Hindlimb unloading alters ligament healing

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Provenzano, Paolo P., Daniel A. Martinez, Richard E. Grindeland, Kelley W. Dwyer, Joanne Turner, Arthur C. Vailas, and Ray Vanderby, Jr. Hindlimb unloading alters ligament healing. J Appl Physiol 94: 314–324, 2003.—We investigated the hypothesis that hindlimb unloading inhibits healing in fibrous connective tissue such as ligament. Male rats were assigned to 3- and 7-wk treatment groups with three subgroups each: sham control, ambulatory healing, and hindlimb-suspended healing. Ambulatory and suspended animals underwent surgical rupture of their medial collateral ligaments, whereas sham surgeries were performed on control animals. After 3 or 7 wk, mechanical and/or morphological properties were measured in ligament, muscle, and bone. During mechanical testing, most suspended ligaments failed in the scar region, indicating the greatest impairment was to ligament and not to bone-ligament insertion. Ligament testing revealed significant reductions in maximum force, ultimate stress, elastic modulus, and low-load properties in suspended animals. In addition, femoral mineral density, femoral strength, gastrocnemius mass, and tibialis anterior mass were significantly reduced. Microscopy revealed abnormal scar formation and cell distribution in suspended ligaments with extracellular matrix discontinuities and voids between misaligned, but well-formed, collagen fiber bundles. Hence, stress levels from ambulation appear unnecessary for formation of fiber bundles yet required for collagen to form structurally competent continuous fibers. Results support our hypothesis that hindlimb unloading impairs healing of fibrous connective tissue. In addition, this study provides compelling morphological evidence explaining the altered structure-function relationship in load-deprived healing connective tissue.

hindlimb suspension; biomechanics; disuse; collagen; bone; muscle

STRESS REDUCTIONS DUE TO MICROGRAVITY (7, 14, 27, 33, 41, 48, 52, 57), hindlimb unloading (2, 8, 18, 47, 49, 51), bed rest (6, 28, 29, 43, 46), or immobilization (1, 3–5, 13, 53) result in suppression of skeletal and connective tissue homeostasis and formation and a reduction in their mechanical properties. These reductions in the musculoskeletal system can result in a profound decline in functional ability as well as increased risk of future pathology (24, 25, 30, 50). Of further concern is that remobilization does not ensure complete recovery of preimmobilization mechanical properties of ligamentous tissue (53). Compounding this problematic decline of normal tissue under stress reduction is the fact that wound healing is also impaired under microgravity and immobilization (15, 19, 23, 26, 44). These issues become more problematic as the duration of prolonged spaceflight increases and the probability of prolonged bed rest grows with the expanding aged population. Methods to understand and counteract musculoskeletal degeneration and impaired wound healing require further study.

Stress reduction is known to adversely affect fibrous connective tissues, such as ligament and tendon. Stress shielding in normal patellar tendon results in a decrease in tensile strength and elastic modulus (31, 36, 59). In addition, immobilization in the form of rigid, external joint fixation is reported to result in lower ultimate load and stiffness in healing rat Achilles tendon compared with transected tendons without immobilization. In rhesus monkeys, Noyes (35) examined the effects of 8 wk of immobilization (whole body casting) and 5 and 12 mo of reconditioning on anterior cruciate ligaments. Results of his study indicate a significant decrease in ultimate load in immobilized ligaments at 8 wk. Neither 5 nor 12 mo of reconditioning resulted in full recovery of anterior cruciate ligament ultimate load. Amiel and colleagues (3) studied biochemical changes associated with joint immobilization by using a stainless steel pin to immobilize the knee joint of rabbits. One end of the pin was inserted into the tibia while the other end was hooked over the proximal end of the femur. After pin fixation, the animals were allowed unrestricted cage activity. This protocol substantially immobilized the joints; however, it did not completely shield the joint from load. Results of their study indicate a reduction in total collagen, increased collagen turnover, and increased collagen syn-
thesis and degradation rate, with degradation rate exceeding the synthesis rate. Using the same pin technique, Woo et al. (53) later examined the effect of immobilization on rabbit medial collateral ligaments (MCLs). Nine and twelve weeks of immobilization resulted in a significant reduction in ultimate load, energy at failure, and changes in failure mode. Although these studies examine joint immobilization, they do not examine the effect of substantial joint stress reduction on the healing ligament.

To simulate weightlessness, the National Aeronautics and Space Administration (NASA)-Ames institute developed the hindlimb suspension model (56, 58). Hindlimb suspension provides a noninvasive model for tissue disuse. Like microgravity or confined bed rest, this model eliminates weight bearing and tissue loading from ground reaction forces during mobilization, which allows substantial hindlimb stress reduction. Hindlimb suspension for 3 wk reduces the ultimate stress and tangential modulus of rat Achilles tendon (2). In addition, Vanderby et al. (51) examined rat hindlimbs after 7 days of hindlimb suspension and reported reduced body weight, reduced soleus muscle mass, as well as reduced ultimate load and stress in the MCL. Significantly reduced MCL ultimate load, stiffness, and stress were also present after 2 wk of hindlimb suspension in rat (49). Although the above studies have examined changes in normal tendon and ligament after hindlimb suspension, they do not examine the effect of hindlimb suspension (i.e., complete loss of ground reaction force) on healing fibrous connective tissues such as ligament.

The purpose of this study is to test the hypothesis that stress reduction through hindlimb unloading delays and impairs the wound-healing response in fibrous connective tissue, such as ligament. To examine this hypothesis, changes in the mechanical properties and microstructural morphology of healing fibrous connective tissue will be studied by using a rat MCL model. Mechanical properties are examined at both the low-load physiological range and failure range. Histology and scanning electron microscopy (SEM) are performed to elucidate information regarding the structure-function relationship of the ligament tissue. In addition, changes in hindlimb muscle and bone, as well as total body mass, are examined to more completely show the physiological effects of hindlimb suspension.

MATERIALS AND METHODS

Animal preparation. This study was approved by the institutional animal use and care committee and meets National Institutes of Health guidelines for animal welfare. Sixty male Sprague-Dawley rats (245 ± 5 g) were used as an animal model. These animals were divided into two time groups (3- and 7-wk healing), each consisting of 30 rats. Each time group was composed of three subgroups, each containing 10 randomly selected animals: sham control, ambulatory healing, and hindlimb suspended healing. Each rat in the ambulatory healing and hindlimb suspension groups underwent bilateral disruption of the MCL. Experimental results show that mechanical properties in healing MCLs are equivalent after either unilateral or bilateral surgical disruption (45). Rats were anesthetized with an isoflurane anesthetic administered by face mask by using a nonrebreathing delivery system. The incision area was clipped and aseptically prepared for surgery. All surgeries were performed aseptically. A skin incision ~8 mm long was made on the medial side of the stifle, and fascia was incised to expose the MCL. The MCL was exposed and transected at the joint line. The wounds were closed with suture. Sham control animals were subjected to identical surgical procedures without MCL rupture. All animals were given an analgesic (Tylenol/acetaminophen) in their water for 72 h postsurgery. Sham control and ambulatory healing animals were allowed unrestricted cage movement. Hindlimb suspended healing rats were subjected to hindlimb unweighting (24 h after surgery), which induced stress reduction by eliminating ground reaction force by using the noninvasive tail suspension protocol of the NASA-Ames center (32, 34, 56, 58). Only the hindlimbs were under a 12:12-h light-dark cycle in a room at 24°C. Rats were fed Purina rat chow, watered ad libitum, and checked twice daily for overall health, skin incision healing, food and water consumption, and the condition of their tails (the harness should prevent slippage without restricting circulation). After either 3 or 7 wk, the animals were euthanized. Of the 20 ligaments per group, 6 were separated for a separate unreported study and will not be included here. The remaining 14 ligaments were distributed as follows. In each of the three treatment groups for each time point, six ligaments were used for biomechanical testing, four were used for histology, and four were used for electron microscopy.

Tissue harvest and biomechanical testing. Immediately after death, animal hindlimbs were placed in plastic bags and stored at −80°C until testing. It has been shown that postmortem storage by freezing does not change the biomechanical properties of ligament (55). On the day of testing, hindlimbs were thawed at room temperature. Tissue harvest and testing were performed by using methods similar to those previously described (38, 40). Extraneous tissue was carefully dissected away to expose each MCL. The femur-MCL-tibia complex was then removed, with care taken not to disturb the ligament insertion sites. Ligaments were kept moist in Hank’s physiological solution at 25°C to prevent dehydration (pH = 7.4), and cross-sectional area was measured optically. The femur-MCL-tibia complex was then placed into a custom-designed tissue bath system with special structures to hold the femur and tibial sections of the sample along the longitudinal axis of the MCL where all fibers appear to load simultaneously (~70° flexion). Optical markers (silicone-impregnated grease) were placed onto the ligament tissue near the insertion sites for strain measurement. The tissue bath containing the MCL samples was inserted into our custom-designed testing machine. A small preload of 0.1 N was applied to obtain a uniform zero point (i.e., to start the tests from the same relative position). In displacement control, ligaments were preconditioned (~1% strain for 10 cycles) and pulled to failure at ~10%/s. Strains and strain rates were calculated from videotape (described below) after displacement controlled tests. Strain rates were consistently within 5% of the target value. During and after the test, the failure location was recorded and examined. Digital images of the location of structural failure were analyzed to assess failure location. For the case of a potential tibial avulsion, the ruptured tibial end of the tissue was examined for bone.
Tissue displacement was obtained by using video dimensional analysis with 10-μm resolution (at this level of magnification) to measure the change in distance between markers. The change in distance between optical markers was calculated by analyzing stored digital frames with NIH Image software by using a custom macro to calculate the change in x-y coordinate center (centroid coordinates) of each marker. Force (resolution of 0.005 N) was displayed on the video screen and synchronized with displacement. From displacement data, strain was calculated as the change between stretched length and gauge length (8.5 ± 0.4 mm (mean ± SD) at preload) divided by gauge length. Stress was calculated as the force divided by the initial area. Stress-strain curves were created and fit with the microstructural model presented by Hurschler and co-workers (21). Biomechanical parameters for comparison (n = 6 ligaments per group) are ultimate force, ultimate strain, strain at failure, and stretch at the toe-to-linear region transition (described below).

Modeling physiological low-load behavior. The ligament stress-strain curve displays nonlinear strain-stiffening behavior in the toe region, after which a more linear region is encountered. The model described in this section quantifies the transition from the toe region to the linear region. This model has been previously described in detail elsewhere (22). A brief description will be included here. Stress-stretch data were fit with the probabilistic microstructural model of Hurschler et al. (21), which utilized a Weibull probability density function (PDF) to represent collagen fiber recruitment and, hence, load-bearing capability as the tissue is stretched and the characteristic crimp pattern of the collagen fiber is removed. The model has the form

\[ \sigma(t) = \int_{t}^{\infty} P(\lambda) \sigma_{25}(\lambda) d\lambda, \quad \lambda > \gamma \]  

(1)

where \( P(\lambda) \) is the Weibull PDF as a function of the straightening stretch ratio, defined as the tissue stretch (\( \lambda_t \)) at which an individual fiber begins to bear load (\( \lambda_m = \) fiber length/tissue reference length), \( \sigma_{25} \) is the longitudinal normal stress in a fiber, and \( \gamma \) is the location parameter of the Weibull distribution and defines the onset of fiber loading (22). Hence, the stress in the tissue is defined by the overall tissue stretch (stretch ratio = \( \lambda_t \)) and the load-bearing contribution of the fiber population. After the data have been well described (\( R^2 > 0.98 \)) by the model, parameters from the Weibull PDF are input into the Weibull cumulative distribution function (CDF), 

\[ F(\lambda) = \int_{0}^{\infty} P(\lambda) d\lambda = \int_{\lambda}^{\infty} \frac{\beta}{\delta} \left( \frac{\lambda - \gamma}{\delta} \right)^{\beta-1} \exp \left[ -\left( \frac{\lambda - \gamma}{\delta} \right) ^{\beta} \right] d\lambda \]  

(2)

where \( \beta \) and \( \delta \) are the shape and scale parameters of the Weibull distribution. After integration, Eq. 2 can be algebraically manipulated to yield the inverse CDF

\[ \lambda_t = \delta \left[ -\ln(1 - F) \right]^{1/\beta} + \gamma \]  

(3)

which defines the stretch ratio at which any fraction of the fibers (F) have been recruited. Based on preliminary studies, the 85th percentile provides a good representation of complete fiber recruitment and will be used in this study. Hence, from Eq. 3, we are able to determine the stretch (and hence stress) at which the tissue exits the nonlinear strain-stiffening toe region and enters a more linear portion of the curve.

Preparation for histology and SEM. Immediately after tissue harvest, samples for histology (n = 4 ligaments per group) were fixed in formalin. After standard histology procedure, 10-μm sections underwent hematoxylin and eosin staining. Slides were coverslipped and viewed with light microscopy. Ligament preparation for SEM (n = 4 ligaments per group) was similar to that previously described (39). MCLs were exposed, and tissues were drip fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4; GSCB, Electron Microscopy Sciences, Fort Washington, PA) at the same knee angle as mechanical testing. After drip fixation, MCLs were removed and soaked in fixation solution for at least 1 h. The ligaments were then dehydrated through a series of ethanol/H2O solutions (30, 50, 75, 90, 100, and 100% ethanol). After dehydration, MCLs were immersed in liquid nitrogen and subsequently placed on a precooled microscope slide. Under a dissecting scope, MCLs were initiated by using a microsurgical scalpel blade. The frozen specimens were carefully fractured in the sagittal plane of the knee joint and then transferred to the to the tibial end. The femoral end was marked for reference. Samples were then critical point dried (Samdri model 780A, Tousimis Research, Rockville, MA), mounted on 10-mm SEM mounting blocks (JEOL 840, SPI Supplies, Structure Probe, West Chester, PA), gold-palladium sputter coated to 195 angstroms, and stored in a vacuum container. The ligaments were imaged with SEM (JOEL-JSM 6100). At low magnification (approximately ×25), the ligament was aligned by orienting the femoral end of the ligament to the top of the screen. The residual and scar regions were identified in all healing ligaments. At a magnification of ×250, the morphology of normal and scar tissue was examined, and images were stored digitally.

Analysis of bone mineral density and strength. To assess how injury and disuse affected bone morphology and strength, the rat femur was examined. In six limbs per group, from separate animals that were not used for biomechanical testing, femurs were carefully harvested and kept hydrated with PBS (1×) solution. A Noland XCT Research M system (STRATEC XCT-960A pQCT, Stratec Medizintechnik GmbH, Pforzheim, Germany), which employs an X-ray source (47 kVp, 40 keV, beam width 8 keV, 0.3 mA) that produces a fan beam with an effective width of 2.5 mm, was used to characterize geometry and bone mineral densities of the femurs. The samples were placed in a custom-designed apparatus to hold the bones horizontally in the machine while being scanned by using voxel size B (148 μm). The femurs were scanned at midshaft and at the widest point of the femoral head. The scan lines were modified by using the scout view feature. Analysis was performed with separation mode 1, and images were stored at a resolution of 640 × 480 pixels. Total (cortical plus trabecular) bone mineral density was recorded at the femur midshaft and in the femoral head. To examine bone strength, the above femurs were mounted in a custom frame and tested destructively in three-point bending. All specimens were tested at room temperature at a rate of 0.1667 mm/s. Force was recorded by using Labtech Notebook digital acquisition software. Maximum load was examined for comparison between groups.

Measurement of muscle weight. The gastrocnemius and tibialis anterior muscles were examined. In six limbs per group, from separate animals that were not used for biomechanical testing or SEM, muscles were carefully harvested and kept hydrated with PBS (1×) solution. These muscles were taken from the same limbs as the femurs discussed in Analysis of bone mineral density and strength.
carefully blotted dry to remove any excess fluid and immediately weighed on a Metler balance scale.

Statistical analysis. Statistical analysis was performed by using a two-way ANOVA followed by pairwise comparison to examine treatment effect. Data were log transformed to better conform to the assumptions of ANOVA. The level of significance was set at 0.05, and analysis was performed with SAS PROC MIXED (SAS Institute, Cary, NC). Fisher’s protected least significant difference test was used as the post hoc multicomparison test.

RESULTS

There were no wound infections or other apparent complications associated with surgery. All of the mobilized animals (sham control and ambulatory healing) returned to normal cage activity. The health of suspended healing animals was carefully monitored, and no complications with surgery or suspension were observed. Initial body weights were carefully selected so that the groups were not different. After surgery, body weight in the control sham and ambulatory healing groups proceeded naturally. However, suspended healing animals had an initial decrease in body weight followed by attenuated gains in body weight increase over the study period (Figs. 1 and 2). At tissue harvest all healing ligaments showed a bridging of the rupture gap with translucent scar tissue. During MCL harvest in the 3- and 7-wk suspended animals, tissue adhesion was present, and in some animals the tissue surrounding the surgical site appeared to heal less thoroughly. In addition, the femur and tibia were noticeably “softer” and more fragile, requiring care during harvest of the femur-MCL-tibia complex.

Examination of failure location in the ligaments revealed that sham tissues fail primarily in the tibial third of the ligament at both 3 and 7 wk, whereas in healing tissues from both ambulatory and suspended animals the majority of tissues failed in the scar region (Table 1). Only one tissue per time group failed by tibial avulsion in suspended animals, which indicated that the dominant decrease in mechanical properties is because of changes in ligamentous tissue and not the bone-ligament insertion. Substantial differences in tissue mechanical properties are present between sham

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**Table 1. Failure location in rat groups**

<table>
<thead>
<tr>
<th>Failure Location</th>
<th>Scar Region</th>
<th>Midsubstance (Sham Only)</th>
<th>Tibial Third of the Ligament</th>
<th>Tibial Avulsion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Week 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham control</td>
<td>N/A</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Ambulatory healing</td>
<td>5</td>
<td>N/A</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Suspended healing</td>
<td>5</td>
<td>N/A</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Week 7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham control</td>
<td>N/A</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Ambulatory healing</td>
<td>4</td>
<td>N/A</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Suspended healing</td>
<td>4</td>
<td>N/A</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

N/A, not applicable.
and suspended healing animals after 3 wk of healing ($P = 0.0001$ and $0.0001$, respectively) and after 7 wk of healing ($P = 0.0104$ and $0.0001$, respectively). Ligaments from suspended animals had ultimate stress values that were significantly lower than ambulatory animals at both 3 and 7 wk ($P = 0.0156$ and $0.0029$, respectively). In fact, ultimate stress values after 7 wk of healing in suspended animals was comparable to 3-wk values in the ambulatory animals, which revealed a decrease in rate of recovery. Full pairwise statistical comparisons for ultimate stress can be found in Fig. 5. Strain at failure was not significantly different between any time or treatment groups: $0.075 \pm 0.034$, $0.079 \pm 0.019$, and $0.082 \pm 0.028$ for 3-wk sham control, ambulatory healing, and suspended healing groups, respectively, and $0.086 \pm 0.014$, $0.082 \pm 0.016$, and $0.084 \pm 0.011$ for 7-wk sham control, ambulatory healing, and suspended healing groups, respectively. Elastic modulus was determined from fitting the data with a microstructural model (21). All data were fit well: $R^2 > 0.98$. Elastic modulus (Fig. 6) was significantly decreased between sham control and both ambulatory healing and suspended healing animals after 3 wk of healing ($P = 0.0002$ and $0.0001$, respectively) and after 7 wk of healing ($P = 0.0102$ and $0.0001$, respectively). Tissues from suspended animals had modulus values that were significantly lower than ambulatory animals at both 3 and 7 wk ($P = 0.0082$ and $0.0173$, respectively). In addition, after 7 wk of healing, the mean modulus value in the suspended group was less than the mean modulus in the ambulatory animals after 3 wk of healing. Full pairwise statistical comparisons for elastic modulus can be found in Fig. 6. Examination of the stretch ratio at the toe region to linear region transition revealed no significant differences between any groups (Fig. 7). However, the stress required to transition from the toe region to the linear

control, ambulatory healing, and suspended healing (Fig. 3). Maximum force (Fig. 4) was significantly different between sham control and both ambulatory healing and suspended healing animals after 3 wk of healing ($P = 0.0001$ and $0.0001$, respectively) and after 7 wk of healing ($P = 0.0064$ and $0.0001$, respectively). In addition, maximum force in ambulatory and suspended healing MCLs was significantly different in the 3-wk group ($P = 0.0011$). After 7 wk of healing, maximum MCL force was substantially reduced in the suspended animals compared with ambulatory animals, but not significantly ($P = 0.1433$). Full pairwise statistical comparisons for force can be found in Fig. 4. Ultimate stress (Fig. 5) was significantly decreased between sham control and both ambulatory healing and suspended healing animals after 3 wk of healing ($P = 0.0002$ and $0.0001$, respectively) and after 7 wk of healing ($P = 0.0102$ and $0.0001$, respectively). Tissues from suspended animals had modulus values that were significantly lower than ambulatory animals at both 3 and 7 wk ($P = 0.0082$ and $0.0173$, respectively). In addition, after 7 wk of healing, the mean modulus value in the suspended group was less than the mean modulus in the ambulatory animals after 3 wk of healing. Full pairwise statistical comparisons for elastic modulus can be found in Fig. 6. Examination of the stretch ratio at the toe region to linear region transition revealed no significant differences between any groups (Fig. 7). However, the stress required to transition from the toe region to the linear

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region was significantly decreased between sham control and both ambulatory healing and suspended healing animals after 3 wk of healing \((P = 0.0005\) and \(0.0001,\) respectively\) and after 7 wk of healing \((P = 0.0277\) and \(0.0001,\) respectively; Fig. 8). Transition stress in ambulatory healing animals improved almost twofold from 3 to 7 wk but was not quite significant \((P = 0.0638).\) Suspended animals showed no improvement in transition stress from 3 to 7 wk \((P = 0.9142),\) indicating a lack of recovery of physiological low-load behavior in suspended animals. In addition, ambulatory animals had significantly larger transition stress after 7 wk than suspended animals \((P = 0.0064).\)

Representative hematoxylin and eosin sections (Fig. 9) revealed extracellular matrix disorganization and hypercellularity after injury in tissues from both ambulatory and suspended animals. Ligaments from sham control animals showed the characteristic crimp pattern, which is associated with normal ligament, with fibroblasts in between collagen bundles (Fig. 9, A and B). In contrast to ambulatory animals, which revealed typical scar morphology, tissues from suspended animals after 3 wk of healing revealed pockets of cell clusters and collagen fibers turning back on themselves, often forming voids within the tissue (Fig. 9, E and F). After 7 wk of healing, ambulatory animals showed improvements in collagen fiber orientation and decreases in cellularity compared with morphology at 3 wk (Fig. 9D). Suspended animals at 7 wk showed substantial improvement in collagen fiber bundle formation and decreases in cellularity (Fig. 9F). However, the majority of bundles were not oriented along the longitudinal axis of the ligament. Collagen fiber bundles in suspended animals were often oriented in directions other than the longitudinal axis of the ligament with some bundles aligning in the transverse orientation. In addition, different fiber bundles in suspended animals were oriented in different directions, creating discontinuities and voids when two differently oriented bundles came in contact (Fig. 9F).

SEM supported the histological finding that tissues from suspended animals had good collagen fiber bundle formation but poor bundle orientation, which resulted in discontinuities and voids (Fig. 10, A–C). Sham control tissues showed collagen fiber bundle alignment along the longitudinal axis of the ligament with fibers having characteristic crimp morphology (Fig. 10A). Collagen fibers in scar tissue from ambulatory animals were randomly oriented at 3 wk with substantially improved alignment and orientation (i.e., more axial) by 7 wk (Fig. 10B). Yet collagen fibers in ambulatory animals had not regained normal structure and organization after 7 wk. Ligaments from suspended animals had good fiber bundle and aggregation (demonstrated by fiber grouping and bundle width) at both 3 and 7 wk, but the majority of bundles were not oriented along the longitudinal axis of the ligament with bundles oriented in separate directions, which created discontinuities and voids. These voids and discontinuities were created when bundle ends came in contact with the side of a bundle oriented in a different direction (Fig. 10C).

Bone and muscle measurements are shown in Table 2. Sham and ambulatory animals had no significant differences within a time group for a particular property, yet properties in ambulatory healing tissues were often reduced compared with tissues from sham animals. Midshaft total bone mineral density was reduced in the suspended animals at 3 wk, but not significantly until 7 wk. Bone mineral density in the femoral head, bone strength, gastrocnemius weight, and tibialis anterior weight were all significantly reduced in suspended animals compared with sham and ambulatory groups for both time points.

![Fig. 7. Stretch ratio at the transition from the nonlinear strain-stiffening toe region to the linear region of the stress-strain curve in ligament (means ± SE). Transition stretch ratio was not significantly different between any study groups. Statistical comparison for any group can be obtained from the intersection of group numbers in the subset table.](image)

![Fig. 8. Stress at the transition from the nonlinear strain-stiffening toe region to the linear region of the stress-strain curve in ligament (means ± SE). Hindlimb unweighting significantly alters low-load behavior in healing tissue. After 7 wk, suspended animals showed no improvement in low behavior, whereas ambulatory animals revealed an almost twofold increase in transition stress. Statistical comparison for any group can be obtained from the intersection of group numbers in the subset table.](image)
DISCUSSION

Results of this study support our hypothesis that stress reduction due to hindlimb unweighting substantially alters the healing of fibrous connective tissue in rats. In suspended animals, only one tissue at each time group failed by tibial avulsion, whereas the remaining femur-MCL-tibia complexes failed in the ligament proper. These results show impaired ligamentous healing from hindlimb unloading that can occur with spaceflight or prolonged bed rest. Maximum force, ultimate stress, and elastic modulus in healing tissues were all significantly reduced with hindlimb unweighting. Stress at the toe-to-linear region transition was substantially reduced in suspended animals compared with ambulatory animals and showed no recovery from 3 to 7 wk. These changes may be due in part to altered tissue organization during healing. Microscopy and histology revealed altered cellular and extracellular matrix organization in suspended animals that was substantially different from characteristic scar organization in ambulatory animals. In addition, total bone mineral density, bone strength, and muscle weight were reduced in hindlimb suspended animals.

Results of this study are consistent with previous studies that have shown a reduction in the biological and mechanical properties of normal ligaments after immobilization (3, 5, 35, 53) and hindlimb suspension (49, 51). Woo and co-workers (19, 23, 54) examined the effects of immobilization with pin fixation, but not complete stress deprivation of the joint after MCL rupture, and concluded that long-term immobilization impedes MCL healing. However, in their studies, ambulatory animals did not undergo surgical repair of the MCL, whereas surgical repair was performed on immobilized ligaments, making it difficult to directly compare their work and data presented herein. Yet, regardless of the surgical procedure, stress reduction (through joint immobilization or hindlimb suspension) diminishes healing of fibrous connective tissue leading to impaired tissue function. In addition, Woo et al. (54) reported increased varus-valgus laxity in healing animals that was recovered in ambulatory animals but not in immobilized animals. These results are in agreement with results obtained by using mathematical modeling of the toe region. Stress-deprived animals required a substantially lower magnitude of stress to leave the physiological toe region and enter the linear portion of the stress-strain curve and revealed no recovery of these low-load properties after 7 wk (Fig. 8).

The fact that low-load properties are not recovered in remobilized animals raises an interesting question in light of previous work in our laboratory. We previously identified a strain-based tissue damage threshold in normal rat MCLs of ~40–60% of the failure strain (38). Because suspended animals require lower amounts of stress to enter linear region of the stress-strain curve
and, hence, the damage threshold (which may be lower in healing tissue), remobilization in the form of normal activity may result in further tissue microdamage leading to difficult or incomplete recovery of presuspended properties. Further microstructural and mechanical studies are required to understand the residual effects of suspension on healing tissues in remobilized animals.

Results of this study agree, in general, with structure-function studies in fibrous connective tissue that reveal a strong relationship between altered extracellular matrix structure and organization after injury and reduced mechanical properties. Previous studies have shown that extracellular matrix flaw size reduces with healing time and has a strong association with changes in failure stress and modulus (42), whereas changes in collagen fibril diameter in scar tissue do not correlate well with increased mechanical properties over the time course of healing (17). In studies utilizing SEM, Padgett and Dahners (37) reported a larger number of matrix defects in immobilized healing rat MCLs than in mobilized tissue, whereas Frank et al. (16) reported better matrix alignment in immobilized rabbit MCLs. These studies (16, 37) do not necessarily conflict with each other. The study by Frank et al. (16) examined the extracellular matrix at a relatively high magnification ($\times$4,000), whereas the work by Padgett and Dahners (37) was performed at lower magnification ($\times$100). Evaluation at high magnification (relative to collagen fiber organization) does not readily allow identification of voids and defects, and Frank et al. (16) do not give information on overall matrix organization. Regardless, it is possible that both studies support the results obtained in this study using histology and

Fig. 10. A: scanning electron micrograph of sham control tissue at 3 wk (2 left panels) and 7 wk (2 right panels). Collagen fibers are oriented along the longitudinal axis of the ligament and possess characteristic crimp morphology. B: scanning electron micrograph of ambulatory healing tissue at 3 wk (2 left panels) and 7 wk (2 right panels). Collagen fibers are randomly oriented at 3 wk with substantially improved alignment and orientation along the longitudinal axis of the ligament by 7 wk. However, collagen structure, organization, and alignment have not regained normal appearance. C: scanning electron micrograph of suspended healing tissue at 3 wk (2 left panels) and 7 wk (2 right panels). At 3 wk, tissue shows good fiber bundle formation and aggregation, but fiber bundles are not well oriented along the longitudinal axis of the ligament with fiber bundles of different orientations coming together without fiber continuity to form discontinuities (arrows) and voids (V) in the extracellular matrix. The 3-wk micrographs show regions where misaligned bundles do not connect (arrows). After 7 wk, collagen bundle formation and aggregation continue to improve, yet voids (V) and discontinuities (arrows) are still present between misaligned collagen fiber bundles.
SEM. In this study, stress reduction from hindlimb suspension altered scar morphology and resulted in voids and discontinuities where well-formed collagen bundle ends (demonstrated by fiber grouping and bundle width) came in contact with the side of a bundle oriented in a different direction. These results are in agreement with work by Padgett and Dahners (37) that increased voids are present in immobilized tissues. Because of their use of only 10 images per tissue at higher magnification, Frank et al. (16) may have been viewing these well-formed bundles that we have shown to be grossly or slightly misaligned (Fig. 10) to create discontinuities and voids in stress-altered tissue. Hence, Frank et al. may have viewed slightly misaligned, well-formed bundles, which led to the conclusion of increased matrix alignment in immobilized tissues.

Examination of the altered structure in tissue from suspended hindlimbs leads to an interesting question: How do such high-quality collagen fiber bundles form in ligaments with substantially reduced mechanical stress and yet not form a continuous force coupling with surrounding well-formed collagen bundles? Birk and Trelstad (10) studied tendon morphogenesis in chick embryos and showed that formation of collagen fibrils and fibril bundles occur within cytoplasmic recesses of fibroblasts. In addition, Birk and co-workers (9, 11, 12) showed that discontinuous fibril segments can fuse to form longer fibril in developing embryonic chick tendon. During development, these fibril fusions can occur through collagen fibril end-to-end fusion (20). Previous examination of the transition from scar to residual tissue in MCLs from ambulatory mature animals has shown collagen fibril and fiber continuity between scar and residual tissues (39). Hence, new fibrils join continuously with residual fibrils in ambulatory animals and are suspected to be a necessary means of transferring force between newly formed scar tissue and residual tissue. This continuity is largely not present in suspended animals. As such, we propose collagen bundle discontinuities in suspended tissue are a major contributor to the reduced mechanical properties of healing tissue in suspended animals. Interestingly, stress levels associated with normal ambulation appear to be unnecessary for the formation of fiber bundles (orchestrated by fibroblasts first with the formation of the collagen fibrils), but this stress appears necessary to align and promote fusion between new fiber bundles to form structurally competent fiber continuity. However, further study is required to understand the mechanisms of collagen formation and organization in scar tissue and to answer the above-stated question. In addition, further studies into the biochemical composition of the matrix and more intense examination of cell behavior and organization need to be performed to better understand the root causes of the altered composition- and structure-function changes associated with fibrous connective tissue disuse.

Several limitations must be borne in mind when considering the results of this study. First, the rat model may be limited in its ability to model adult humans under stress-altered conditions such as space-flight or prolonged best rest. However, the results of this study are consistent with related studies that show reduction in ligament, bone, and muscle properties with disuse. Second, the rat hindlimb suspension model does not inhibit muscle activity, hence some knee motion and joint loading can occur. Observation and tissue atrophy suggest that such motion and loads are substantially reduced. Third, transected ligaments do not replicate stretch-induced injuries, but they are very reproducible for comparative purposes. Fourth, the effects of short periods of therapeutic exercise, such as passive and active motion, to counteract the effects of limb disuse were not studied. Such treatments would be more similar to conditions of human tissue healing. Fifth, low-load behavior was described from an ex vivo preloaded condition, and further understanding of the ex vivo vs. in situ reference position is required. Sixth, sample preparation for SEM can induce a shrinkage artifact. However, all specimens were handled and prepared in the same controlled manner. Because the morphological portions of this study are qualitative in nature, shrinkage error most likely had little effect on our observations. Last, although further biological, biochemical, and histomorphometric studies are ongoing, further understanding of the biological and physical mechanisms driving the compelling changes associated with tissue disuse is required.

Despite these limitations, our results support our hypothesis that stress reduction through hindlimb unloading impairs healing of fibrous connective tissue. Maximum stress, ultimate stress, elastic modulus, and physiological low-load behavior were altered by hindlimb suspension. Examination of extracellular matrix organization revealed novel and important changes in

Table 2. Bone and muscle properties

<table>
<thead>
<tr>
<th>Bone and Muscle Properties</th>
<th>Sham Control</th>
<th>Ambulatory Healing</th>
<th>Suspended Healing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midshaft total bone density, mg/cm³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 3</td>
<td>897.7 ± 20.4</td>
<td>903.3 ± 29.8</td>
<td>837.6 ± 41.5</td>
</tr>
<tr>
<td>Week 7</td>
<td>929.0 ± 22.8</td>
<td>953.8 ± 36.5</td>
<td>841.4 ± 14.5*</td>
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<tr>
<td>Femoral head total bone density, mg/cm³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 3</td>
<td>695.0 ± 10.0</td>
<td>654.1 ± 13.3</td>
<td>574.1 ± 12.4*</td>
</tr>
<tr>
<td>Week 7</td>
<td>758.7 ± 16.4</td>
<td>702.1 ± 33.0</td>
<td>626.8 ± 19.3*</td>
</tr>
<tr>
<td>Bone strength, N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 3</td>
<td>116.9 ± 9.9</td>
<td>104.6 ± 5.3</td>
<td>62.8 ± 5.4*</td>
</tr>
<tr>
<td>Week 7</td>
<td>130.4 ± 3.7</td>
<td>119.2 ± 13.5</td>
<td>94.6 ± 3.8*</td>
</tr>
<tr>
<td>Gastrocnemius weight, mg</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Week 3</td>
<td>2,258.2 ± 73.0</td>
<td>2,160.5 ± 83.9</td>
<td>1,607.9 ± 57.4*</td>
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<tr>
<td>Week 7</td>
<td>2,710.0 ± 26.6</td>
<td>2,469.5 ± 215.5</td>
<td>1,817.5 ± 77.0*</td>
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<td>Tibialis anterior, mg</td>
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<tr>
<td>Week 3</td>
<td>815.1 ± 30.2</td>
<td>748.2 ± 31.9</td>
<td>646.0 ± 37.9*</td>
</tr>
<tr>
<td>Week 7</td>
<td>940.1 ± 29.8</td>
<td>858.2 ± 63.3</td>
<td>670.2 ± 57.8*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significant (P < 0.05) difference from both sham control and ambulatory healing groups.
stress-deprived tissues with voids and discontinuities between well-organized, but misaligned, collagen bundles in suspended animals. This morphology was substantially different from matrix organization in ambulatory animals. This morphology was substantially different from matrix organization in connective tissues. Effects of immobilization on joints. Clin Orthop 219: 28–37, 1987.


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