automatically on the specimen (Figure 2). The horizontal scan time between these two dark lines

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* Precision Tool and Instrument Co., Thornton Heath, Surrey, England.

was reported by the VDA as an output voltage. As the distance between the two lines increased during tensile stretch, the output voltage increased proportionately. Therefore, the tensile strain of the specimen could be automatically determined. The advantages of using the VDA system to measure the tensile strains are: 1) The measurement does not rely on external attachment to the specimen to measure tensile strain, 2) Therefore ligament slippage at the clamp cannot contribute to errors in soft tissue strain measurements, 3) video information is video-taped for repeated strain analyses. This method of strain analysis is accurate, reproducible, automatic, and not time consuming. The system also eliminates certain human errors which can occur, for example, in the technique of making serial photographs to record strain.

All tests were performed with the ligament submerged in a saline solution bath at 37°C. An Instron crosshead rate, i.e., 2 cm/min, was used. The test procedure consisted of cycling the specimen to approximately 2 per cent strain for ten cycles as preconditioning, and the load-strain curve of the eleventh cycle was recorded. Knowing the cross-sectional area of the specimen, and with the strain determined by the video dimensional analyzer system, the mechanical properties (stress-strain curve) of the lateral collateral ligament substance were then determined.

Sampled tissue for biochemical analysis included the patellar tendon and medial collateral ligament. Uniformity of tritium labeling for each tissue type (left versus right) was also determined in a separate group of five injected, but unpinned, rabbits. The time period (including post injection time) and the dose for this group were identical with that used in the pinned group. Immediately following dissection the medial collateral ligaments and patellar tendons were weighed and then defatted in a mixture of ether-acetone (50:50). The defatted tissue was dried to constant weight and ground in a Wiley Mill.

A small aliquot of dried tissue (2–4 mg) was taken for determination of hydroxyproline (HP) content (Woessner 1961), after which 15–20 mg of each tissue sample was hydrolyzed in 3N p-toluene sulfonic acid *in vacuo* for 24 hours at 108°C. Prior to chromatography, an aliquot of the hydrolysate was sampled for determination of its HP content. Isolation of HP (labeled and unlabeled) was achieved by ion-exchange liquid chromatography of the hydrolysate on a column (62 × 0.9 cm) of spherical cation-exchange beads. Thus, the exact amount of collagen applied to the column was known. The amount of HP that elutes from the column was determined by combining the fractions of the hydroxyproline peak for measurement of HP content (95.5 ± 0.5 percent of the amount of HP applied was recovered). Following chromatography, 200 μl from each of the fractions comprising the hydroxyproline peak were mixed separately with 8 ml of scintillation cocktail and counted for 50 minutes on a Packard Tri-carb Liquid Scintillation Spectrophotometer (Mechanic 1974). Using the above procedures, total hydroxyproline, specific radioactivity, and total radioactivity were measured for the medial collateral ligament and patellar tendon.

The repeated and prolonged labeling of collagen during a stage of active growth makes it possible to determine both loss of collagen and gain of collagen during a subsequent period of immobilization (Klein et al. 1977). Collagen synthesized during the period of time that the injections are administered contains a tritium label and is therefore referred to as old (radioactive) collagen. Conversely, collagen synthesized during the subsequent period of immobilization

Table 1. A comparison of collagen mass and radioactivity between left and right limbs from unpinned (normal) rabbits

Normal group (unpinned)	Dry weight (mg)*	Collagen mass* (μg Hydroxyproline)	Specific radioactivity* (cpm/μg Hydroxyproline)
Medial Collateral Ligament			
right	26.4±1.6	2,512±199	4.13±.36
left	27.6±1.9	2,648±251	4.18±.39
percent difference	+3.9±2.6	+5.0±3.6	+1.0± .9
paired t-test**			
Patellar Tendon			
right	158.9±11.9	16.735±1021	3.87±.30
left	154.6± 8.1	15,965±1475	3.84±.31
percent difference	–3.2± 3.4	–4.6±2.3	–0.9±1.0
paired t-test**			

* Data expressed as mean ± S.E.

** Student's t-test was performed on all categories. Only significant differences are indicated by appropriate *P* values.

contains no label and is referred to as new (non-radioactive) collagen.

Since collagen mass and specific radioactivity are not statistically different between the left and right limbs of unpinned (normal) animals, each collateral ligament and patellar tendon from the immobilized left knee has a contralateral control – the non-immobilized right knee (Table 1). The amount of old collagen existing prior to immobilization can then be calculated by measuring the amount of labeled collagen present in the tissue from the opposite (contralateral) limb (Klein et al. 1977). The amount of new collagen added is then calculated by subtracting the amount of old (radioactive) collagen remaining from the total mass of collagen present in the immobilized tissue. The amount of old collagen lost after a period of immobilization can also be determined by comparing the amounts of radioactive collagen present in the tissue from the immobilized and contralateral limbs. Both the loss and gain of collagen are expressed as a percentage of control values so that a mean from several animals can be calculated. The following equations illustrate the calculations necessary for determination of old collagen mass, new collagen and loss of old collagen (Klein & Lewis 1972).

$$1.\ \text{Mass of old (radioactive) immobilized collagen} = \frac{\text{Total radioactivity immob. tissue}}{\text{Total radioactivity control tissue}} \times \text{Mass of control collagen}$$

$$2.\ \text{Mass of new (non-radioactive) immobilized collagen} = \text{Total mass immobilized collagen} - \text{Mass of old immobilized collagen}$$

$$3.\ \text{Degradation of old immobilized collagen (percent)} = 1 - \frac{\text{Total radioactivity of immobilized collagen}}{\text{Total radioactivity of control collagen}} \times 100$$

In order to determine the extent to which tritiated proline (liberated from the degradation of radioactive collagen following the period of labeling) is reutilized in the synthesis of new collagen, the distribution of radioactivity in soluble and insoluble collagen fractions was measured on separately pooled samples of control and immobilized collateral ligaments and patellar tendons. The pooling of tissue was necessary in order to accumulate enough tissue for extraction in neutral salt solutions. Extraction in 1 *M* NaCl, pH = 7.4, was performed with constant stirring for 48 hours at 4°C. This was followed by extraction of the residue with 0.2 *M* Na citrate, pH = 3.6, and then 2 percent acetic acid under the same conditions. All extracts were dialyzed against 200 vol. of 1 percent acetic acid and lyophilized. The remaining insoluble residue was dissolved in 1 percent acetic acid and lyophilized. These four fractions were hydrolyzed as described above and analyzed for hydroxyproline content and specific radioactivity.

RESULTS

Biomechanical studies

The load-deformation curves of the control and immobilized lateral collateral ligaments demonstrate general nonlinear behavior as do most biological soft tissues. The stiffness of the tissues was small at lower loads and gradually increased with increasing strain. Large differences exist between the control and immobilized lateral collateral ligaments (LCL) (Figure 3). The trend of data obtained is similar to the medial collateral ligament-bone complex of the rabbit knee obtained earlier (Woo et al. 1979).

To determine that such reduction in stiffness characteristics secondary to immobilization is a result of loss in mass, or loss in mechanical prop-

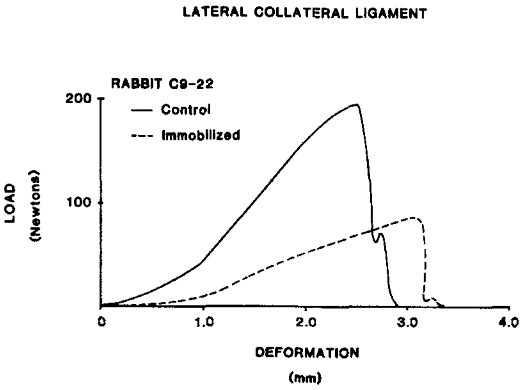

Figure 3. Load deformation curves from the lateral collateral ligaments (LCL) of the control and immobilized rabbit's knee.

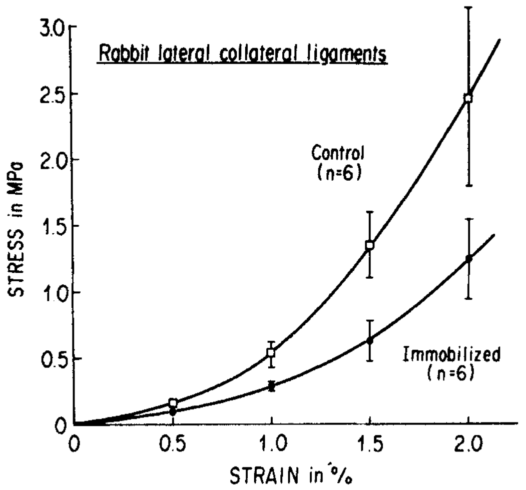

Figure 4. Mechanical properties (stress-strain) from the lateral collateral ligaments of the control and immobilized rabbit's knee.

erties, or both, one must examine the size of the ligament as well as the stress-strain curves. Comparison of the cross-sectional area measurements showed the immobilized ligaments to have a slightly, but not statistically significantly lower cross-sectional area than the controls (3.5 ± 0.3 mm^2 versus 4.1 ± 0.5 mm^2, respectively, $P > 0.10$). Therefore, the majority of stiffness reduction of the LCL must be due to the change in ligament substance itself, rather than simple atrophy, since the mechanical properties (stress-strain curves) of the immobilized lateral collateral ligament were significantly inferior to those of the control lateral collateral ligament (Figure 4).

Biochemical studies

In the group of unpinned rabbits (normal animals), the mean differences in dry defatted tissue weight, collagen mass and specific radioactivity between right and left knees were found to be not statistically significant (Table 1). In the group of pinned rabbits there was likewise no statistical difference in dry weight of collagen mass between the left (immobilized) and the right (non-immobilized) limb for either the medial collateral ligament or patellar tendon. However, in this group there was a significant decrease in specific radioactivity in the tissue from the immobilized knee. This was observed for both the medial ligament and the patellar tendon. The decreases were –10.5 percent and –11.8 percent, respectively (Table 2).

All immobilized animals showed a significant loss of old radioactive collagen in both the medial ligament and patellar tendon from the immobilized joints. The mean losses ± standard error were 14.0 percent ± 5.8 percent and 6.1

Table 2. A comparison of collagen mass and radioactivity between left (immobilized) and right (free) limbs from pinned rabbits

Immobilized group	Dry weight (mg)*	Collagen mass* (µg Hydroxyproline)	Specific radioactivity* (cpm/µg Hydroxyproline)
Medial Collateral Ligament			
right	25.7±1.4	2,502±156	5.82±.31
left	24.5±1.2	2,369±129	5.22±.35
percent difference	–2.6±3.4	–0.4±2.1	–10.5±2.0
			$P<.05$
Patellar Tendon			
right	155.2±2.5	16,153±496	5.82±.51
left	157.5±5.1	16,621±885	5.14±.51
percent difference	+1.5±3.1	+3.7±3.1	–11.8±2.6
			$P<.05$

* Data expressed as mean ± S.E.

** Student's t-test was performed on all categories. Only significant differences are indicated by appropriate P values.

Table 3. Percentage changes in the rates of collagen turnover for the immobilized medial ligament and patellar tendon

	Medial collateral ligament	Patellar tendon
Percent gain in new collagen	11.3±3.4 P[t]<0.05	8.7±2.8 P[t]<0.05
Percent loss of old collagen	14.0±5.8 P[t]<0.05	6.1±2.6 P[t]<0.05
Percent change in collagen mass	−2.1±7.7 P[t]>0.5	2.7±4.1 P[t]>0.5

[t] Paired t-test → Immobilized vs contralateral control.

percent ± 2.6 percent, respectively. Concurrent with the loss was a gain in new collagen mass amounting to a mean value ± standard error of 11.3 percent ± 3.4 percent in the medial ligament and 8.7 percent ± 2.8 percent in the patellar tendon (Table 3). Since no statistical change in total collagen mass was observed, an apparently complete replacement of degraded (radioactive) collagen with newly synthesized (non-radioactive) collagen is indicated. It is seen in Table 3 that a larger loss of collagen occurred in the medial collateral ligament than in the patellar tendon. This was accompanied by an equally large gain in new collagen. Thus, it appears that the rate of collagen turnover is greater in the immobilized medial collateral ligament than in the immobilized patellar tendon.

Table 4. Radioactivity profile for soluble and insoluble collagen fractions extracted from H^3-proline labeled rabbits

Collagen extract*	Specific activity (cpm/μg)	% of total radioactivity
1 *M* NaCl, pH = 7.4		
Control	2.94	0.3
Immobilized	2.85	0.3
0.2 *M* Na citrate, pH = 3.6		
Control	6.68	1.0
Immobilized	6.35	1.3
2% acetic acid		
Control	7.52	5.4
Immobilized	6.92	4.3
Insoluble residue		
Control	8.29	93.3
Immobilized	7.60	94.1

* Pooled patellar tendon, medial collateral ligament.

The distribution of radioactivity in soluble and insoluble collagen extracted from pre-labeled medial collateral ligaments and patellar tendons (pooled tissues) is shown in Table 4. Specific radioactivity and percentage of total radioactivity have been calculated for the neutral salt soluble, citrate soluble, acid soluble, and insoluble residue fractions of control and immobilized tissue. It is seen that the level of radioactivity is notably smallest in the neutral salt soluble fraction (most recently synthesized collagen) and increases sequentially with each subsequent extraction. The small amount of radioactivity in the salt soluble fraction could be due to partial degradation and solubilization of more mature (more highly radioactive) collagen, or perhaps to limited reutilization of tritiated proline liberated from the complete degradation of old radioactive collagen. In either case the amount of radioactivity observed in the neutral salt soluble fraction is small and the values are approximately equal for control and immobilized tissue.

These data support the formulation of terminology used to describe the maturity of the collagen and the relationship of maturity to its level of radioactivity whereby mature collagen (less extractable) is referred to as "radioactive collagen" and recently synthesized collagen (easily extractable) is referred to as "non-radioactive collagen".

DISCUSSION

This study shows that disuse of the knee joint as a result of rigid immobilization produces a change in the rate of collagen turnover in the medial collateral ligament and patellar tendon. An increased rate of both collagen synthesis and degradation without a statistical change in collagen mass has been demonstrated in these two tissues. Thus, a net increase in the amount of newly synthesized collagen occurs in this tissue from an immobilized joint. This last fact correlates with our previous studies which revealed an increased concentration of reducible collagen cross-links in immobilized periarticular connective tissue (Akeson et al. 1977). The correlation finds basis in the fact that newly synthesized collagen forms intermolecular reducible cross-links as an early step in the maturation of collagen fiber. The above analysis is supported by the fact that the mechanical properties of the immobilized lateral collateral ligament (LCL) substance were much inferior when compared to its contralateral control as demonstrated by the stress-strain curves. The newly synthesized collagen fibers in the immobilized ligaments must be laid down in a haphazard, haystack manner because of absence of the usual controls on orientation of matrix imposed by physical forces. Furthermore, the accelerated collagen turnover of the immobilized tissue indicates that these less mature collagen fibers should be more pliable and thus possess lower stiffness properties to resist the tensile loads.

In a study employing a different method of immobilization (e.g. denervation of one limb in a rat model), Klein et al. (1977) similarly reported an increase in the rate of collagen turnover for the medial collateral ligament from the denervated limb. This was accompanied by a small but significant net loss of collagen mass. Quantitatively, the rates of synthesis and degeneration are comparable to the results presented in our study. Our results likewise suggest a small but not statistically significant net loss of collagen mass in the immobilized ligaments.

Klein et al. (1980) reported a significant loss of collagen mass in the anterior cruciates and lateral menisci from a 12-week immobilized dog knee. Since the point at which atrophy of collagen mass begins is unknown, these discrepancies with our results may be due in part to the fact that our period of immobilization was of shorter duration; also it is possible that degradation of collagen is greater in other tissues such as menisci and cruciate ligaments whose physiological functions are different from those of the patellar tendon and medial ligaments.

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